

## RESEARCH COMMUNICATION

# Immunological Profile of Arsenic Toxicity: A Hint Towards Arsenic-induced Carcinogenesis

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### Abstract

Arsenic (a Group I carcinogen in humans) contamination and poisoning of human populations in different parts of Southeast and Eastern Asia, including West Bengal and Bangladesh, has become a major environmental concern. Arsenic intoxication affects diverse human organs including the lungs, liver, skin, bladder and kidney. This metalloid acts as a promoter of carcinogenesis, exerting toxic effects on the immune system. The present study was aimed at investigating arsenic-induced carcinogenesis and effects on the immune system in an animal model. Tumors were induced using ethylnitrosourea (ENU) and arsenic was used as a promoter. To investigate specific effects on the immune system, cytokine (TNF- $\alpha$ , IFN $\gamma$ , IL4, IL6, IL10, IL12) production of lymphocytes was evaluated by FACS. The damaging consequences of treatment were assessed by evaluating the specific programmed cell death cascade in lymphocytes, assessed by FACS readings. The results revealed that under arsenic influence, and more so with arsenic+ENU, marked neoplastic changes were noted, which were corroborated with histological changes, cytokine modulation and apoptosis hinted at marked neoplastic changes.

**Keywords:** Apoptosis - arsenic - caspase - cytokine - ENU

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### Introduction

Arsenic has been listed as a carcinogen on the Toxics Release Inventory list as per the Office of Pesticide Programs List of Chemicals Evaluated for Carcinogenic Potential, U.S. EPA, March 15, 2002. Again, Arsenic and Lead have been classified among the most Commonly Released Toxic Chemicals in Nevada, according to latest U.S. EPA Report released in March, 2009.

Arsenic poisoning, due to its environmental pervasiveness has become a worldwide environmental concern as millions of people are exposed to arsenic through contaminated drinking water in India, Bangladesh, Taiwan, and some parts of Mongolia and China and some parts of South America. Arsenic affects human organs including lungs, liver, skin, bladder, kidney, the CNS and the hemopoietic system (Blot et al., 1979; Schwartz, 1997; Kaalia and Flora, 2005).

One of the most worrying consequences of chronic arsenic poisoning is the carcinogenic effect of arsenic on humans. The element is classified as a Group I carcinogen in humans by the International Agency for Research on Cancer (IARC, 1987, 2004) and US Environmental Protection Agency (EPA, 1988) as a known human carcinogen. Ingested Arsenic in drinking water causes

lung, colon and bladder cancer risks while low dose Arsenic exposure causes skin cancer. Arsenic causes genomic instability, causing gene mutations that lead to carcinogenesis, the DNA repairing enzymes being especially vulnerable (Hughes, 2002). DNA repair activity is inhibited by arsenic, but the inhibition is not a direct action of arsenic on repair enzymes (Hughes, 2002). Because arsenic can elicit many diverse effects, more than one mechanism may be involved in its carcinogenic effects. The inability of arsenic to act as a classical mutagen (point mutations) had hindered research on defining the mechanisms of action for arsenic (Hughes, 2002).

Acute, subchronic & chronic arsenic exposure suppresses B & T lymphocyte maturation, induces their apoptosis and that of macrophages, and suppresses T cell specific cytokine expression by directly interfering with JAK-STAT pathway. (Yu et al., 1998; Vega et al., 1999; Cheng et al., 2004). Oncogenesis results in immune suppression, which can further aggravate the tumor. It has also been proved by various researchers that the depressed immunity can trigger the neoplastic growth. Hence a vicious cycle is generated between the immune system and cancer. Though the role of arsenic has been established as a carcinogen/ co-carcinogen and its immunotoxic role

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has also been delineated, but the effect of arsenic induced carcinogenesis on the immune system remains to be worked out.

Efforts to produce an animal model for carcinogenesis failed previously as Arsenic, when used alone was not really a carcinogen per se, but a co-carcinogen or promoter of carcinogenesis, i.e., it promoted the carcinogenic action of other known carcinogens such as cigarette smoke and UV radiation. (Wang et al., 2002). Studies support the hypothesis that arsenic may act as a co-carcinogen- not directly causing cancers, but allowing other substances, such as cigarette smoke and ultra-violet light, which can cause mutations in DNA more effectively. This approach has been modified by later workers and animal models for studies on arsenic carcinogenesis using a known carcinogen along with arsenic have been put forward (Rossman et al., 2002; Cohen et al., 2007). While some workers used Dimethyl arsenic acid (DMA) (15) or UVR (Rossman et al., 2002), we administered Ethyl nitrosourea (ENU), a chemical carcinogen in conjunction with arsenic to study carcinogenesis in mice. Furthermore, earlier workers focused on one afflicted organ type such as skin (Rossman et al., 2002) or urinary bladder (Cohen et al., 2007), whereas we tried to address the mechanistic queries by which the different afflicted organs proceeded on to carcinogenesis in a chronological order.

In the present study histological studies of tissue sections of different organs of the different animal groups were done to corroborate the study with carcinogenic induction. Since the immune status is completely devastated in the face of a carcinogenic attack, we aimed at the assessment of cytokine modulations in conditions of Arsenic induction as well as a combined regimen of ENU and arsenic induction against healthy controls age matched, which would hint at the immune status of the afflicted animals, an indicator of carcinogenesis. Furthermore, since cancer growth is accentuated in a weakened immune milieu, death of lymphocytes which account for such a state was assessed by the assessment of the different death proteins.

## Materials and Methods

### Animals

Healthy newborn Swiss albino mice of both sexes along with the mother were maintained in our Laboratory for the purpose of investigations. 6 animals in each group were weaned at 30 days of age and housed separately in isolated cages. All animals were fed autoclaved Hind Lever pellet and water ad libitum and housed in a room with ambient temperature of 22°C in 12 h light/darkness cycle. Mice were examined daily and weighed weekly throughout the experimental period. Maintenance and animal experiment procedure strictly followed 'Principles of Laboratory Animal Care' (NIH Publication No. 85-23, revised in 1985) and also local 'ethical regulations'.

### Arsenic dosimetry

Sodium metaarsenite [ $\text{NaAsO}_2$ ] (Mol wt. - 129.91) was taken as the source of Arsenic. 50 ppm of Sodium Meta arsenite was administered in drinking water (1 liter

water contained 86.72 mg of  $\text{NaAsO}_2$ ) of the animals after the animals attain adulthood 2 months of age onwards.

### ENU dosing of animals for cancer induction

N'-N'-Ethyl nitrosourea (ENU) was freshly prepared by dissolving 10 mg/ml in sterile, saline and adjusting the pH to 4.5 with crystalline ascorbic acid. ENU was injected intraperitoneally (i.p.) to newborn rats (3-5 days old) with a dose of 80mg/kg body weight. (Druckray et al., 1966; Lantos, 1972; Koestner et al., 1977; Mukherjee et al., 2004).

### Animal grouping

Since mice reach adulthood within 5-6 weeks, they become fully grown adult mice and were kept as age matched controls for all the groups, viz., 2, 3 and 4 months respectively. Thus, a single control group sufficed for the entire study. The animals were grouped in batches of 6 for each experimental group, which are as follows:

- i.) Normal control (N),
- ii.) ENU dosed animals.
- iii.) Arsenic dosed animals. Animals were sacrificed after 2 months (Group IIIa), 3 months (Group IIIb) and 4 months (Group IIIc) after arsenic dosing.
- iv.) ENU+Arsenic dosed animals. Animals neonatally treated with ENU were administered Arsenic as above and sacrificed after 2 months (Group IVa), 3 months (Group IV b) and 4 months (Group IV c) after arsenic+ENU dosing.

### Histological examination

Tissue sections from brain, liver, spleen, kidney, G.I. Tract, Testes and lungs from the different groups of animals were collected after dissecting the viscera and were prepared for routine histological studies. Collected tissues were fixed in 10% formal-buffer overnight, dehydrated and finally embedded in paraffin through histokinet processing. Tissue sections were cut at 5  $\mu\text{m}$  thickness and finally stained with routine haematoxyline/eosine.

