CHAPTER – II
MATERIALS & METHODS
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Throughout the whole course of investigation different Reagents as raw material were used:

1. Percoll
2. PBS (Gibco)
3. Biological Response Modifiers (BRMs):
   (i) Interleukin-2 (IL-2, Mouse recombinant, SIGMA).
   (ii) Interferon-gamma (IFN-γ, Mouse recombinant, Dako).
   (iii) Non-specific corpuscular antigen, sheep erythrocytes (SRBC).
5. Phosphate Buffer Saline (PBS, pH 7.2).
6. Normal Saline (0.9% NaCl).
7. Heparine (Sigma, USA, Tissue Culture).
8. Dextran 8% (Sigma, USA).
11. Pyridine
12. Fluorochrome (Hoechst 33342), Sigma USA.
13. Signal Transduction kit (Boehringer, Mannheim, Germany).
14. N-N: Ethyl nitrosourea (ENU), Sigma, USA.

Instruments:
1. Inverted microscope.
2. Laminar Flow (Horizontal).
The investigations were carried out in the experimental model with induced leukaemia and following administration of specific and non-specific biological response modifiers (BRMS).

ANIMALS: ANIMAL SPECIES AND SUITABILITY:

Although leukaemia induction studies have been made in rats in general other attempts have also been made to induce haematological malignancies in animals like mice, guinea pigs and Chinese hamster. But considering the easy maintenance and genetic similarity with human mice have been considered as the best suitable one for use as experimental model. Inbred swiss albino mice of both sexes formed the animals models for inducing experimental leukaemia. Animals were treated with carcinogen within 10 days after brith. These were maintained with standard balanced diet and clean water ad libitum.

Introduction of Leukaemia in Young Mice: Young mice aged 7-10 days N-N' ethylnitrosourea (ENU) was injected (I.P.) at a dose rate of 50 mg/kg body weight and they were observed for 6-8 months time. Leukaemia induction was investigated through peripheral blood smear study (tail vain puncture). The smear was stained with standard Leishman stain.

Application of Biological Response Modifier: Interleukin-2 (IL-2, Mouse recombinant, SIGMA), interferon-gamma (IFN-γ, Mouse, Recombinant, Dako) and a non-specific corpuscular antigen sheep crythrocytes (SRBC) have been administered either separately on in combination. These were administered in normal control group of mice.
and also in mice 4 months following application of ENU. The dose schedule was as follows: IL-2 5 µg/kg, IFN-γ 5 µg/kg B.W. and SRBC .25 ml of 7% (PCV/saline volume). These were also administered in combinations of two and three alternatively.

**Survival Study:** This was done by calculating the number of day the animal survived after ENU/Biomodulators applications.

**Groups Maintained:** The following groups were, therefore, maintained for the purpose of investigations:

1. ENU control (E): served as leukaemic control mice.
2. Animals treated with ENU receiving, IL-2, six months following ENU injection (E2).
3. Animals treated with ENU, receiving interferon-gamma six months following ENU administration (Eγ).
4. Animals treated with ENU, receiving SRBC six months after ENU administration (ES).
5. Animals receiving IFN-γ, IL-2 in combination six months following ENU administration (Eγ 2).
6. Animals receiving IFN-γ and SRBC, six months following injection of ENU (EγS).
7. Animals receiving IL-2 and SRBC six months following application of ENU (E2S).

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8. Animals receiving IL-2, IFN-γ and SRBC six months following application of ENU(E 2γ s).

For each group a corresponding normal control has been maintained as above.

**Spleen Cell Preparation:** After sacrifice of the animals, spleen portions were collected aseptically from them into normal saline. After collection, they were placed on the wiremesh and (180 Mess) and massaratted by a piston of a syringe. Then the total mass was washed by normal saline and RPMI 1640 media for twice or thrice into a petridish. Single cell suspensions were prepared by mixing thoroughly by a pasteur pipette.

**Isolation of Spleen Derived Cells:** From the single cell suspension of spleen cells, mature lymphocytes, macrophages and neutrophils were isolated through density gradient methods described as bellow:

**Isolation of Macrophages:** After preparing the single cell suspensions of spleen in the petridish, cells were kept into incubator for 30 minutes for adherence of macrophages. The non-adherent cells were collected into centrifuge tube and left for the isolations of lymphocytes and neutrophils. The adherent cells were washed thrice by PBS-EDTA and finally washed by PBS. Then the total sample was collected into centrifuge tube and spin for 10 min at 700 rpm. Supernatant was discarted and pellet washed by PBS and then suspended into RPMI 1640 media and cells were counted for immune parameters.

**Isolation of Macrophages (mϕ) from Mouse Abdomen:** For the PLN assay, adequate number of macrophages are required. To serve the purpose peripheral macrophages are isolated from the mouse abdomen. In this
case 1% warm starch solution is injected into mouse peritoneum 5 days before the date of isolation. On the day of isolation, animal is sacrificed and abdomen is opened through a small hole and macrophages are collected by wash off with warm (40°C) saline. By using the smooth edged pasteur pipette tip, macrophages are aspirated carefully to avoid blood contamination. 

**Isolation of Lymphocytes & Neutrophils:** The nonadherent cell suspensions were washed by normal saline and the pellet was suspended into non FBS-RPMI 1640 media. Then the whole solution was placed on percoll for the isolation of lymphocytes and neutrophil through percoll density gradient at the interface of 1.077 and 1.089 density. Then the cells were collected into separate centrifuge tube for different cell layers. Cells were washed by PBS twice a time and then suspended into non FBS-RPMI media for immune parameter.

In case of animals treated with the biomodulators like IL-2, IFN-γ and SRBC, spleen was isolated on seventh day after the treatment and in case of combination treatment spleen was collected on the 10th day.

**Studies on Cell Mediated Immune Parameter:** The functional assay was carried out with the cells obtained in each mentioned group. Apart from the absolute cell count from the individual yield the immunological efficacy of these cells were carried out through,

1. Spontaneous E-rosetting (Hudson and Hay, 1993)

2. Cytotoxicity assay through Fluorochrome (Hoechest 33342) release phenomenon (Chaudhuri et al, 1998)
3. PMN mediated phagocytic burst by NBT reduction assay (Chaudhuri et al, 1991)

4. Leucocyte adherence inhibition test by macrophages (Hudson and Hay, 1997). The above methods were carried out with slight modifications to the original workers as mentioned.

5. PLN assay and antigen presentation (Chaudhuri et al, 1993)