The problems encountered in bone marrow transplantation are attempted to be solved through re-infusion of autologous bone marrow stem cells or progenitor cells or those derived from the cord or peripheral blood (1-3). In many areas, success was partial and sometimes of greater benefit. In recent trends the subject of cytotherapy is largely being taken over for many cases requiring therapeutic manoeuvre with stem or progenitor cells (4). Application of cytokines and growth factors have been found to elicit multiple responses on the pluripotent cells in terms of mobilization and release of the cells from the bone marrow compartments to the peripheral end (5-7); the combination with cytokines like interferon gamma (IFN-γ) was reported to be selective for stem cell mobilization in leukemic patients (8-9). Several other reports using a combination of growth factors, cytokines and chemotherapeutics have been documented to show that stem cell mobilization from the bone marrow to the peripheral blood may be manipulated. Here we emphasized on the cellular distribution within the bone marrow compartment with special reference to migrational efficacy densitywise under the conditions and to assess the functional potentiality of such cells with reference to immune functions with immunomodulatory capacity of SRBC in particular. Sheep Red Blood Cells (SRBC) were found to possess an immunopotentiating role towards a number of immunocytes including lymphocytes of different subsets, polymorphonuclear neutrophils (PMN), macrophages and even the dendritic cells (DC) (10-13). This non-specific Biological Response Modifier (BRM) has been found to act through its ligand system towards a suitable receptor resembling CD-2 molecule (T11) (14-15). The present study is aimed at delineating the cellular status within the bone marrow cavity with respect to density specific compartmentalization vis-a-vis the functional efficacy of such compartmental cells with experimentally induced leukemia and that following application of SRBC. Three salient perspec-
tives have been focused in the present study: (1) Relative immunocompetence of the bone marrow cells against target (2) Kinetics and functional maturation in terms of compartmental migration and (3) Role of exogenous BRM like SRBC on the above system.

Materials and Methods

Animals. Inbred Swiss albino mice of both sexes (maintained in our own laboratory, 30 year old inbred colony) formed the animal model for inducing experimental leukaemia. Animals were treated with carcinogen within 7-10 days after birth. These were maintained with standard diet and clean water ad libitum.

Induction of leukemia in young mice. To young mice age 7-10 days, N-N'-Ethylnitrosourea (ENU) (16-17) was injected (i.p.) at a dose of 80mg/kg. body weight and they were observed for 6-8 months time. Leukemia induction was investigated through peripheral blood and bone marrow smear study using standard Leishman stain.

Application of Biological Response Modifiers (BRMs). Sheep blood (SRBC) was freshly collected aseptically and maintained in Alsevar's reagent (shelf life 3-4 weeks). Use of SRBC for the purpose of immunopotentiation and anti-tumour activity has been described previously (10-13). In the present study 0.25ml of 7% (PCV/saline volume) SRBC was inoculated (i.p.) to each animal constituting the following groups:

- Gr. I - Normal mice (sham control) (0.25ml Saline injected i.p.) [N]
- Gr. II - Normal mice injected with 0.25ml of 7% SRBC, i.p. [NS]
- Gr. III - Animals injected (i.p.) with N-N' Ethylnitrosourea (ENU) 80mg/kg. body weight as leukemic control [E]
- Gr. IV - ENU induced leukemic mice injected (i.p.) with 0.25ml of 7% SRBC [ES], six months following application of ENU. Animals of the concerned groups were subjected for investigation seven days after SRBC challenge.

Survival study of the above groups of animals was carried out together with the manifestations including acute toxicity, if any, secondary infections, etc.

Preparation of bone marrow cells. 180 days after ENU treatment animals were sacrificed to isolate the long bones (femurs, tibia and fibula). On the 7th day after treatment of SRBC, mice from the respective groups of animals were sacrificed for bone marrow preparation. This was done by flushing RPMI 1640 media with the help of a syringe and 26-gauge needle and the cells were mixed well with repeated pipetting and finally harvested in RPMI 1640 media.

Enrichment of bone marrow derived pluripotent cells. The cells from different groups of animals (N, NS, E, ES) were separated from the whole bone marrow population through percoll density gradient at the interface of 1.050 and 1.077 density (18). Cells thus prepared were washed in PBS and finally transferred to RPMI media and the cell distribution at the low density (LDC) and the high density region (HDC) were noted by counting the cells for individual groups of animals. This provided an index of cell migration at the different compartments as determined density-wise.