### Antibodies

Mice monoclonal antibody specific for IL-4, IL-6, IL-10, IL-12, IFN- $\gamma$ , CD95 (Fas), CD178 (Fas L), Bcl-2 were purchased from BD Biosciences, USA. Mice monoclonal antibody for Bax and TNF- $\alpha$  was purchased from Santacruz Biotechnology, Santacruz, California. Mice monoclonal antibody specific for Caspase-8 raised against epitope mapping at the N terminus caspase 8 p20 subunit of human origin used for detection of the precursor of caspase-8 were purchased from Santacruz Biotechnology, Santacruz, California. Mice monoclonal antibody specific for Caspase-3 raised against epitope corresponding to amino acids 1-277 representing the full-length precursor form of caspase 3 of human origin and rat monoclonal antibody specific for Caspase-9 raised against epitope corresponding to amino acids 315-397 mapping within the C terminus of caspase-9 of human origin used for detection of the precursor of caspase -9 were purchased from Santacruz Biotechnology, Santacruz, California.

The primary and the secondary antibodies were diluted in PBS/azide (1% PBS 1% sodium azide in PBS) plus 10 % autologous mice serum (Mukherjee et al., 2002).

#### *Labeling of cells and flow cytometric analysis*

Single color analysis was used for all the antibodies. For the surface antigen CD178,  $1 \times 10^6$  cells/ml were directly incubated with the primary antibodies for 45 mins at 4°C. The cells were then washed and resuspended in PBS after which they were stained with the secondary antibody FITC, in the next step (30 mins. at 4°C in dark). After washing, cells were fixed with 1ml ice-cold 1% paraformaldehyde in PBS (pH 7.2) overnight at 4°C in dark. For intracellular antigens Bcl-2, Bax, p53, caspase-8, caspases-9 and Caspase-3, 4 batches of  $1 \times 10^6$  cells/ml were fixed in 0.5 ml of 0.3% ice cold paraformaldehyde in PBS (30 mins at room temperature). Cells were then washed with PBS and permeabilized by being gently resuspended in 1% Triton-X 100 in PBS and incubated at 37°C for 30 mins. The samples were then labeled with the corresponding primary and secondary antibodies and fixed as described above. After overnight incubation, samples were taken out and resuspended in PBS and kept in ice on dark and analyzed within 1 hour. Immunofluorescence was performed in a FACS caliber using Expo 32 software (Beckman Coulter, USA). For each sample, 40,000 events were scored. For surface and intracellular antigen labeling, the Ig control sample values were subtracted from all other sample values to remove FITC background fluorescence.

## Results

#### *Histological studies*

**Brain:** Histological sections of the brain tissue from Normal, ENU group, Arsenic dosed & Arsenic +ENU animals show no specific variation. In the 4<sup>th</sup> month in arsenic fed animals injected with ENU proliferative oligodendroglial cells were visible along with angiogenic vessels.

**Liver:** In comparison with normal and ENU group liver, slight vacuolar changes with ballooning degeneration are observed at few places in 2<sup>nd</sup> month which is further aggravated by appearance of the necrotic bodies in few places in 3<sup>rd</sup> month of arsenic and arsenic+ENU group. All the conditions further deepen in the 4<sup>th</sup> month. Vacuolar changes & ballooning degeneration is very prominent in the ENU induced arsenic dosed animals. Multinucleation, as well as cells in various stages of division were a prominent features observed in many areas of the slides in both Arsenic and ENU+Arsenic groups. Vacuolar changes were observed in the ENU group in the 4<sup>th</sup> month.

**G. I. Tract:** In comparison to the normal G. I. tract slight mucosal epithelial degeneration & inflammation is seen in 2<sup>nd</sup> month, followed by hypertrophied musculature in the 3<sup>rd</sup> month & extreme atrophied epithelial cells & degenerative necrotic changes in the 4<sup>th</sup> month of arsenic dosing. Arsenic + ENU condition is associated with extreme degenerative changes & complete rupture of the epithelial cells, as also cells in various stages of division. Some slight neoplastic changes also appear in the 4<sup>th</sup> month of Arsenic and ENU+Arsenic dosing.

**Kidney:** In comparison to the normal and ENU Kidney, 2 months Arsenic dosing shows tubular changes. Lymphocytic infiltration occur in the 3 month which proliferates in the 4<sup>th</sup> month. Necrotic changes observed in both As & Arsenic+ENU animals. The latter group shows heavy interstitial inflammation in the cortical glomerulus with slight neoplastic changes.

**Testes:** In comparison to the normal and ENU testis section, there is no identifiable change in the 2<sup>nd</sup> & 3<sup>rd</sup> month of Arsenic dosing while in the 4<sup>th</sup> month spermatogonial cells shows maturational arrest & atrophy of the germinal cells. In Arsenic +ENU condition seminiferous tubule atrophy associated with germinal cell disruption is noted.

**Spleen:** Disruption of the epithelia becomes noticeable from the 2<sup>nd</sup> month onwards in ENU+Arsenic and Arsenic group. In the 3<sup>rd</sup> month, these effects further intensify and multinucleated cells in various stages of division become a prominent feature in the fourth month in both groups. No changes in the ENU group were observed.

**Lungs:** In comparison to the normal lung, mild inflammation is seen in 2 month, beginning of bronchial inflammation is seen in 3 month followed by heavy alveolar bleeding in the 4 month of Arsenic dosing. Dense fibrotic changes are observed in the 4<sup>th</sup> month & bronchial and peribronchial inflammation & lobular changes in alveoli are prominent in ENU injected Arsenic dosed animals. No changes in the ENU group compared to normal were noted.

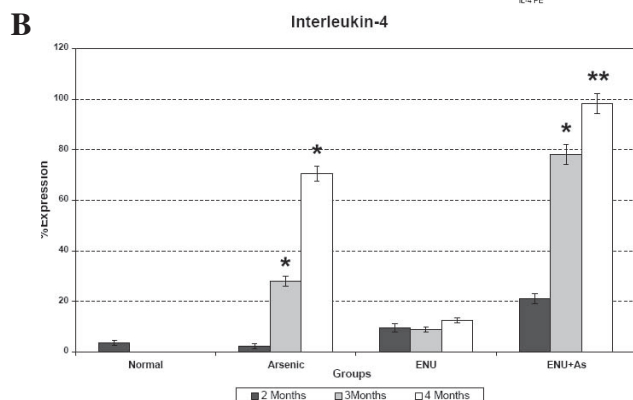
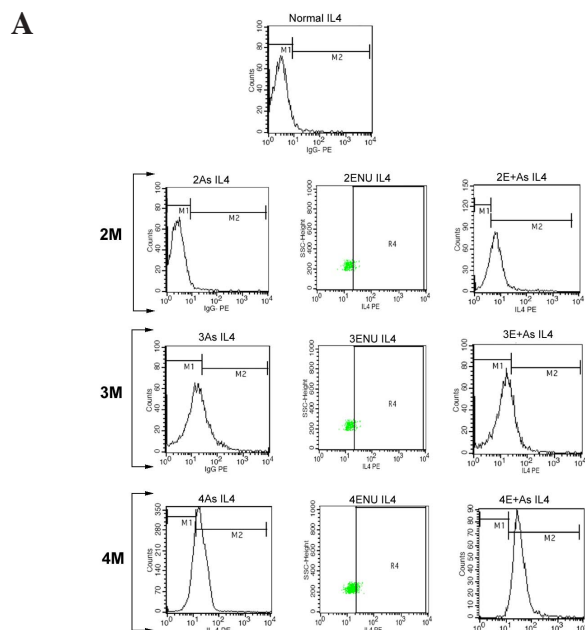
#### *Study of cytokine production*

As cytokines are the mediators of immune functions, the nature and extent of cytokine production is determined for execution of immune action in the body. This study helped us to find out the effect of Arsenic and ENU in controlling immune action by altering cytokine production.

#### *Th-2 cytokines*

**Interleukin 4** (Figure 1): In arsenic treated animals, IL-4 shows a higher value in 2<sup>nd</sup> month ( $2.87 \pm 1.21$ ), which increased remarkably ( $P < 0.0001$ ) with increased arsenic load in the 3<sup>rd</sup> month ( $27.75 \pm 2.62$ ). The observation continued to the 4<sup>th</sup> month ( $70.23 \pm 3.71$ ) showing that IL-4 production again increased sharply ( $P < 0.0001$ ). On the other hand, progressive increase in IL-4 production was noted in case of animals dosed with Arsenic as well as ENU, with highest values in the 4<sup>th</sup> month ( $98.39 \pm 4.86$ ). The IL-4 status showed a heightened activity in case of Arsenic+ENU group over and above the Arsenic group. The observations in the ENU group did not show any heightened response compared to other groups.

