Brain tumor inhibition in experimental model by restorative immunotherapy with a corpuscular antigen

Soven Kumar Gangopaddhyay\(^1\), Susobhan Sarkar\(^2\), Zarina Begum\(^3\), Swapna Chaudhuri\(^*\) & Samaresh Chaudhuri\(^2\)

\(^1\)Immunology Section, Department of Pathology, University College of Medicine, 244B, A.J.C. Bose Road, Kolkata 700020, India
\(^2\)Cellular & Molecular Immunology Lab, Department of Physiology, University College of Medicine, 244B, A.J.C. Bose Road, Kolkata 700020, India
\(^3\)Section of Immunology, Department of Haematology, School of Tropical Medicine, C.R. Avenue, Kolkata 700073, India

Received 17 December 2002; revised April 2003

In view of the advances in our understanding of anti-tumor immune response, it is now tempting to contemplate the development of immunotherapies for malignant brain tumors, for which no effective treatment exists. Immunotherapy, with agents known as biological response modifiers (BRMs) are thus gaining increasing interest as the fourth modality of treatment. A non-specific BRM, sheep erythrocytes (SRBC) when administered (ip, 7\% PCV/V, 0.5 ml) in a group of animals at the end of seventh month of ethylnitrosourea 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 cells isolated from tissue excised from the tumor susceptible area of brain of SRBC treated animals. Moreover, histological examination of brain from animals showed immunomodulatory role of SRBC in experimentally induced brain tumor. 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. The observations suggest that SRBC down regulate 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. Thus, a corpuscular antigen, can potentiate CMI response in experimentally induced brain tumor animal model, in which response induced in the periphery are able to mediate anti-tumor effects in the brain.

Keywords: Anti-tumor immune response, Brain tumor, Corpuscular antigen, Immunotherapy, Restorative immunotherapy

Treatment of brain tumors presents unique challenge because of unique aspects of their biology and anatomic site, and their overall poor response to current therapeutic approaches involving surgery, radio therapy and chemotherapy which cause several adverse effects in addition to the lesion in the brain.\(^1\)\(^-\)\(^4\) 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).\(^5\) The impaired cell-mediated immunity observed in patients with malignant brain tumors appears to result from immuno-suppressive factors\(^6\)\(^-\)\(^9\), which have been implicated for the decrease in T-cell cytotoxicity and proliferation potential and 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)\(^10\)\(^-\)\(^12\), 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, restorative immunotherapy with local and systemic delivery of biological response modifiers (BRMs), and adoptive cellular therapy.\(^13\)\(^-\)\(^14\) In experimental animal models, such attempts are directed towards manipulating the immune systems 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\(^15\)\(^-\)\(^17\)
and that sheep red blood cells can activate and enhance T-lymphocytes proliferation\(^8\) and can also augment IFN-\(\gamma\) production by lymphocytes\(^9\) it is possibly the crucial point to consider sheep red blood cell (SRBC) as an immuno-provocating agent. SRBC has been shown to exert a strong immunostimulatory and anti-tumor property in different experimental animal models\(^{20-22}\), which could be explained in terms of immuno-modulation.

In the present study, restorative immunotherapy has been attempted with sheep red blood cells (SRBC) in brain tumors, experimentally induced with ethyl nitrosourea (ENU) in animals. In order to explore the immunostimulatory and anti-tumor property of sheep red blood cells in brain tumor, attempts have been made 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 orders to ascertain its anti-tumor property conclusively.