Flowcytometric (FACS) analysis of CD34+ cells. Following fixation in 1% paraformaldehyde HDC and LDC of all the groups of animals were incubated with FITC conjugated rat anti-mouse CD34 monoclonal antibody (1µg/10⁶ cells, Pharmingen, U.S.A.) for half an hour in dark. This was followed by final washing of the cells with PBS in about 1ml. dilution for analysis in FACS Calibur (Becton Dickinson, USA) using Cell Quest software and a total of 10,000 events were acquired for analysis.

Studies on cell mediated immune parameters. The functional efficacy of the bone marrow derived cells isolated as above has been carried out in each group as follows:

1. Absolute cell count in terms of cells per 5mg. (wet weight) of the tissue (Flushed out BM cells were soaked in blotting paper and weight taken in tare paper as per standard technique).

2. Spontaneous E-rosetting as per the method described by Kinchington and Ng (19): Briefly, 0.25ml. of 7% SRBC was allowed to react with 0.25ml. of 4x10⁶/ml, effectors (HDC/LDC) at 37°C for 15min. and finally centrifuged and kept at low temperature (4°C) overnight.

3. Cytotoxicity assay by fluorochrome (H33342) release phenomenon as developed in our laboratory (7, 11, 12, 20): this consists of lysing the H33342 labeled target tumour cells (Dalton's Lymphoma) by effector cells at a ratio of 1:10, and measuring the fluorochrome released in the supernatant following 18 hrs. of incubation at 37°C.
4. Phagocytic burst by NBT reduction assay using Dalton’s Lymphoma cells as the target (7, 11); the effector: target ratio has been maintained at 10:1. The HDC and LDC cells were incubated for 18 hrs. at 37°C in the presence of Nitroblue tetrazolium chloride (NBT) and lyopolysaccharide (LPS). Reduced phagosomes were extracted with pyridine and the reading taken at 530µm.

Statistical Analysis. Statistical analysis included the mean ± standard deviation of data and student’s “t” test for comparing the level of significance (‘p’ values).

Results

Leukemia induction, effect of BRM and the survival study. ENU could induce a mixed type of leukemia (Fig. 1a, 1b) within a period of 6-8 months following carcinogen administration. The results showed acute death of 15% animals within a period of 7-10 days while others developed secondary infections (25%) and associated symptoms leading to leukemic development in about 60% of animals. Appearance of leukemic blasts both in peripheral blood and bone marrow revealed predominantly lymphoblastic cells and a fraction of myeloblasts as well. These animals had a mean survival time of 150±45 days compared to a normal healthy control of 440±35 days (Fig. 2). Many of these showed secondary infections with diverse haematological picture.

Effect of non-specific BRM, SRBC. Sheep erythrocytes (SRBC) when applied in-vivo showed significant protection towards the total survival even after leukemia induction. The leukemic animals receiving SRBC also showed recovery from secondary infections and associated manifestations, which pointed towards modulations in immunological functions under the instance. About 90% of the leukemic animals were protected (Fig. 2).

Cellularity at density specific compartments: Flowcytometric analysis. The cells harvested as LDC (1.050) and those at HDC (1.077) were screened through fluorescence activated cell sorter (FACS) to

![Fig. 1 - A - Appearance of leukemic blasts in the peripheral blood smear of mice six months following ENU administration in young age (7-10 days). Lymphoblastic cells were predominant with occasional presence of myeloblasts (X400). B - A number of malignant blast cells are seen in the bone marrow smear of ENU induced mice, most of which represent lymphoblasts (X1000).](image)

![Fig. 2 - Survival data of different groups of mice including that of experimental leukemia and effect of SRBC on both control and leukemic group. SRBC showed significant survival benefit in leukemic mice as well as normal mice.](image)
identify the respective distribution of CD34+ cells at the respective compartments. Simultaneous counting of HDC and LDC also supported the above data showing the normal migratory approach from LDC to HDC as evident from data (Table I). It is apparent that the HDCs always have a greater drag of cell population compared to those at LDC. In leukemic animals the cell distributions are originally low at both the compartments with a higher shift of LDC cells to HDC. SRBC could not apparently modify such mobilization although cellularity at both the compartments are very high compared to ENU induced leukemic counterpart (Table I). Relative distribution of pluripotent bone marrow cells has also been authenticated from studies under flowcytometry where LDC (37%) under normal condition showed a higher distribution of CD34+ cells than the HDC (23.4%)(7).

A drastic diminution of CD 34 positivity has been noticed in both LDC and HDC of ENU treated animals (Fig. 3a) (9.05% and 4.32% respectively) whereas, application of SRBC in-vivo significantly elevated extent of CD 34+ cells both in LDC and HDC in the group concerned (Fig. 3b) (44.02% and 41.11% respectively).