**Interleukin-10** (Figure 2): In the Arsenic group, progressive increase ( $P < 0.0001$ ) of Interleukin-10 (IL-10) production from the 2<sup>nd</sup> ( $2.92 \pm 1.18$ ) to the 3<sup>rd</sup> month ( $31.86 \pm 2.95$ ) and from the 3<sup>rd</sup> to the 4<sup>th</sup> month ( $49.37 \pm 2.85$ ) is noted. However, surprisingly, IL-10 levels show a sharp rise ( $P < 0.0001$ ) in 2<sup>nd</sup> month ( $91.3 \pm 3.65$ ) of the ENU+Arsenic group, followed by steady downfall in production in the 3<sup>rd</sup> ( $47.27 \pm 3.09$ ) through the 4<sup>th</sup> months ( $40.16 \pm 2.81$ ), such that levels were lowest in the 4<sup>th</sup> month.



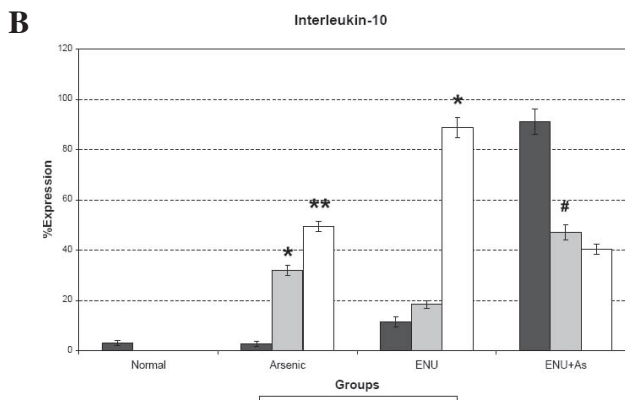
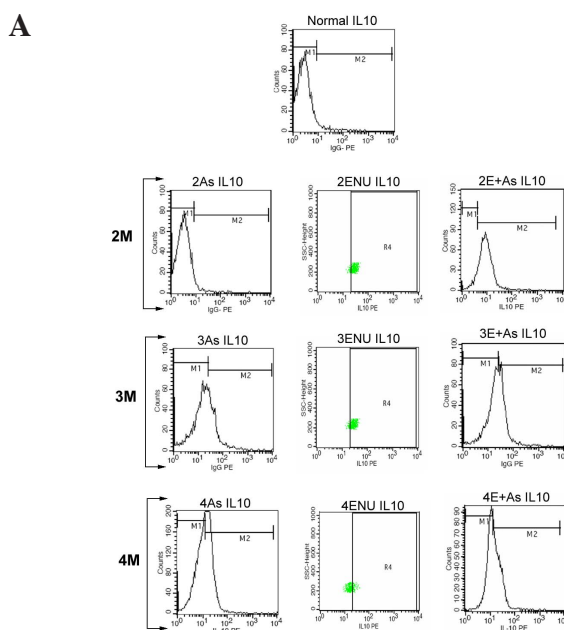
**Figure 1. Study of Intracellular Expression of IL-4.** A Represents Flow cytometric Analysis of IL-4 Percent Positive Cell in Histogram (2&4 Months) and in Scattergram (3<sup>rd</sup> Month) of a Single Representative Experiment. B Represents Graphical Form (Bar Diagram) of IL-4 Expression

In the ENU group, IL-10 levels indicated slight increases in the 2<sup>nd</sup> month ( $11.46 \pm 1.87$ ) and 3<sup>rd</sup> month ( $18.36 \pm 1.72$ ) but in the 4<sup>th</sup> month ( $88.66 \pm 3.17$ ), production suddenly shot up to a high level.

**Interleukin 6** (Figure 3): IL-6 levels in the case of Arsenic fed animals showed a significant ( $P < 0.05$ ) decrease in the 2<sup>nd</sup> month ( $2.27 \pm 1.16$ ) compared to normal ( $15.1 \pm 2.17$ ). However, production shot up to significantly ( $P < 0.001$ ) high levels in the 3<sup>rd</sup> month ( $70.5 \pm 3.25$ ) followed by still greater increase in the 4<sup>th</sup> month ( $96.4 \pm 4.39$ ). In the Arsenic+ENU group, levels are high to begin with in the 2<sup>nd</sup> month ( $89.8 \pm 4.27$ ). This followed drops in IL-6 production in the 3<sup>rd</sup> month ( $58.72 \pm 3.49$ ) and an insignificant ( $P > 0.05$ ) downfall in the 4<sup>th</sup> month ( $53.54 \pm 3.77$ ). During the period of the study, the animals in the ENU group maintained a steady level of IL-6 production significantly ( $P > 0.05$ ) below controls.

#### Th-1 cytokines

**Interleukin 12** (Figure 4): In case of ENU administered animals, IL-12 levels leaped up from the 2<sup>nd</sup> month ( $11.27 \pm 2.06$ ) to significantly ( $P < 0.0001$ ) high levels in the 3<sup>rd</sup> month ( $98.32 \pm 4.54$ ) and stayed high in the 4<sup>th</sup> month



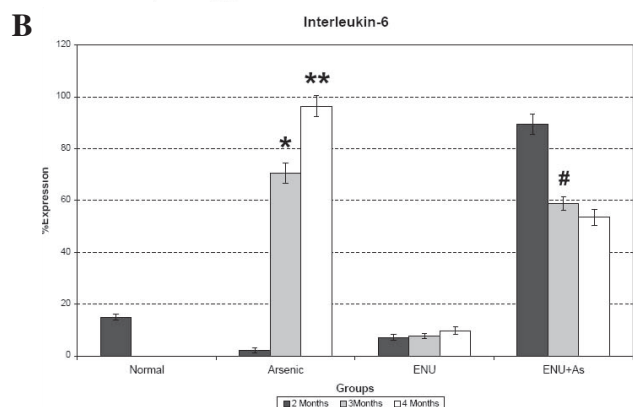
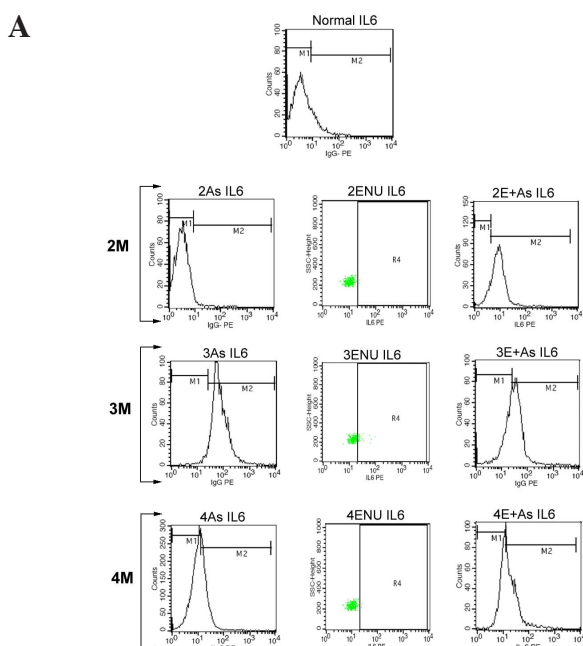
**Figure 2. Study of Intracellular Expression of IL-10.**

A Represents Flowcytometric Analysis of IL-10 Percent Positive Cell in Histogram (2&4 Months) and in Scattergram (3<sup>rd</sup> Month) of a Single Representative Experiment in which Expression Patterns of IL-10 of ENU, Arsenic and ENU+Arsenic Induced Animals were Studied Against Normal Healthy Controls. B Represents Graphical Form (Bar Diagram) of IL-10 Expression

( $97.31 \pm 4.86$ ). In the Arsenic group also, IL-12 levels rose significantly ( $P < 0.001$ ) in the 3<sup>rd</sup> ( $28.34 \pm 2.49$ ) and 4<sup>th</sup> months ( $46.26 \pm 2.91$ ) though the fold increase of IL-12 production was not as steep as in the case of the ENU group. In the animal group under the combined effect of ENU+Arsenic, levels of IL-12 were high in the 2<sup>nd</sup> month ( $79.58 \pm 3.83$ ) to begin with. An insignificant ( $P > 0.05$ ) decrease was observed in the 3<sup>rd</sup> month ( $77.76 \pm 4.34$ ) followed by a significant ( $P > 0.05$ ) decrease in the 4<sup>th</sup> month ( $72.96 \pm 4.49$ ).