Materials and Methods

**Animals** — Healthy new born Druckrey rats (120) of both sexes, aged 7±3 days, originally supplied by Central Drug Laboratory, Calcutta, India and maintained subsequently in the Laboratory were used. These animals formed following five experimental groups of 24 each: (I) normal untreated controls (N), (II) A-ENU: with inoculation of an acute dose of 50+30 mg/kg body weight of ENU; (III) F-ENU: with inoculation of fractionated doses of 15 mg/kg body weight/week with a total of 120 mg/kg body weight in 8 consecutive weeks; (IV) SRBC-A-ENU: animals prepared as per gr. (II) and received 7% SRBC (0.5 ml, ip.) at the end of 7th month of ENU administration and; (V) SRBC-F-ENU: animals prepared as per gr. (III) and received SRBC (7%), 0.5 ml, ip., at the end of 7th month ENU treatment. The 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. Rats were examined daily and weighed weekly throughout the experimental period. Progressive neurologic signs and alterations of body weight were considered as criteria for animal selection. Maintenance and animal experiment procedure strictly followed “Principles of laboratory animal care (NIH)” and also local “ethical regulations”.

**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 (ip) 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 second subsequent week respectively. Animals (48) of another batch were injected, (ip) on the same day with equal fractionated doses of 15 mg/kg body weight per week with total 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\(^{20-25}\).

**Administration of SRBC** — The methods have been described elsewhere\(^{20,21}\). Briefly, 7% SRBC suspension was prepared through PCV/(N) saline volume dilution. 0.5 ml of it was inoculated (ip) 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** — Of all the animals prepared as above, observations were made to account for the total number of days survived by individual animals and the mean survival time in each group were determined. Further, progressive neurologic signs and weight loss were taken into account in selecting the animals for the tumor development study.

**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 histokin processing. Sections were cut at 5 μm thickness and finally stained with routine haematoxilin/eosine.

**Growth kinetics of tumors** — Small portions of tumor susceptible areas of cerebral cortex and subcortical white matter from all groups of animals were taken as the sample of tissue culture; primary explant technique was employed\(^28\). After the steady state is achieved, the growth kinetics of the cells as maintained from all the above groups of animals were studied in terms of Proliferation Index (PI)\(^28\), which was calculated from the ratio of counts at 24 hr to that at “0” hr.; and also by a fluorescent dye, Hoechst 33342 uptake\(^28\), where counts were taken in a spectrofluorimeter (Beckman, USA) using a scanning wavelength in between 400-500 nm (365 nm excitation/435 nm emission)\(^28\).

**Studies on immune function** — Spleen cells isolated from animals of different groups treated as above
were subjected for cellular immune response studies. For each study, cells from normal group of animals served as normal control.

**E-Rosette formation**—Lymphocytes were separated from spleen cell suspension on a percoll density gradient elut method\(^3\). Lymphocytes (0.25 ml of 3-4×10⁶/ml) were mixed with 0.25 ml of 1% (PCV/saline vol.) sheep erythrocytes (SRBC) and incubated at 37°C for 15 min. Following brief centrifugation, the preparation was kept overnight at 4°C. Number of rosettes formed were counted per 200 lymphocytes and expressed as rosette %.

**Cytotoxic efficacy of splenic lymphocytes by HO-33342 release assay**—A newer approach to this method using a fluorochrome dye Hoechst 33342 (HO33342, Sigma, USA) which binds to DNA of cells irreversibly without leakage until lysed was adopted\(^3\). Tumor cells (target; a steady glial tumor line, syngeneic in nature) were labeled with HO-33342 fluorochrome dye (6 μl/10⁴/ml) for 15 min at 37°C and excess was washed off (total incorporation). Cytotoxicity assay was performed by maintaining an effector (splenic-lymphocytes)-target ratio at 10:1 through an incubation (37°C, 4% CO₂-air environment) period of 18 hr. Fluorochrome released as per target lysis (experimental lysis) measured in a spectrofluorimeter (Hitachi, Tokyo) provided an index of cytotoxic efficacy of effectors\(^3\). A labeled target group maintained alone serves for spontaneous lysis.

\[
\text{Cytotoxic efficacy} = \frac{\text{Experimental lysis} - \text{Spontaneous lysis}}{\text{Total incorporation}} \times 100
\]

