Immunotherapeutic Approach of Experimental Brain Tumor with a Corpuscular Antigen

Swapna Chaudhuri*, Soven Kumar Gangopaddhyay*, Susobhan Sarkar1 and Samaresh Chaudhuri2

1Cellular & Molecular Immunology Lab, Department of Physiology
University College of Medicine, 244B, A.J.C. Bose Road, Kolkata – 700020
2Section of Immunology, Department of Haematology
School of Tropical Medicine, C.R. Avenue, Kolkata – 700073

Young Druckrey rats of both sexes ageing 5-10 days were injected with fractionated and acute doses of N-N' ethyl nitrosourea intraperitoneally. This required an optimum period of 6-8 months to develop brain tumor with a mean survival of 190 days. Sheep erythrocytes (SRBC) when administered (ip. 7% PCV/V, 0.5 ml) in a group of animals at the end of seventh month of ENU administration, resulted in significant increase in the mean survival time (>350 days). Studies conducted for growth kinetics pattern with proliferation index and fluorochrome (HO – 33342) uptake techniques at the tissue culture level exhibited a regulatory inhibition of the cell excised from the tumor susceptible area of brain of SRBC treated animals compared to those of untreated tumor controls. Further probe into the mechanisms involving immunological investigations at the cellular level in these animals indicated an augmented and potentiated cell mediated immune response (CMI) as evidenced by enhanced spontaneous rosette forming capacity and cytotoxic activity of lymphocytes and neutrophil (PMN) mediated phagocytosis respectively. Moreover histological studies demonstrated reversion of neoplastic glial features to normal glial features in SRBC-treated tumor bearing animals. The observations suggest that SRBC down regulates malignant growth pattern of experimental brain tumors either by an immunologically enhanced killing of tumor cells and/or by directly inhibiting the tumor growth possibly via a stimulated cytokine network.

Keywords: Cytokine network, Ethyl Nitrosourea (ENU), Experimental Brain Tumors, Growth Kinetics, Immuno-modulation, Sheep erythrocytes

Treatment of brain tumor presents unique challenge because of unique aspects of their biology and anatomic site, and their overall poor response to current therapeutic approaches involving surgery, radiotherapy and chemotherapy which cause several adverse effects in addition to the lesion of the brain (Bartel et al. 1973, Diengdoh & Booth 1976, Nakagaki et al. 1976, Glantz et al. 1999). The apparent lack of efficacy demonstrated by conventional therapies has presented a search for potentially beneficial alternative form of therapies. One of the most promising approaches for the treatment of malignant brain tumor is immunotherapy.

Patients with malignant brain tumors exhibit a broad suppression of cell-mediated immune response (CMI) [Dix et al. 1999]. The impaired cell-mediated immunity observed in patients with malignant brain tumors appears to result from immuno-suppressive factors.
secreted by tumor (Roszman & Brooks 1980, Bodmer et al. 1989, Kuppner et al. 1989, Maxwell et al. 1992, Hishii et al. 1995), which have been implicated for the decrease in T-cell cytotoxicity and proliferation potential, systemic cytokine dysfunction. These findings when taken together with the observation that activated lymphocytes are able to infiltrate effectively through the blood brain barrier (BBB), (Bradley 1966, Selma 1996, and Weller et al. 1996) suggest the feasibility of applying immunotherapeutic approaches.

Numerous attempts have been made to treat malignant brain tumors using a variety of immunologically based strategies, including passive immunization, vaccination with tumor cells, and restorative immunotherapy with local and systemic delivery of biological response modifiers (BRMs), adoptive cellular therapy (Gillespie & Mahaley 1995, Pollack et al. 2000). In experimental animal models, such attempts are directed towards manipulating the immune system to generate an overall effective anti-tumor immune response.