**Interferon- $\gamma$**  (Figure 5): IFN- $\gamma$  levels shot up to higher values in the 3<sup>rd</sup> month ( $47.25 \pm 3.69$ ) in the Arsenic group after being in a near normal level ( $1.63 \pm 0.59$ ) in the 2<sup>nd</sup> month ( $2.78 \pm 1.05$ ). A further significant ( $P < 0.05$ ) increase was noted in the 4<sup>th</sup> month ( $58.63 \pm 3.52$ ). The Arsenic+ENU group showed an increase from the 2<sup>nd</sup> ( $39.6 \pm 3.3$ ) to the 3<sup>rd</sup> month ( $44.23 \pm 3.65$ ) followed by a significant ( $P < 0.0001$ ) increase in IFN- $\gamma$  production in the 4<sup>th</sup> month ( $85.17 \pm 4.23$ ). The ENU group did not show any significant ( $P > 0.05$ ) alterations in IFN- $\gamma$  production





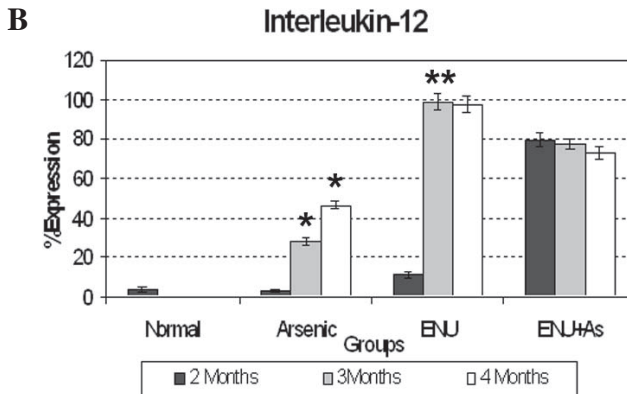
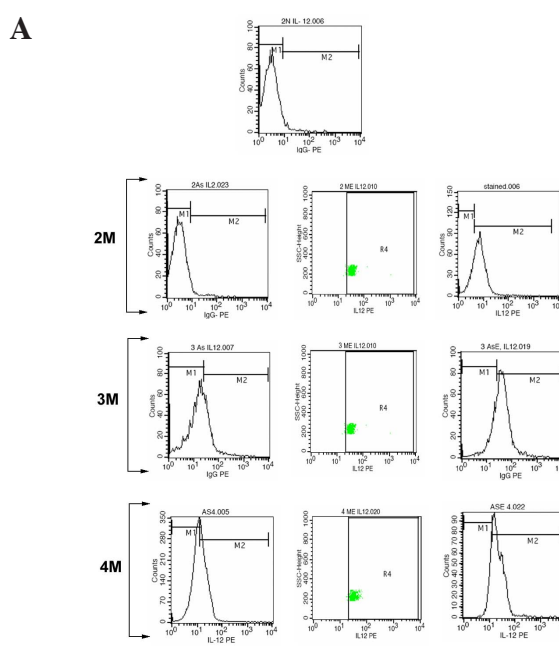
**Figure 3. Study of Intracellular Expression of IL-6.** A Represents Flowcytometric Analysis of IL-6 Percent Positive Cell in Histogram (2&4 Months) and in Scattergram (3<sup>rd</sup> Month) of a Single Representative Experiment. B Represents Graphical Form (Bar Diagram) of IL-6 Expression

in different months though the levels were significantly ( $P<0.05$ ) higher than normal.

**Tumor Necrosis Factor- $\alpha$**  (Figure 6): Progressive increase in TNF- $\alpha$  production from the 2<sup>nd</sup> through the 3<sup>rd</sup> and 4<sup>th</sup> month was a steady occurrence in all three groups under study compared to the normal. However, while such an increase was not significant in the ENU group, in the Arsenic group, levels rose in the 3<sup>rd</sup> month from a near normal level ( $7.56\pm1.84$ ) in the 2<sup>nd</sup> month followed by a steeper increase in the 4<sup>th</sup> month. But, the most profound changes were noted in the ENU+Arsenic group, where the progressive increases from 2<sup>nd</sup> ( $54.24\pm2.45$ ) to 4<sup>th</sup> ( $87.56\pm4.45$ ) month exceeded the production values in the other groups.

#### Analysis of the Extrinsic Apoptotic Pathway Estimation of Death proteins

**Fas-L** (Figure 7): Progressive increase in FasL production was noted in the Arsenic group from 2<sup>nd</sup> ( $5.78\pm1.21$ ) to the 4<sup>th</sup> ( $61.14\pm3.41$ ) month. In the ENU group, no significant alterations were observed between the different months, whereas the levels were significantly ( $P<0.0001$ ) higher compared to normal ( $31.53\pm3.02$ ).

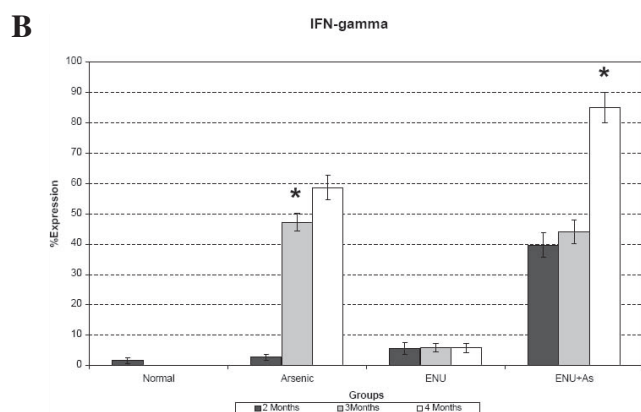
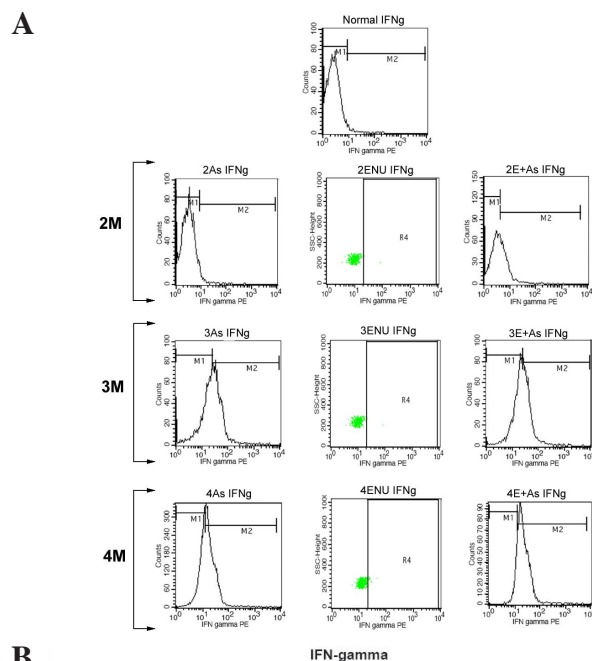


**Figure 4. Study of Intracellular Expression of IL-12.** A Represents Flowcytometric Analysis of IL-12 Percent Positive Cell in Histogram (2&4 Months) and in Scattergram (3<sup>rd</sup> Month) of a Single Representative Experiment. B Represents Graphical Form (Bar Diagram) of IL-12 Expression

In the ENU+Arsenic group the 2<sup>nd</sup> ( $54.58\pm3.84$ ) month showed high FasL values compared to normal with progressive further increase in the 3<sup>rd</sup> ( $79.56\pm4.54$ ) and 4<sup>th</sup> ( $95.45\pm3.26$ ) months.

**Caspase 8** (Figure 8): In the Arsenic group, Caspase 8 synthesis stayed close to normal ( $2.58\pm1.75$ ) in the 2<sup>nd</sup> month ( $3.52\pm1.6$ ). Synthesis began to increase in the 3<sup>rd</sup> month ( $29.35\pm2.38$ ) and highest values in this group were obtained in the 4<sup>th</sup> month ( $73.68\pm3.92$ ). The ENU group did not show any significant ( $P>0.05$ ) deviation between 2<sup>nd</sup> and 3<sup>rd</sup> month. In the ENU+Arsenic group, Caspase 8 levels were increased in the 2<sup>nd</sup> month ( $43.15\pm3.53$ ). The effect was further heightened in the 3<sup>rd</sup> month ( $71.38\pm4.25$ ) with highest values being shown in the 4<sup>th</sup> month ( $83.35\pm4.67$ ).