**PMN-mediated phagocytosis**—Neutrophils (PMNs) were isolated from the splenic tissues from different experimental groups by percoll density gradient (density 1.089) and were allowed to phagocytose the target brain tumor cells (from a steady glial tumor line, syngeneic in nature) in presence of nitroblue tetrazolium chloride at the effector:target ratio of 100:1. The preparation was incubated for 18 hr at 37°C in 4% CO₂-humidified atmosphere. Finally, the reaction was stopped by adding 0.1N chilled HCl and pellet was extracted with boiling pyridine for the reduced blue formazan. Reduction of yellow NBT to blue formazan indicated the extent of phagocytic burst at 530 nm by effectors concerned. Thus, results were evaluated from the extent of oxidative burst as revealed for blue formazan formed (relative O.D.)\(^3\).

**Statistical analysis**—Statistical analyses of the data were based on standard deviations of means and level of significance were followed through Student's t test. All results were evaluated statistically by applying the SPSS-PC package (Version 9.0,SPSS, Chicago, Illinois, U.S.A.). A probability of <0.01 was considered statistically significant.

**Results and Discussion**

The present study has provided several experimental evidences to conclude that administration of sheep red blood cells (ip) in experimentally induced brain tumor, can potentiate the cell mediated immune response (CMI) resulting in an effective tumor regression through effector mechanisms. Such therapeutic efficacy has been evidenced by the improvement of survival in animals (Fig. 1) as well as by histological findings (Fig. 2).

Administration of 7% SRBC (0.5 ml) in ENU treated animals revealed some interesting results: total survival period significantly increased (Fig. 1), 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 (Fig. 2), where the reversion of neoplastic glial features to normal glial features has been shown to occur in

![Fig. 1 — Survival data — survival data of ENU induced rats (A-ENU and F-ENU) compared to normal animals (N). Mean survival period was significantly decreased in tumor bearing animals (A-ENU, F-ENU). SRBC administration demonstrated significant increase in mean survival period in tumor bearing animals, for both A-ENU and F-ENU group, although the best protective effect was observed in F-ENU-SRBC group.](image-url)
Fig. 2—Histological evidences showing normal brain tissue with the presence of few astrocytes, oligodendrocytes and few neurons (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 (b), and hyper proliferation of the oligodendroglia cells with the presence of mitotic figures were observed with the fractionated doses of ENU (F-ENU) (c). Significant decrease in the number of oligodendroglial cells was observed with the SRBC administration in A-ENU group(d), 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(e) ×400.
SRBC-treated ENU-induced animals. This finding is well corroborated with the immunological findings as well as growth kinetic study.

Histological studies show normal glial cell populations in white matter of cerebral cortex with few astrocytes oligodendrocytes and a few neurons (Fig. 2a). 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.2b), and hyper proliferation of the oligodendroglia cells with the presence of mitotic figures were observed with the fractionated doses of ENU (F-ENU) (Fig. 2c). Significant decrease in the number of oligodendroglial cells was observed with the SRBC administration in A-ENU group, however, the best effect was noted in the F-ENU group, where reversion of neoplastic glial features to normal glial features was observed following SRBC administration (Fig. 2d & e).

Malignant cells of the brain have been shown to be regulated by SRBC. As found with other studies 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 the tumor cells both in terms of their proliferation index (PI) (Fig. 3a) and per cent fluorescence uptake (HO-33342) (Fig. 3b & c) of cells at 24 hr culture. The degree of malignancy as determined by the above methods also corresponded well with the higher morbidity rate in the group of animals concerned. These results were comparatively more pronounced in the animals receiving acute doses of ENU rather than those receiving fractionated doses. It seems likely from the present data that SRBC has the capability to inhibit or block tumor 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 oncogene expression. Several in vitro studies have revealed that all these manifestations can be achieved by interferon type I (alpha and beta) and type 2 (gamma) depending on the type of tissues or tumor line involved. However, there are ample supports in favour of the facts that interferons generated in vivo can induce gene activation causing initiation of latent endoribonuclease and thus interfering with tumor replicating processes; spontaneous action of interferon in vivo can induce re-expression of characteristic differentiation phenotype marker; moreover, in vivo