The unique observation that T-cells are capable to form spontaneous rosettes with sheep red blood cells (Pang et al. 1976, Bernard et al. 1987, Selvaraj et al. 1987, Ogawa et al. 1995) and that sheep red blood cells can activate and enhance T-lymphocytes proliferation (Ebert 1985) and can also augment IFN-γ production by lymphocytes (Wilkinson & Morris 1984) is possibly the crucial point to consider sheep red blood cell (SRBC) as an immuno-provocating agent. In our previous study, SRBC has been shown to exert a strong immunostimulatory as well as anti-tumor property in different animal models (Chaudhuri et al. 1991, Chaudhuri et al. 1993, Roy et al. 1997), which could be explained in terms of immuno-modulation.

In the present course of investigation, restorative immunotherapy has been attempted with sheep red blood cells (SRBC) in brain tumors, experimentally induced with ethylnitrosourea (ENU) in animals. The objective of the study is to explore the immunostimulatory and anti-tumor property of sheep red blood cells in brain tumor, with attempts to evaluate its role in the growth kinetics of experimental brain tumors. A consequent survival study and immunological parameters have been conducted to hint at the mechanisms involved. Moreover, histological study has been carried out in order to ascertain its anti-tumor property conclusively.

Materials and Methods

Animals

Healthy new born Druckrey rats (120 gm) of both sexes and aged 7± 3 days old, originally supplied by Central Drug Laboratory, Calcutta, India were used as the experimental model and maintained subsequently in our laboratory for the purpose of investigations. The animals consisting of 24 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 liubitum. Rats were examined daily and weighed weekly throughout the experimental period. The animals were grouped as following:

(I) Normal untreated, healthy, age matched (7 months old) control groups (N)

(II) Acute-ENU group (A-ENU) is the loading dose with intraperitoneal inoculation of dose of 50+30 mg/kg body weight of ENU.

(III) Fractionated- ENU (F-ENU) fractionated dose of ENU (15 mg/kg body weight) were inoculated each week for consecutive eight weeks with a total of 120 mg/kg body weight.

(IV) SRBC-A-ENU consisted of animal grouping prepared with acute dose of ENU (A-ENU) and then receiving a single dose of SRBC (7% PCV/Vol; 0.5 ml) intraperitoneally at the end of 7th month of ENU administration.

(V) SRBC-F-ENU: rats of this group were induced with brain tumors with fractionated dose of ENU (as per gr. III) and then receiving a single dose of intraperitoneal SRBC at the end of 7th month.

Induction of brain tumors: ENU administration

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 to 48 animals with an acute dose of 50 mg/kg body weight in the first week after birth and 30 mg/kg body weight in the subsequent week respectively. Animals (48) of another batch were injected, intraperitoneally on the same day with fractionated doses of 15 mg/kg body weight per week with total of 120 mg/kg in 8 consecutive weeks. From the above two groups, 24 animals each were kept for SRBC treatment to constitute group IV and group V animals, whereas, the rest 24 animals each in acute and fractionated ENU groups served as group I and group II animals respectively (Druckrey et al. 1966, Koestner et al. 1971, Lantos 1993, Lantos et al. 1997, Barth 1998).

Administration of SRBC

These methods have been described previously (Chaudhuri et al. 1991, Chaudhuri et al. 1993). Briefly, 7% SRBC suspension was prepared through PCV/(N) saline volume dilution, 0.5 ml of it was inoculated intraperitoneally to 24 animals each belonging to A-ENU and F-ENU respectively to serve as group IV (SRBC-A-ENU) and group V (SRBC-F-ENU) animals.
Survival study

Among the animals of each group the total days of survival of each individual was recorded. This data were incorporated into Kaplan Meier section of SPSS statistical software package (Version 9.0, SPSS, Chicago, Illinois, U.S.A) and were represented as follows: Group I N as Factor 0, Group II A-ENU as Factor 1, Group III F-ENU as Factor 2, Group IV A-ENU-SRBC as Factor 3, Group V F-ENU-SRBC as Factor 4. The survival functions were graphically represented as X-axis denoting days of survival and Y-axis denoting cumulative frequencies of survival (Fig. 1a). The comparative body weights of individual rats of each group were weighed monthly and plotted with mean weight of each group against months (Fig. 1b). Further, neurological signs like rotatory movement, restriction of movement and also alopecia, weight loss and alteration in the size of the animals were registered. Physical symptoms matched with histological findings were taken accounts for selection of rats bearing brain tumors.