**Functional efficacy of bone marrow derived cells.**

The cells isolated as HDC and LDC from different groups of animals were assayed for immunological efficacy from three different aspects; namely, (a) spontaneous E-rosette formation, (b) cytotoxic capacity and (c) phagocytic efficacy.

(a) Spontaneous E-rosette formation: Bone marrow cells of leukemic mice both at HDC and LDC showed a significant decrease in spontaneous rosette forming capacity compared to that found in normal mice (P<0.001) (Fig. 4a, 4b). Groups of normal animals receiving SRBC (NS) showed a decrease in rosette forming capacity both at LDC and HDC level, the decrease being more significant in the latter. In ENU induced leukemic mice SRBC, however, induced an increased rosette forming capacity in both the compartments (P<0.001).

(b) Cytotoxic efficacy: As evident from data (Fig. 5a, 5b) the cytotoxic capacity of the BMC at both LDC and HDC were found to be reduced (P<0.001) in leukemic mice. SRBC administration in normal mice showed an increased cytotoxicity against the target by LDC but a significant decrease in HDC. On the contrary, in leukemic mice cytotoxic capacity is only moderately elevated in LDC from that of the leukemic group whereas in HDC the cytolytic capacity is increased in a highly significant manner (P<<0.001). Thus, SRBC showed differential effects on separate group of cells.

(c) Phagocytosis: Phagocytic capacity as conducted by NBT reduction test revealed a greater efficacy in HDC group of normal mice than the LDC counterpart (Fig. 6a, 6b). In leukemic mice the phagocytic efficacy was found negligible in LDC group due to insufficient cell number in that compartment, whereas, in HDC the leukemic animals showed an increase in phagocytic capacity from that of the normal animals. Sheep erythrocytes were found to stimulate the phagocytic efficacy of both HDC and LDC of leukemic mice in a highly significant manner (P<0.001) but not in normal counterparts which is rather suppressive.

**Discussion**

Cells in bone marrow microenvironment have long been considered to express an interchanging migratory activity in mammalian system. A number of hypotheses have been put forward to explain the mobilization of bone marrow derived cells (BMC) with respect to maturation both structurally and functionally (21-22). In the present context, the cells from the bone marrow cavity of different groups of mice have been isolated using modified density gradient elutriation technique originally proposed by Moore and Belmont (18). As revealed from flowcytometric analysis (FACS) carried out previously, the method satisfactorily provided an enriched CD34+ population (37%) at the low density region considered as LDC and a high density region (23.4%) considered as HDC (7). The results obtained in the present investigation showed that leukemic development due to ENU administration significantly reduced CD 34+ cell population in both the compartments in the animals concerned. The most striking
observation is the increase in CD 34 positivity at a highly significant level at both LDC and HDC of leukaemic animals receiving SRBC. Apparently the effect was found to be very much protective (P<0.01). Consequently, absolute cell counts of the BMC of different groups of animals showed some interesting correlation (Table I) : considering the compartmentalization of BMC at LDC and HDC a spontaneous migration of cells from low to higher density has been noticed. Hints to such a possibility have already been discussed (23). A number of data have revealed that at stable phase in normal healthy individuals a low density compartment retains a high concentration of stock cells in the bone marrow matrix which upon emergency trigger, migrate to the high density region along with the course of differentiation. Following application of ENU a larger shift of the cells have been noticed from LDC to HDC although total cellularity
(CD 34+) is drastically reduced (9.05% and 4.32% respectively) (P<0.001) compared to that from normal under the event. SRBC when applied had little or insignificant effect in compartmental migration in normal animals (NS) and in leukaemic mice (Table 1); but in leukaemic mice SRBC could significantly (P<0.001) raise the cellularity (CD 34+) both in LDC and HDC (44.02% and 41.11% respectively) compared to the leukaemic control. (Fig. 3a, 3b). It is difficult to underline the exact event whether the transmigratory maturation together with accelerated self renewal system of the BMC is operative under the circumstances. These data, however, suggested that SRBC as a biological response modifier could operate the BMC

![Figure 4](image1.png)  
**Fig. 4** - A - Spontaneous E-rosette forming capacity of low density cells (LDC) of bone marrow from different groups of mice showing effect of SRBC on normal and leukaemic groups: the normal group suffers a significant decrease in E-rosette forming capacity (NS, P<0.001) whereas in leukaemic group SRBC significantly elevates E-rosette forming capacity (ES, P<0.001).  
B - Spontaneous E-rosetting capacity of high density cells (HDC) of bone marrow of different groups of mice: shows significant decrease of E-rosetting capacity in leukaemic mice (E) and that following SRBC administration in normal group (NS); SRBC stimulated the rosetting capacity of the HDC of leukaemic group in highly significant manner (ES, P<<0.001).