**Caspase 3** (Figure 9): A significant ( $P<0.0001$ ) increase was noted in Caspase 3 production in the Arsenic group in the 3<sup>rd</sup> month ( $29.57\pm2.89$ ) above that observed in the 2<sup>nd</sup> month ( $1.89\pm0.56$ ) which was nearly close to normal ( $3.05\pm1.01$ ). A slight increase occurred in the 4<sup>th</sup> month ( $31.1\pm2.91$ ) compared to the 3<sup>rd</sup> month. Levels of Caspase 3 in the ENU+arsenic group began to rise significantly ( $P<0.0001$ ) right from the 2<sup>nd</sup> month ( $36.8\pm3.09$ ). This



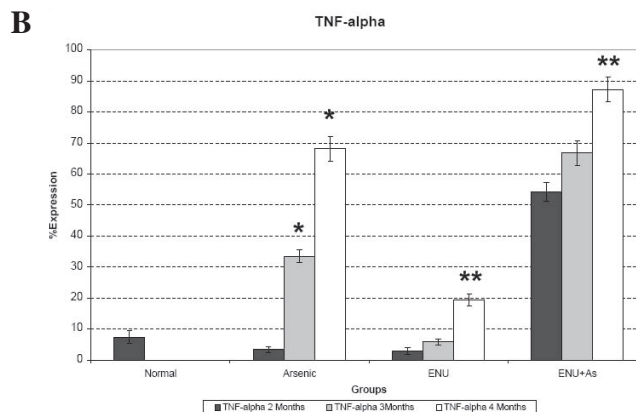
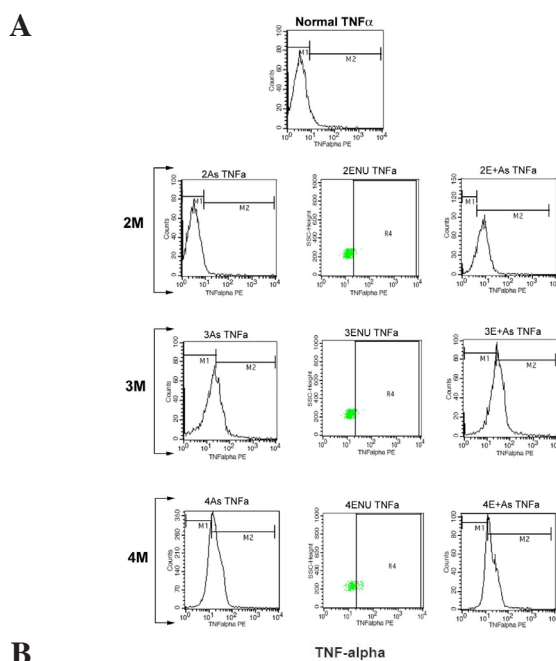
**Figure 5. Study of Intracellular Expression of IFN- $\gamma$ .** A Represents Flowcytometric Analysis of IFN- $\gamma$  Percent Positive Cell in Histogram (2&4 Months) and in Scattergram (3<sup>rd</sup> Month) of a Single Representative Experiment. B Represents Graphical Form (Bar Diagram) of IFN- $\gamma$  Expression

rose to very high levels in the 3<sup>rd</sup> month ( $82.72 \pm 3.86$ ) which remained high in the 4<sup>th</sup> month ( $84.1 \pm 4.13$ ). The value obtained in the 2<sup>nd</sup> month of the ENU group though showed slight increase from normal, the rise was not high enough to be of any significance ( $P > 0.05$ ). However, values in the ENU group for 3<sup>rd</sup> month ( $11.9 \pm 2.23$ ) and 4<sup>th</sup> month ( $13.2 \pm 2.05$ ) were significantly ( $P < 0.05$ ) higher than normal.

#### Estimation of the Intrinsic Apoptotic Pathway

**Bcl2** (Figure 10): Bcl2 levels which were initially high in the 2<sup>nd</sup> month ( $40.39 \pm 2.36$ ) of the Arsenic group fell sharply back to normal levels in the 3<sup>rd</sup> month ( $4.92 \pm 1.28$ ), which was maintained in the 4<sup>th</sup> month ( $5.5 \pm 1.6$ ). In the ENU group, higher values were obtained for the 2<sup>nd</sup> month ( $13.9 \pm 1.93$ ). But these came down to near normal levels in the 4<sup>th</sup> month. Greatly increased values of Bcl2 were obtained in the 2<sup>nd</sup> month ( $62.6 \pm 3.82$ ) of the ENU+Arsenic group, which fell only slightly in the 3<sup>rd</sup> month followed by a significant ( $P < 0.01$ ) drop in the 4<sup>th</sup> month ( $26.66 \pm 2.4$ ).

**Bax** (Figure 11): Though Bax production was significantly ( $P < 0.0001$ ) greater than normal ( $4.15 \pm 1.37$ ) in the 3<sup>rd</sup> month ( $34.23 \pm 2.31$ ) and slightly higher still in



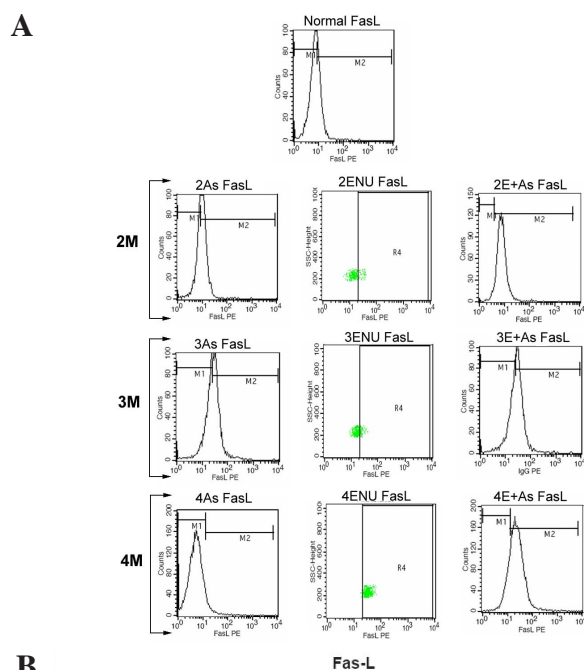
**Figure 6. Study of Intracellular Expression of TNF- $\alpha$ .** A Represents Flowcytometric Analysis of TNF- $\alpha$  Percent Positive Cell in Histogram (2&4 Months) and in Scattergram (3<sup>rd</sup> Month) of a Single Representative Experiment. B Represents Graphical Form (Bar Diagram) of TNF- $\alpha$  Expression

the 4<sup>th</sup> month ( $37.78 \pm 2.15$ ) of the Arsenic group. Bax results were progressively higher from 3<sup>rd</sup> ( $78.76 \pm 4.09$ ) to 4<sup>th</sup> month ( $97.48 \pm 5.79$ ) in the ENU+Arsenic group with highest values observed for the 4<sup>th</sup> month. The ENU group did not show significant alteration in different months although levels were significantly ( $P < 0.05$ ) higher compared to normal.

**Caspase 9** (Figure 12): In the Arsenic group Caspase 9 levels were significantly higher in 3<sup>rd</sup> ( $17.35 \pm 1.62$ ) and 4<sup>th</sup> month ( $30.75 \pm 2.33$ ) than normal ( $3.28 \pm 1.36$ ), highest values being for the 4<sup>th</sup> month. In 4<sup>th</sup> month ( $60.13 \pm 4.12$ ) of ENU group, Caspase-9 expression was significantly ( $P < 0.0001$ ) increased compared to other groups. The ENU+Arsenic group showed a rise in the 2<sup>nd</sup> month ( $31.49 \pm 2.31$ ) but the rises in the 3<sup>rd</sup> ( $92.26 \pm 5.37$ ) and 4<sup>th</sup> months ( $94.3 \pm 4.9$ ) are greatly increased.