Histological Studies

Portions of brain tissues from respective group of animals were prepared for routine histological studies: tissues were fixed in 10% formal-buffer overnight and finally dehydrated and embedded in paraffin through histokinet processing. Sections were cut at 5μ thickness and finally stained with haematoxylin / eosin.

Growth Kinetics of Tumors

In most animals injected with ENU, macroscopic tumors were visible in cerebral cortex and sub-cortical areas. The areas of microscopic tumors were latter confirmed with histological data and found to develop a whitish coloration compared to the light pinkish hue of cerebral cortex. These areas of cortical and sub-cortical region were selected out to be the tumor susceptible areas. Brain tissues were collected after 7th month of tumor induction for group II and III animals. In groups of IV and V six days after SRBC injection in 7 months old brain tumor induced animals. Normal controls were maintained for 7 month old untreated rats.

Figure 1a. Survival study by Kaplan Meier: Survival data of ENU induced rats (A-ENU and F-ENU) compared to normal animals (N). Survival function was significantly decreased in tumor bearing animals (A-ENU, F-ENU). SRBC administration demonstrated significant increase in survival function in tumor bearing animals for both A-ENU and F-ENU group, although the best protective effect was observed in F-ENU-SRBC group.
Figure 1b. Comparative study of body weight: Comparative analysis of body weight (in grams) of the animals from the different experimental animal groups. The body weight of ENU induced animal group decreases gradually and maximum decrease was monitored in the A-ENU group. Whereas after SRBC administration gain of body weight to near normal value, were demonstrated in both the ENU groups that received SRBC.

Small portions of tumor susceptible areas of cerebral cortex and sub-cortical white matter from all groups of animals were taken as the sample of tissue culture; primary explant technique was employed which is briefly described as follows: chopped portion (1 mm) were bathed in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) in culture dish (60 mm, Corning, USA) and incubated overnight at 37°C followed by increase in the media volume upto 5 ml within a week. After obtaining visible explant growth, they were dispersed with PBS-EDTA-Trypsin (0.25% in PBS), washed repeatedly and finally reinoculated at 5 x 10^5 cells/ml at separate culture dishes. Cells were grown in continuous subculture for attaining a steady repopulation condition.

**Proliferation index of the cultured cells**

After the steady state is achieved, the growth kinetics of the cells as maintained from all above groups of animals were studied in terms of Proliferation Index (PI): 2.7 x 10^4 cells from each of the group of animals were freshly inoculated in 3 ml culture media as before and incubated for 24 hours at 37°C with 4% CO_2 and humidified atmosphere. At the end of 24 hour the cells were briefly washed with trypsin-EDTA and finally washed off with PBS. Cells were resuspended in 1 ml media (RPMI-1640) in each case and counts were taken in a cell counter. PI was calculated from the ratio of counts at 24 hrs to that at ‘0’ hr.

**Fluorimetric assessment of growth kinetics**

Hoechst 33342 (HO33342), a fluorescent dye (Sigma, USA) was diluted to 1 mM in deionised water and stored in the dark at 4°C upto 2 months. For the present setup, a working dilution of 6μg/ml was found to stain satisfactorily a fraction of 2 x 10^8 cells in culture/incubation within 15 minutes. Thus for each experimental protocol 6μg of HO-33342 dye was administered in culture dishes containing 2.7 x 10^4 cells for each group and incubated at 37°C with 4% CO_2 environment for 24 hr. Cells were washed off with PBS-EDTA briefly and then extensively to remove excess dye. The cells were suspended in 2 ml portions of PBS and counts taken in a spectrofluorimeter (Beckman, USA) using a scanning wavelength in between 400-500 nm (365 nm excitation/435 nm emission) (Chaudhuri et al. 2000).