![Fig. 3](image-url)

Fig. 3—(a): Cell proliferation Index (PI) of Gial 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 tumor groups.(b): Fluorescent spectra of HO-33342 uptake of Gial cells under variable conditions (vide text). The spectra shows a significantly higher dye uptake in tumor induced gial cells and near normal values following SRBC treatment in tumor groups.(c): Cytokinetic Index of Gial 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.
resistance to growth factor receptor expression by tumor cells is also pronounced by interferon. It seems reasonable to argue that SRBC by stimulating in vivo elaboration of IFN-γ can down regulate the growth character of the brain tumor cells through modulation of gene expression at different levels. The possibility whether the SRBC mediated stimulation to astrocytes in brain might have interplay in tumor inhibitory process is a subject of further research.

Mechanism of anti-tumor activity of sheep erythrocytes studied earlier showed some immunomodulatory functions as being responsible for tumor inhibition in the host concerned. In the present context, studies conducted with the splenic lymphocytes (SL) and PMNs in different groups of experimental animals revealed interesting correlations as well: spontaneous E-Rosetting, the cytotoxic efficacy of SL and phagocytic capacity of PMNs were significantly decreased in all the corresponding ENU groups of animals (Figs 4-6) 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 6 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 fractionated doses of ENU was found to be significantly protective; the SL and PMNs show significantly higher cytotoxic (Fig. 5) and phagocytic efficacy (Fig. 6) respectively, the stimulation being more in animals receiving fractionated doses of ENU; may 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. Precisely, 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.

In the present course of investigation, the significant enhancement of E-rossette formation in ENU-treated animals following administration of sheep red blood cells (Fig. 4) suggests lymphocyte activation and proliferation. Presumably, the up regulation of CD2 (T11) molecules occurring in individual lymphocytes indicated by super rosette formation and 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 (unpublished data). The active component of sheep red blood cell which is presumed to be responsible for such activity has been found to be a cell surface glycoprotein molecule, known as T11 Target Structure (T11TS) or sheep lymphocytes form of LFA-3 or more currently CD58 that binds with the E-receptor or CD2 (T11) molecules of the lymphocytes has been shown to 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 erythrocyte receptor protein.
Administration of sheep red blood cells (SRBC) in tumor-bearing animals augmented the cytotoxic efficacy of lymphocytes (Fig. 5). The non-specific activity carried out by lymphocytes possibly involves CD8+, CD4+ T-lymphocytes and natural killer cells (NK cells), which were presumed to be activated following SRBC administration, since they were all CD2+ cells—showing activation of CTL and NK function through sheep erythrocyte receptor (CD2, T11)52.

Finally, the enhancement of phagocytic activity of PMN (Fig. 6) seemed to occur by SRBC activated CD4+ lymphocytes indirectly through cytokine network (neutrophil activating factor). Chaudhuri et al.54 have shown that tumoricidal phagocytic activity is increased in SRBC treated animals55. Moreover, direct activation of PMN phagocytic activity with SRBC is also possible, through CD2 receptor reported to be present on its surface56.

Although immunomodulatory activities are exhibited by many agents13,54-56, those mediated through SRBC were found to be unique in that they have direct stimulatory effects on lymphocytes. 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 of myeloid lineage. Moreover, activation of CD4+ lymphocytes with SRBC also implies activation of immunological network indirectly through cytokine network, recruiting various immune cells. Further, it is now known that activated-T cells can enter the brain across the blood–brain barrier, thus activation of CD2+ lymphocytes (CD4+, CD8+, NK) with sheep red blood cells at the peripheral extent in an immunosuppressive state is an important finding, that facilitates infiltration of activated lymphocytes across blood brain barrier, favoring antigen presentation by the APC cells of the brain, particularly microglia and generating an effective anti-tumor immune response (unpublished data).

Acknowledgement

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

References