## Discussion

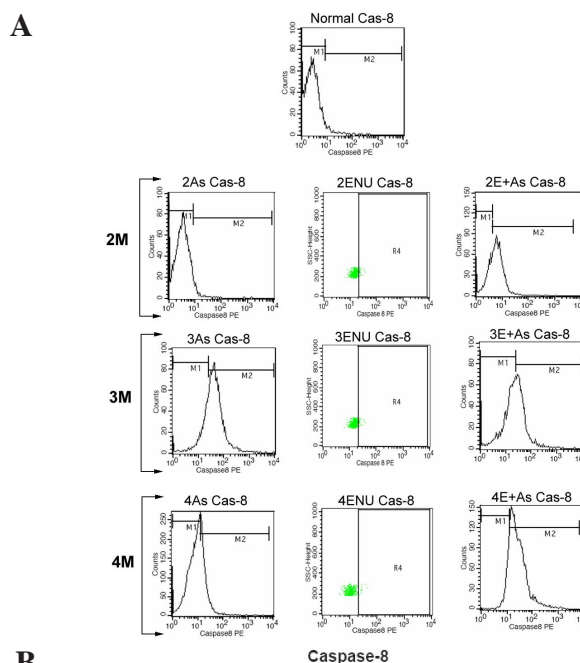
It is well established that ENU causes tumors in the central nervous system (Lantos, 1993; Druckray et al., 1966; Koestner et al., 1977; Lantos et al., 1972). In the present study, absence of any definitive changes



**Figure 7. Study of Expression of the Intrinsic Apoptotic Protein Fas-L.** A Represents Flowcytometric Analysis of Fas-L Percent Positive Cell in Histogram (2&4 Months) and in Scattergram (3<sup>rd</sup> Month) of a Single Representative Experiment. B Represents Graphical Form (Bar Diagram) of Fas-L Expression

in the brain in Arsenic group indicated that Arsenic is non-carcinogenic to the brain tissue, although it acts as a carcinogen or a co-carcinogen elsewhere in the body. However, appearance of proliferative oligodendroglia as well as new angiogenic vessels in the 4<sup>th</sup> month hints at tumor development (Folkman, 1995). This may infact be the result of ENU causing carcinogenic changes in the CNS as expected rather than due to the co-carcinogenic effect of Arsenic. This holds good especially as changes in the Arsenic group did not indicate carcinogenesis.

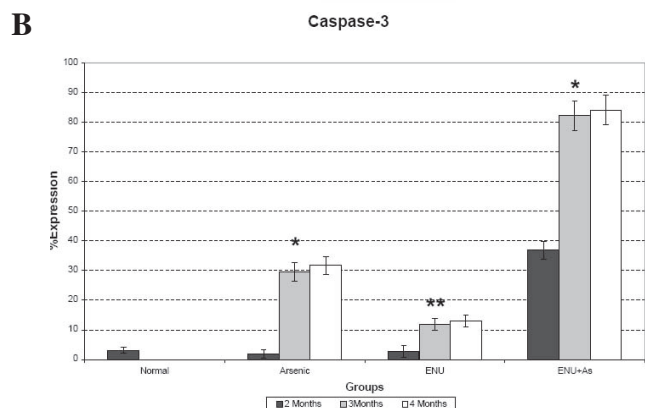
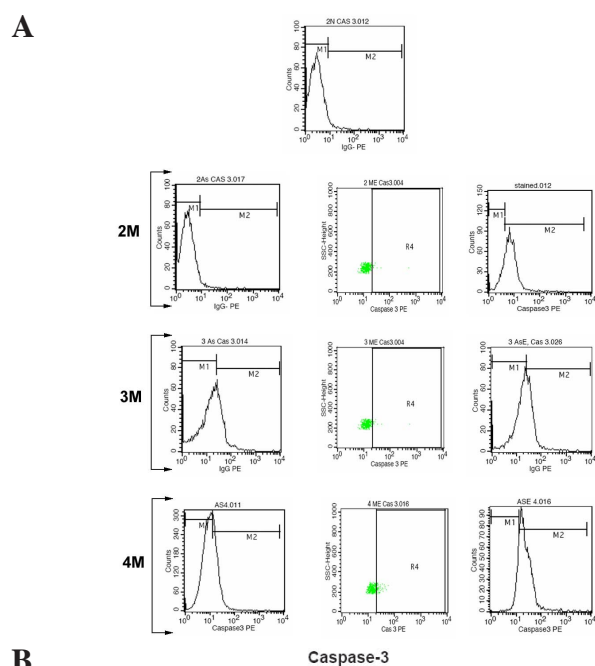
In the liver, the carcinogenic agent, ENU, aided in its task by the promotional effect of Arsenic; carcinogenic development seems to corroborate our hypothesis that Arsenic was in fact a Promoter of Carcinogenesis. Progressive pictures of dividing cells in the 3<sup>rd</sup> and 4<sup>th</sup> month of this group further highlights the carcinogenic changes with multiplicity of nuclei in several areas of the tissue section becoming a prominent feature of the 4<sup>th</sup> month. The slight neoplasticity observed in the 4<sup>th</sup> month of Arsenic dosing probably points at a weaker carcinogenic potential of Arsenic, which may develop with continued exposure over longer time periods.



**Figure 8. Study of Intracellular Expression of Caspase 8.** A Represents Flowcytometric Analysis of Caspase 8 Percent Positive Cell in Histogram (2&4 Months) and in Scattergram (3<sup>rd</sup> Month) of a Single Representative Experiment. B Represents Graphical Form (Bar Diagram) of Caspase 8 Expression

Mild carcinogenic changes start setting in in the 4<sup>th</sup> month of ENU+Arsenic in the G.I. tract with visible multinucleation but here the observed tissue damage is mostly due to necrosis related degenerative changes and epithelial cell rupture. The mild carcinogenic changes as observed in Arsenic dosed animals in the 4<sup>th</sup> month as above hints at the latent carcinogenicity of Arsenic due to prolonged exposure.

In the kidney tubular changes and lymphocytic infiltration as evidenced in the 2<sup>nd</sup> month of Arsenic dosing points to the fact that the body is trying to launch an immune attack against metalloids generated toxicity. The changes are mostly of a degenerative nature, resorting to mass cell killing by necrosis, effects of which are further amplified in the 3<sup>rd</sup> & 4<sup>th</sup> month. Necrotic changes were observed ENU+Arsenic animals as well. The heavy interstitial inflammation in the cortical glomerulus is a probable sign of the defensive action of the immune cells towards the disruptive effects of the damaging chemicals. Mild neoplastic changes with cells in various stages of division again elucidate the carcinogenic effect induced jointly by the two chemicals working in conjunction.

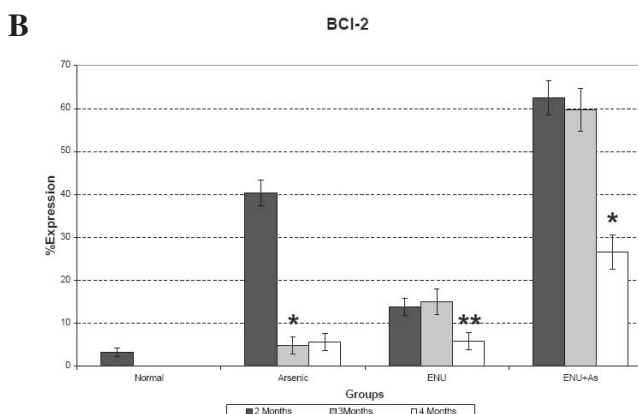
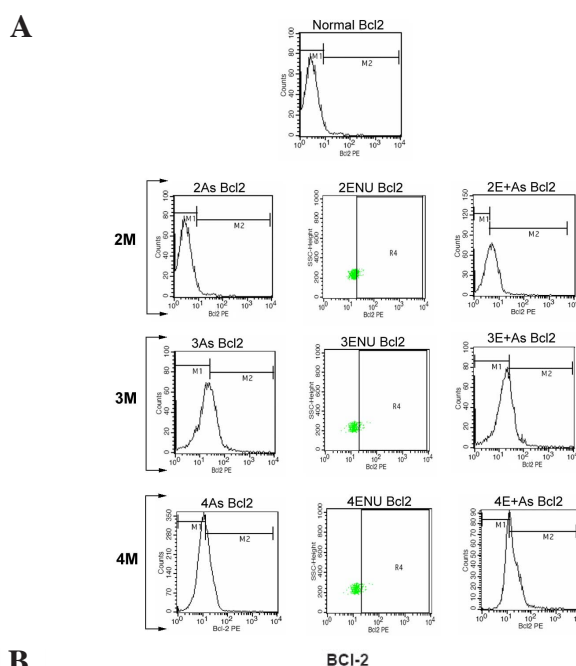


**Figure 9. Study of Intracellular Expression of Caspase 3.** A Represents Flowcytometric Analysis of Caspase 3 Percent Positive Cell in Histogram (2&4 Months) and in Scattergram (3<sup>rd</sup> Month) of a Single Representative Experiment. B Represents Graphical Form (Bar Diagram) of Caspase 3 Expression

Dose dependent growth arrest and atrophy in testes points towards the fact that at lower exposure to Arsenic, the testes remains comparatively unaffected. It is only with a higher degree of exposure that these changes become apparent in the testes. In Arsenic +ENU condition seminiferous tubule atrophy is associated with germinal cell disruption, though carcinogenic changes are not seen to occur in case of this organ.