**Studies on immune function**

**E-rosette Formation**

Lymphocytes were separated from spleen cell suspension on a percoll density gradient elutriation method (Raha et al. 1990). 0.25 ml of 3-4 x 10^6 cells/ml lymphocytes were mixed with 0.25 ml of 1% (PCV/saline Vol) sheep erythrocytes (SRBC) and incubated at 37°C for 15 minutes. Following brief centrifugation, the preparation was kept at 4°C overnight. Number of rosettes formed were counted per 200 lymphocytes and expressed as rosette %.
Cytotoxic efficacy of splenic lymphocytes by HO-33342 release assay

This method consisting of using HO-33342 fluorochrome (Sigma, USA) labeled target tumor cells (brain tumor cells) instead of C51 labeled target. Briefly, 5 x 10⁶ target cells were incubated with 6 μg of the dye in 1ml of saline/PBS for 20 minutes at 37°C. Excess of the fluorochrome washed off and cytotoxicity assay was performed with the splenic lymphocytes of each group isolated by percoll density gradient against labeled target at a ratio of 100:1. Cells were subjected to 18 hrs incubation in CO₂ environment and 37°C temperature. The fluorochrome released in the supernatant from lysed targets gave the index of percent cytotoxicity. A comparative activity of the splenic CTL was also carried out against fluorochrome labeled Dalton’s lymphoma (Chaudhuri et al. 2000, Law et al. 2001).

Phagocytic capacity of polymorphonuclear neutrophils (PMN)

The method has been discussed previously (Chaudhuri et al. 1991). Briefly, PMNs isolated by percoll density gradient were allowed to phagocytose the target brain tumor cells in the presence of nitro blue tetrazolium chloride at the effector: target ratio of 100:1. The preparation was incubated for 18 hours at 37°C in 4% CO₂ humidified atmosphere. Finally, the reaction was stopped by adding 0.1 N chilled HCl and pellet was extracted with boiling pyridine for the reduced blue formazan. The intensity of color provided a direct measure of extent of Phagocytosis at 530 nm (Chaudhuri et al. 1991, Hudson & Hay 1989).

Results

Survival Studies

All the survival functions were statistically significant with a slight deviation in cumulative survival within the groups. In the acute ENU group although there is a slight deviation within the group but it is not statistically significant. The survival functions between the normal and ENU groups (both A-ENU and F-ENU) showed a significant deviation which is statistically significant. Among both ENU groups the survival functions again differ significantly. But in both ENU induced groups after application of SRBC the survival rate showed a profound increase and reached the near normal value. The average weight of the baby rats about 7 days old and all the groups averaged to about 2 ± 0.5 gms. Upto the 4th month all the animals grew up steadily maintaining the same weight profile but changes started occurring in between the 4th and 5th month of age in the different group of animals. Though the weight of the rats of the normal group increased steadily upto 5th month, after which the steep rise slowed down to maintain the static weight varying from 125±5 gms to 135±8 gms. But the weight of animals belonging to A-ENU & F-ENU groups dropped down significantly from 5th month onwards and dropped down to 60±7 gms in ENU and 58±6 gms in A-ENU respectively. But in the A-ENU-SRBC & F-ENU-SRBC group the weight of the animals started rising up significantly after the 7th month, the end of which a single dose of SRBC has been administered and both the groups reached normal levels after 11 months.

After a single dose of SRBC injection the physical symptoms like rotatory movements decreased, new hair growth appeared in denuded regions and the movements of animals became brisk like normal animals.

Histological Evidences

Histological studies show normal glial cell populations in white matter of cerebral cortex with few astrocytes, oligodendrocytes and a few neurons [Fig. 2(a)]. The effect of A-ENU (acute dose) showed grade IV oligodendroglioma with typical honeycomb like appearance and presence of mitotic figures, giant cells and absence of intercellular spacing [Fig. 2(b)], and hyper proliferation of the oligodendroglial cells with the presence of mitotic figures were observed with fractionated doses of ENU (F-ENU) [Fig. 2(c)]. Significant decrease in the number of oligodendrogial cells was observed with the SRBC administration in A-ENU group, however, the best effect was observed in the F-ENU group, where reversion of neoplastic glial features to normal glial features was observed following SRBC administration [Fig. 2(d) & 2(e)].