![Figure 5](image2.png)  
**Fig. 5** - A - Cytotoxic efficacy of LDC of different groups of mice: the leukaemic group shows a significant decrease which following SRBC administration shows moderate degree of stimulation both in normal as well as in leukaemic group (NS and ES respectively).  
B - High density cells of BMC shows a significant decrease of cytotoxic efficacy in normal mice following SRBC administration; whereas, in leukaemic group SRBC stimulated cytotoxicity in a highly significant manner (ES, P<<0.001).
renewal system together with compartmentalization procedure in two distinct functional subdivisions via some cellular/molecular mechanisms involved in the cytokine network (24-26). The flowcytometric analysis of CD34+ cells, however, represented a generic population in general and did not include other lineage specific migration within the bone marrow cavity. Attempts are now being made in our laboratory to characterise the lineage specificity under the event.

Whether the migrational aspect of cells from LDC to HDC under the events were based on the maturational approach of the BMC per se (21-25), has been studied in terms of functional assessment of the two distinct cell types as isolated. One of the most critical approaches of the present investigation is to establish the maturity in terms of functional efficacy of the cells concerned under variable conditions. It seems reasonable to argue that functional efficacy of the BMC might have limitations due to immaturity of the cells with respect to the absence of functional receptors under the condition (3); immunological protections within the bone marrow is, therefore, questionable. In our laboratory, we planned to assess such immunological functions as attributed to lymphocytes in general, like spontaneous E-rosetting, (CD-2 specific), cytotoxicic efficacy, and phagocytic capacity representing polymorphonuclear neutrophils and others. The results in these directions showed that cells in HDC were more responsive than those of the LDCs. The data simultaneously furnished that the cells in general elicit immune responses with capability of providing defence within the bone marrow cavity combating infective procedures.

The functional efficacy of both HDC and LDC in terms of E-rosetting and cytotoxicity were found to be significantly depressed compared to the normal which may be due to the leukemogenic inhibition rather than the direct effect of ENU, as the residual harmful effects of the agent can be ruled out after a long gap of 6-8 months. Similarly, deficient phagocytic efficacy in LDC but not in HDC of leukemic mice can account for both a quantitative and a qualitative deterioration of the BMC under the condition although a moderate increase in phagocytic activity by HDC of leukemic mice has been shown; the reason may be a tumourigenic stimulation of the phagocytes on the contrary. Sheep erythrocytes, considered to play antitumour and immunopotentiating role (10, 11, 12, 20), was administered in normal and leukemic mice with a concomitant protection to the survival period (Fig. 2). Attempts to investigate the immunological functions furnished significantly improved immunological responsiveness in terms of rosetting, cytotoxicity and phagocytic capacity of both LDC and HDC of SRBC treated leukemic mice (ES) compared to the normal counterpart (NS) where SRBC was surprisingly inhibitory on the BMC. The cells of leukemic mice exhibited significant levels of functional protection to the above parameters. Although it poses a somewhat difficult status to explain, it is evident from the data that SRBC exerted a facilitatory trigger towards the immunological

Fig. 6 - A - The phagocytic capacity of LDC of bone marrow of different groups: shows a significant degree of stimulation by SRBC in leukemic group, whereas, in normal mice it was found inhibitory (P<0.001).
B - HDC of different groups represented more responsive phagocytic efficacy in leukemic counterparts; upon SRBC administration the HDC of leukemic mice shows a significant increase in phagocytosis (P<<0.001).
functions of the bone marrow cells particularly in leukemic mice. Whether the stimulatory determinants could find the appropriate receptors on the cells concerned in the leukemic mice under the event, may be the subject of further consideration. Previous studies with SRBC also supported the strong immunological reactivity of the effectors in the face of a growing tumour in animals (10, 11, 12, 20). Possibility of increased cytokine interactions under such conditions can form the other basis of explanation (11). Current studies with the effectiveness of the specific determinate molecule of SRBC, namely the T-11 target structure (T11TS) or Sheep Leucocyte Function Antigen 3 (SLFA3 or CD58)(14, 15, 27) have shown a significant role of the molecule in promoting cell mediated immunity with special reference to T-cell activation and entry to the tumor site with concomitant strong tumou-


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