Progressive epithelial degeneration from 2<sup>nd</sup> to 3<sup>rd</sup> to 4<sup>th</sup> month of both Arsenic and ENU+Arsenic group in the spleen point towards the damaging effects of these chemical systems. Carcinogenic changes, as evidenced by the appearance of multinucleation in both groups shows that perhaps the lymphatic organs are more susceptible than other organs in the body to the threat posed by Arsenic.

The inflammatory changes seen in lungs due to Arsenic induction point to an all-out defense mechanism launched by the immune system to counter the Arsenic attack. Fibrotic changes are indicative of lung damage wherein alveolar tissue is replaced by fibrous tissue. The heavy alveolar bleeding may result due to widespread



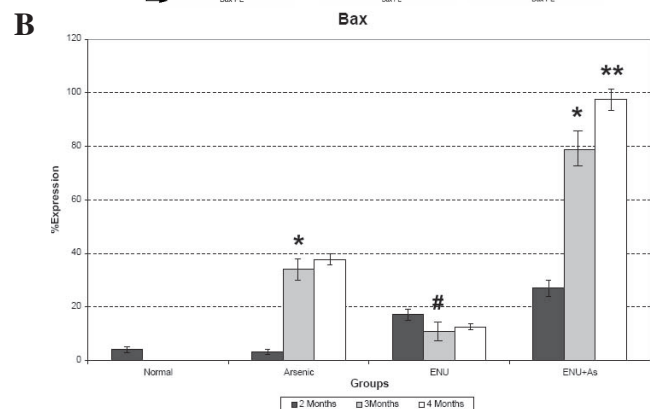
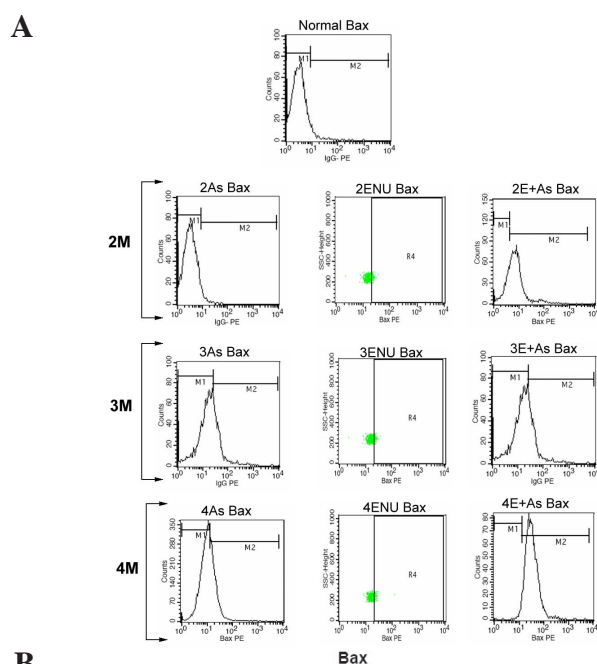
**Figure 10. Study of Intracellular Expression of Bcl-2.** A Represents Flowcytometric Analysis of Bcl-2 Percent Positive Cell in Histogram (2&4 Months) and in Scattergram (3<sup>rd</sup> Month) of a Single Representative Experiment. B Represents Graphical Form (Bar Diagram) of Bcl-2 Expression

lung capillary disruption due to the Arsenic load as well as the more pronounced load of Arsenic and ENU.

So, the liver was the most affected organ with milder neoplastic changes occurring in the G.I. tract and kidney. This probably hints that Arsenic devastates metabolism since the liver is the seat of metabolism in the body. The histological changes further elucidate the fact that Arsenic on prolonged exposure may exhibit carcinogenicity in certain target organs such as liver and G. I. Tract, though this effect is not seen in all organs under study. Thus the histological studies appear to indicate that the carcinogenic potential of Arsenic varies in a dose dependent fashion in certain target organs only, suggesting thereby that though Arsenic may have some cancer inducing properties on prolonged exposure, the relative importance of Arsenic as a Promoter, rather than an inducer of carcinogenesis is of greater significance.

The mixture of cytokines that is produced in the tumor microenvironment has an important role in cancer pathogenesis. Cytokines that are released in response to infection, inflammation and immunity can function to inhibit tumor development and progression. Alternatively, cancer cells can respond to host-derived cytokines



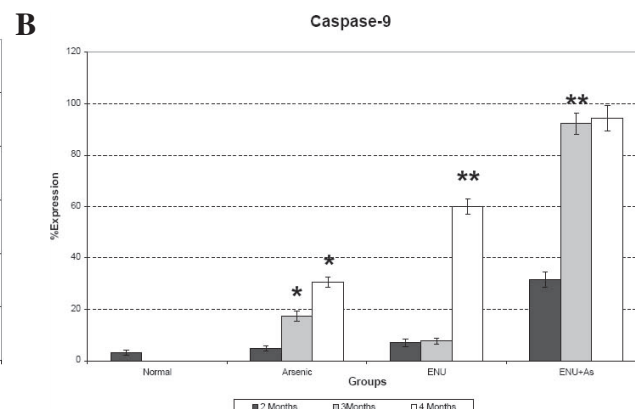
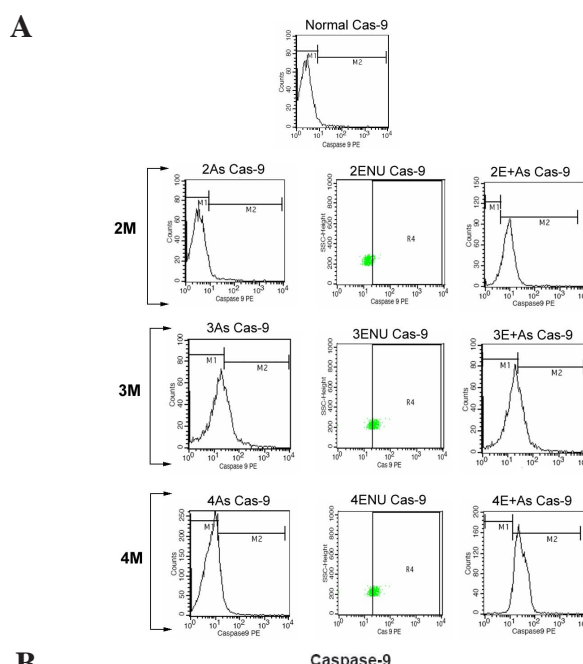


**Figure 11. Study of Intracellular Expression of Bax.**

A Represents Flowcytometric Analysis of Bax Percent Positive Cell in Histogram (2&4 Months) and in Scattergram (3<sup>rd</sup> Month) of a Single Representative Experiment. B Represents Graphical Form (Bar Diagram) of Bax Expression

that promote growth, attenuate apoptosis and facilitate invasion and metastasis (Dranoff, 2004).

The pro-inflammatory Th-1 cytokines when assayed showed a marked attempt by the body to combat cancer development. Cancer progression and/or development produces an inflammatory condition which corroborates the increased inflammatory cytokine production. Accordingly, IL-12 expression (Figure 4) in both ENU and ENU+Arsenic groups were high with progression of exposure time, indicating effective Antigen presentation and a potentiated immune system in general as this job is performed by this cytokine in conjunction with IL-15 (Cavallo et al., 1997; Boggio et al., 1998; Coussens and Werb, 2002). IFN- $\gamma$  levels also corroborated this finding with highest expression in the ENU+Arsenic group followed by the Arsenic group. Increased IFN- $\gamma$  expression (Figure 5) indicates increased MHC-II upregulation and higher tumor antigen presentation. IFN- $\gamma$  also upregulates ICAM-I & other integrin family members, helping in rolling of lymphocytes on the endothelial surface. High TNF- $\alpha$  levels (Figure 6) were expressed in the ENU+Arsenic group (Bach et al., 1997; Kaplan et al., 1998; Street et al., 2001; Ashkenazi, 2002).



**Figure 12. Study of Intracellular Expression of Caspase 9.**

A Represents Flowcytometric Analysis of Caspase 9 Percent Positive Cell in Histogram (2&4 Months) and in Scattergram (3<sup>rd</sup> Month) of a Single Representative Experiment. B Represents Graphical Form (Bar Diagram) of Caspase 9 Expression

Thus the proinflammatory cytokine expression increased maximally in arsenic induced and arsenic animals receiving ENU. Simultaneously high TNF $\alpha$  expression could be also correlated with high apoptosis as was evident by the increase in the apoptotic regulators in the affected animals (Liotta and Kohn, 2001; Coussens and Werb, 2002; Dunn et al., 2002). All these changes in the Th-1 cytokines studied can be correlated with inflammatory responses in the face of a carcinogenic attack (Penn, 1994; Coussens and Werb, 2002).