Growth Kinetics

To investigate the cytokinetic behavior of the ENU induced tumor cells in the rat brain following administration of sheep red blood cells, two different sets of experiments were performed and expressed in terms of PI (proliferation index) (a) and HO-33342 dye uptake by the proliferating cells (b).

(a) Proliferation index (PI): The cell proliferation rate of the glial cells in ENU-treated animals for both acute and fractionated doses were 75±3.42% and 50±2.94% respectively, which were significantly higher (p<0.001) than that of the normal untreated control of 30±2.03%. Administration of sheep red blood cells in such tumor bearing animal group for both acute and fractionated dose was found to reduce the proliferation rate of the glial cells significantly (p<0.001) to almost near normal level [Fig. 3(a)].

(b) Fluorochrome uptake study: Corresponding studies on HO-33342 uptake by cells in culture
Figure 2. a) Histological evidences showing normal brain tissue with the presence of few astrocytes, oligodendrocytes and few neurons; b) The effect of A-ENU (acute dose) showed grade IV oligodendrogliaoma with typical honeycomb like appearance and presence of mitotic figures, giant cells and absence of intercellular spacing; c) Hyper proliferation of the oligodendroglia cells with the presence of mitotic figures were observed with the fractionated doses of ENU (F-ENU); d) Significant decrease in the number of oligodendroglia cells was observed with the SRBC administration in A-ENU group; and e) The best effect was observed in the F-ENU group, where reversion of neoplastic glial features to normal glial features was observed following SRBC administration.
Figures 3. a) Cell proliferation index (PI) of Glial cells before and after induction of tumors and that following application of sheep erythrocyte. The figure shows high counts in tumor induced animals (b,c) and near normal counts in SRBC treated groups; b) fluorescent spectra of HO-33342 uptake of Glial cells under variable conditions (vide text). The spectra show a significantly higher dye uptake in tumor induced glial cells and near normal values following SRBC treatment in tumor groups; and c) Cytokinetic index of glial cells revealed through HO-33342 uptake in different groups of animals (see Text). The quantitative data confirms the tumor reducing effect of SRBC in tumor bearing animals.

Immunological Profile

E-Rosette Formation

To study the effect of SRBC on the proliferative study of lymphocytes in tumor bearing animals, rosette forming capacity of lymphocytes with SRBC were represented a much higher uptake of the fluorochrome in group II (A-ENU) and group III (F-ENU) animal compared to its normal counterpart. In group IV and V animals, the fluorochrome uptake was reduced to 12±2.91% and 12.5±2.85% respectively indicating a near normal value.
performed. In the normal control group the number of rosette forming lymphocytes were found to be 19±4.31% (n=24). The ENU treated animal groups (n=24), both acute and fractionated showed significant decrease (p<0.001) in rosette forming capacity when compared to normal control group. The administration of SRBC in such tumor bearing animal groups increased the number of resetting lymphocytes which were significantly greater (p<0.001) than that of ENU-treated groups and reached almost to the near normal value [Fig. 4].

**CTL-Assay by HO-33342 release**

To investigate whether the administration of sheep red blood cells potentiate the cytotoxic activity of lymphocytes, HO-33342 release assay were carried out with lymphocytes obtained from the spleen and directed against labeled target. The values obtained from the fluorochrome of lysed targets represented in different groups of animals have been presented in (Fig. 5). The results showed a significant drop of cytotoxic activity of splenic lymphocytes (p<0.001) in both acute and fractionated ENU groups of animals (n=24) in comparison to normal untreated control group. The administration of SRBC in such tumor bearing animal groups increased the number of resetting lymphocytes which were significantly greater (p<0.001) than that of ENU-treated groups and reached almost to the near normal value [Fig. 4].

**Phagocytic burst by PMNs**

The results have been presented in (Fig. 6). This showed that PMN mediated phagocytosis is significantly decreased (p<0.001) in acute ENU group (n=24) rather than the fractionated ENU group of animals (n=24). SRBC, when administered, protected the fractionated ENU group of animals more significantly than the acute ENU groups, although the later showed appreciable protection.

**Discussion**

In the present study, several experimental evidences have sustained the conclusion that administration of sheep red blood cells (SRBC) (i.p) in experimentally induced brain tumor can potentiate the cell mediated immune response (CMI) culminating in an effective tumor regression through effector mechanisms. The following observations were made in this study. 1) Survival period of the tumor bearing animals can be prolonged following administration of SRBC. 2) The reversion of neoplastic glial features to normal glial feature could be achieved following administration of sheep red blood cells in ENU treated animals. 3) The immunoregulatory and tumor inhibitory effects of SRBC can be ascertained in terms of its role in growth kinetics of experimental brain tumors. 4) The potentiation of the cell mediated immune response (CMI) in immune suppressed ENU induced tumor bearing animals can successfully be achieved by following administration of sheep red blood cell. The studies of the tumor parameters, growth kinetics and histological findings corroborate with the survival studies.

Under normal condition the peripheral leucocytes enter the blood brain barrier [Selmaj, 1996] but leave the brain in great numbers in absence of antigen. But after tumor development, breaches are found in the blood brain barrier through which brain tumor antigens and other secretory products of the tumor reach the peripheral immune system. These products also reach the periphery through the cervical lymph nodes. At this point though the peripheral lymphocyte become sensitized to the brain tumor antigens, all the peripheral immunocytes are functionally and phenotypically inactivated by the secretory tumor products like PGE-2, IL-10 and TGFβ. The injected SRBC is degraded by the phagocytic cells and its epitope (T11TS) presented on antigen presenting cells stimulate the deactivated sensitized lymphocytes and other immunocytes. Now the activated immunocytes can cross the blood brain barrier and can specifically kill the tumor cells. So the group IV and V animals receiving SRBC doses after tumor growth show better survival benefit compared to the two tumors induced groups.

In our previous studies with fibrosarcoma induced in Swiss mice and immunosuppressed C57B1/6 mice, it was observed that intra-peritoneal SRBC injection at the same dose could inhibit the growth of fibrosarcoma both at preventive and therapeutic levels.
Administration of a single dose of 7% SRBC (0.5 ml) in ENU treated animals revealed some interesting results. Total survival period significantly increased, tumor induction was delayed or absent over the period of one year after ENU administration as observed macroscopically / microscopically. Thus, significant increase in survival period in ENU-SRBC group supports the immunotherapeutic effect of SRBC which has been shown to exert strong anti-tumor property in ENU-treated tumor bearing animals as evidenced through histological findings where the reversion of neoplastic glial features to normal glial features occur following its (SRBC) administration. In Fig. 1b the decrease of weight in tumor bearing animals (both induced with acute and fractionated doses) may result from malnutrition and infection which may be the cause of demise of animals. Thus weight loss due to the malnutrition may be a covariate in the survival study. Regain of weight of rats in both groups IV and V after SRBC administration indicates health promotion due to removal of tumor as revealed by survival and histological findings and removal of secondary infections indicated by the immune potentiating effects as revealed by immune parameter study. As the single dose of SRBC can revert the neoplastic glial features to normal glial features as evidenced from Growth kinetics, Histological studies and Immune parameters, the second or more booster doses were not administered. Moreover, as SRBC acts as an immunostimulator, excessive administration may give rise to autoimmunity.