Assay of the Th-2 cytokines proved that a general state of immunosuppression prevailed in the ENU+Arsenic group over and above that recorded in any of the other groups. As has been documented time and again, this immunosuppression effected by the Th-2 cytokines aids tumor growth (Penn, 1994; Opall and DePaolo, 2000; Hanahan and Weinberg, 2002; Doseff et al., 2003) and such effects are most pronounced in the ENU+Arsenic group. With advancement of exposure time (Months), greater levels of IL-4 (Figure 1) were noted in the ENU+Arsenic group over and above the high levels in the Arsenic group, with highest levels found in the 4<sup>th</sup> month

of ENU+Arsenic induction. This points towards immune suppression in the tumor milieu, which would aid further tumor growth and disease progression (Gascan et al., 1991; Qin et al., 1997). The role of increased IL-10 production (Figure 2) in Arsenic and ENU Groups is confirmative with their immunosuppressive role which is conducive to cancer growth. Due to the antagonistic role of IL-4 and IL-10, increased level of IL-4 has induced an inverse relation with ENU+Arsenic Group from a very high IL-10 level in the 2<sup>nd</sup> month to a significantly decreased level in the 4<sup>th</sup> month. This explains the depressed levels of IL-10 in the 4<sup>th</sup> month of the combined ENU+Arsenic regimen though higher levels have been noted in the 2<sup>nd</sup> month. IL-10 inhibits the synthesis of a number of cytokines such as IFN- $\gamma$ , IL-2 and TNF- $\beta$  in Th1 T-helper subpopulations of T-cells (Opall and DePaolo, 2000; Paul, 1989). Thus these findings also corroborate the depressed immune status that prevails in the face of a carcinogenic attack. IL-6 (Figure 3) is another cytokine with pleiotropic functions (Hilbert et al., 1995). It acts as an acute phase cytokine, found in higher amount when liver and other organs are in stress and also helps in leukocyte migration, effector molecule production etc. (Hilbert et al., 1995; Opall and DePaolo, 2000; Guosheng et al., 2003). Though normal animals show gradual decrease of IL-6 production with progressing age, the animals which were fed arsenic water showed an initial increase of its production in 3<sup>rd</sup> month as compared to 2<sup>nd</sup> month after which in the 4<sup>th</sup> month the production further increases. At the same time, arsenic fed and ENU induced animals showed a very steep rise in IL-6 production from 2<sup>nd</sup> to 3<sup>rd</sup> to 4<sup>th</sup> month in keeping with the devastated conditions in the liver, aiding effective leukocyte migration and effector molecule production as discussed. These observations open up the opportunity of allowing lymphocytes to enter in the site of inflammation in the affected groups (Guosheng et al., 2003). The relative fall in IL-6 values below normal in the ENU group hint that this group is not posed with the serious threat of carcinogenesis. It may be said, therefore that the inflammatory responses as indicated by the activity of the pro-inflammatory Th-1 cytokines as well as the high levels of the anti-inflammatory Th-2 cytokines as observed in our study provide the ideal setting for cancer growth and progression.

These effects are in direct contrast with the a study where a group of workers tested the chemotherapeutic effect of Arsenic Trioxide on Cytokine modulation and found that IL-6 production was significantly increased, however, TNF- $\alpha$  did not show significant variations in Acute Promyelocytic Leukemia cells. Here high IL-6 levels corroborate effective defense against cancer (Saini and Walker, 1998). However, while that study highlighted the chemotherapeutic efficacy of Arsenic trioxide, our's highlights instead the carcinogenic action of Arsenic as a promoter/ inducer of carcinogenesis.

Another study emphasizes that though PHA (phytohemagglutinin) stimulation increased IL-2 expression in arsenic induced Bowen's disease (squamous cell carcinoma), IL-2R  $\alpha$  and  $\beta$  expression as well as sIL-2R expression was not proportionately increased; leading to the conclusion that defective cell mediated immune

function in Bowen's disease results due to impairment of membrane IL-2R expression in lymphocytes after stimulation. (Yu et al., 1998). This study bears a direct testimony to our study where in the absence of proper stimulation, lymphocytes are driven towards apoptotic death in the cases that we examined.

The study of lymphocytic death via both the intrinsic and extrinsic apoptotic pathway provided us with further interesting leads. It is known that Bcl-2 is unique among the proto-oncogenes, as it is localized on the mitochondrial membrane and interferes with programmed cell death independently of its ability to promote cell division. Bcl-2, a prosurvival protein, belongs to a still growing family, the members of which are able to form homo- or heterodimers with one another and also contains members with antagonizing functions for each other such as Bax (Antonsson et al., 2003; Borner, 2003). The Bcl-2-Bax ratio determines whether the internal milieu of the cell proceeds for survival or death (Antonsson et al., 2003). Once the Bax protein is activated, the cell proceeds for death and Cytochrome c is liberated from mitochondria (Martinou and Youle, 2006). Cytochrome c along with Apoptotic activating factor (Apaf) forms the apoptosomal complex (Finucane et al., 1999) where procaspase-9 is recruited and processed to form the active caspase-9 which proceeds to cleave procaspase-3 to active caspase-3 (Porter and Jänicke, 1999; Creagh and Martin, 2001; Acehan et al., 2002; Sohn et al., 2004). Active caspase-3 ultimately results in DNA degradation by cleaving the Inhibitor of Caspase Activated DNase complex from Caspase Activated DNase thereby liberating the latter thereby resulting in DNA fragmentation (Schmitz et al., 2006). In our experiments, the intrinsic apoptotic regulators that were studied were the pro-survival and pro-apoptotic members of the Bcl-2 family: the Bcl-2 and Bax, the Fas Ligand (Fas-L) and also the cysteine proteases. Comparing the Bax-Bcl-2 expression in arsenic induced animals, it could be clearly observed that Bcl-2 expression (Figure 10) fell sharply in the 4<sup>th</sup> month of ENU+Arsenic induction, as well as in the Arsenic group though to a lesser extent than the former from initial corresponding high levels in both groups, whereas Bax expression (Figure 11) shot up to high levels in the ENU+Arsenic group, thereby strongly pushing lymphocytes to commit suicide. In the ENU treated animals a very slight Bcl-2 activity was noted in the 4<sup>th</sup> month, whereas Bax levels in the ENU group indicated a very small fraction of killing. While comparing cysteine proteases, the caspase-8 (Figure 8), caspase-9 (Figure 12) and caspase-3 (Figure 9) data of the normal with the arsenic induced animals it was observed that the caspase activity was much higher in the arsenic and ENU induced animals. FasL levels (Figure 7) also corroborated the fact that lymphocytes were being induced to enter into the apoptotic pathway (Debatin and Krammer, 2004; Siegmund et al., 2005). The involvement of FasL and caspases 8 and 3 (Kuwana et al., 1998) as observed in our experiments showed that lymphocytic killing via the Extrinsic apoptotic pathway was occurring. The executioner caspase 3 showed highest levels in the ENU+Arsenic group. Lower activity in the Arsenic group showed that it is operative to a much lower extent. On the

other hand, Bcl-2, Bax and caspase 9 involvement showed that the Intrinsic apoptotic pathway was also operative in the killing of lymphocytes in the present study. (Sohn et al., 2004; Martinou and Youle, 2006). To sum up, the results showed that in case of the ENU+Arsenic group, lymphocytic killing occurs through both the intrinsic and extrinsic apoptotic pathways, however in the ENU group, which shows heightened caspase 9 activity only, the lymphocytic killing is operative through the intrinsic pathway only. Lymphocytic killing in the Arsenic group follows both pathways as well, but here the effect is moderate as compared to the ENU+Arsenic group. This has been indicated in the results where the expression of apoptotic proteins follows a similar pattern in both groups; however such expression in the Arsenic group is much lesser comparatively.

Lymphocytic death as well as Th-2 cytokine profile in the ENU+Arsenic group presents the picture of a severely depressed immune system, which is uniquely favorable for cancer development. The picture of the Th-1 cytokines provides us with an immune system at war with the carcinogenic invasion. This widespread immune suppression and the marked inflammatory responses were most marked in the ENU+Arsenic group over and above the Arsenic group. These results were seconded by the histological studies where rapidly dividing cells and multinucleation were prominently noticed in the ENU+Arsenic group. Thus, carcinogenesis was highly indicated upon co-induction of Arsenic and ENU.

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