As the objective of our growth kinetic study was to demonstrate the proliferation potentials of the tumorigenic cells in culture and the of SRBC administration in proliferative potentials we avoided the time lapse study as the 0-24 hours study was sufficient to serve the purpose. As found with other studies (Hoshino 1984, Hoshino et al.1972, Raha et all. 1990) the proliferation kinetics of such tumor cells in vitro culture almost conclusively demonstrated the malignant nature of the tumors. The growth kinetics study as conducted in tissue culture system showed a significant increase in proliferation capacity of tumor cells both in terms of their proliferation index (PI) and percent fluorescence uptake (HO-33342) of cells at 24 hr of culture. The uptake of HO-33342 by cells in culture has been introduced on the basis of the binding of the fluorescent material with nuclear DNA which is irreversible and non diffusible from cells in culture when administered in optimal concentrations. The method was found to be suitable for studying growth kinetics of cells in culture as it corresponded well with the cell counting index. The degree of malignancy as determined by the above method also corresponded well with the higher morbidity rate in the group of animal concern. These results were comparatively more pronounced in the animals receiving acute doses of ENU rather than those receiving fractioned doses.

Mechanism of anti-tumor activity of sheepr erythrocytes studied earlier (Chaudhuri et al. 1991, Chaudhuri et al. 1993, Roy et al. 1997) showed that some immunomodulatory functions were responsible for tumor inhibition in the host concerned. These immunomodulatory effects were correlated with the survival benefits of SRBC administration. In the present context, studies conducted with splenic lymphocytes (SL) and PMNs in different groups of animals revealed interesting correlations as well: spontaneous E-Rossetting, the cytotoxic efficacy of SL and phagocytic capacity of PMNs were significantly decreased in all the corresponding ENU groups of animals indicating inhibitory effects of developing intracranial tumor in systemic immunity. Although it raises questions about the residual effects of ENU on the immunocytes concerned; such possibilities can be ruled out as the investigations were conducted more than six months after ENU administration when the immunocytes were represented by fresh progeny after several regenerations. Administration of SRBC on the animals receiving both acute and fractioned doses of ENU was found to be significantly protective, the SL and PMNs were found to show significantly higher cytotoxic and phagocytic efficacy respectively, the stimulation being more in animals receiving fractioned doses of ENU, the reason may be considered to be due to a greater residual suppressive effect of tumor / ENU in acute dose recipient. Corresponding greater survival period in these animals further supported the immunotherapeutic effects of SRBC in animals with ENU induced brain tumor. Previously, the therapeutic results can be considered to be effective through enhanced immune effector mechanism under the influence of SRBC which generate a significant number of activated lymphocytes capable of crossing the blood-brain barrier (Palma et al. 1978, Braddle 1996, Selmaj 1996, and Weller et all. 1996).

In the present course of investigation, the significant enhancement of E-rosette formation in ENU-treated animals following administration of sheep red blood cells suggest lymphocyte activation and proliferation both qualitatively and quantitatively. It has been reported earlier that sheep red blood cells can enhance lymphocyte proliferation (Ebert 1985) and in the present study presumably the up regulation of CD2 (T11) molecules occuring in individual lymphocyte indicated by super rosetting and also quantitative lymphocyte proliferation activity was indicated by significant increase in rosette forming cells (CD2+ lymphocytes). This study also indicates possible up regulation of CD25 (IL-2R) expression and IL-2 liberation from lymphocytes [data communiacted]. Administration of sheep red blood cells (SRBC) in tumor bearing animals augment the cytotoxic efficacy of lymphocytes. The non-speceific lytic activity
carried out by lymphocytes possibly involve CD8+ T-cells and NK cells which were presumed to be activated following SRBC administration. Since activation of CTL and NK function have been found to occur reportedly through sheep erythrocyte receptor (CD2, T11) (Krensky et al. 1984, Siliciano et al. 1985). Finally the enhancement of phagocytic activity of PMN seems to occur by SRBC activated CD4+ lymphocytes indirectly through cytokine network (Neutrophil Activating Factor). Also our previous study has shown that tumorosidal phagocytic activity is increased in SRBC treated animals (Chaudhuri et al. 1991). Moreover, direct activation PMN phagocytic activity with SRBC is also possible, through CD2 receptor reported to be present on its surface (Niiazova & Osipova 1995).

Although immunomodulatory activities are exhibited by many agents (Bergquist et al. 1980, Ishizawa 1981, Gillespie & Mahalay 1985, Nakagawa et al. 1985, Endo et al. 1986, Jacobs et al. 1986), those mediated through SRBC were found to be unique in that they have direct stimulatory effects on lymphocytes. The active component of sheep red blood cell which is responsible for such activity has been found to be a cell surface glycoprotein molecule known as T11 target structure (T11TS) or sheep form of LFA-3 or more currently CD58, which binds with the E-receptor or CD2 (T11) molecules of the lymphocytes (Hunig 1985, Dustin et al. 1987, Selvaraj et al. 1987, Springer et al. 1987, Tifenthaler et al. 1987, Wallener et al. 1987, Sun et al. 1999,) and can send mitogenic stimulus to activate them. Supporting the findings of the previous workers who demonstrated activation of T cells through an alternative pathway through 50 kd T11 sheep erythrocytes receptor protein (Meuer et al.1984, Hunig 1985, Hunig et al. 1986, Denning et al. 1988, Meuer et al. 1989, Kovasu 1990, Semnani et al. 1994, Wingren et al. 1995). Since CD2 (T11) is known to be a pan T cell marker, thus activation of T cells is not restricted to CD4+ and CD8+ T cells only, it also involves NK cells as well. Moreover recent studies have also suggested occurrence of CD2(T11) molecule on macrophages (Mφ) (Gutierrez et al. 1999) and PMN also (Niiazova & Osipova 1995). Thus interaction of T11TS(LFA-3) of Sheep Red Blood Cells With CD2(T11) receptor is supposed to activate a large number of Immunocytes not only of lymphoid but also myeloid lineage. Moreover activation of CD4+ lymphocytes with SRBC also implies activation of immunological network indirectly thorough cytokine network recruiting various immune cells. Further more it is now known that activated T cells can enter the brain through the blood brain barrier. Thus activation of CD4+ and CD8+ lymphocytes with sheep red blood cells at the peripheral extent in an immune suppressive state is an important finding, favoring antigen presentation by the APC cells of the brain particularly microglial and generating an effective antitumor immuneresponse (Begum et al. 2003).

It seems likely from the present data that SRBC has the capability to inhibit or block proliferation not only by potentiation of the CMI response but also, presumably by interfering with the mitotic rate of the malignant cells. This may be possible by (1) inducing a cytostatic effect, (2) differentiation induction, (3) blocking DNA synthesis and growth factor interactions and (4) interacting with cellular gene expression. Several in vitro studies have revealed that all these manifestations can be achieved by interferon type 1 (alpha and beta) and type 2 (gamma) depending on the type of tissue or tumor line involved (Samuel 1987, Balkwill 1989, Clemens 1991,). However there are ample supports in favor of the facts that interferons generated in vivo can induce gene activation causing initiation of latent endoribonuclease and thus interfering with tumor replicating process (Samuel 1987), spontaneous action of interferon in vivo can induce re-expression of characteristic differentiation marker; moreover, in vivo resistance to growth factor receptor expression by tumor cells is also pronounced by interferon (Levy 1988). It seems reasonable to argue that SRBC by stimulating in vivo elaboration of IFNγ (Willkinson & Morris 1984) can down regulate the growth character of the brain cells through modulation of gene expression at different levels (Stark 1988). The possibility whether the SRBC mediated stimulation to astrocyte in brain might have interplay in tumor inhibitory processes is a subject of further research. Attempts are now being made to isolate and analyze the integral part(s) of SRBC that can held responsible for exerting such modulatory effects.

Acknowledgement
The work was supported by grants obtained from the Department of Science and Technology, Ministry of Science and Technology, Government of India and R.D. Birla Smarak Kosh. Bombay, India.

References


Niajazova OP, Osipova SO, Badalova NS, & Dekkhankhodzhaeva NA. Early and late E-rosette forming neutrophils in persistent lambliaisis. Medical Parazitology (Moskow) 1: 43-44.


Roszman TL, & Brooks WH. 1990. Immunobiology of primary intracranial tumors III. Demonstration of a


