Sheep erythrocyte demonstrated better effect than IL-2 and IFN-γ as biological response modifier against glioma in experimental model


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Received 25 May 2004; revised 9 February 2005

The significant insights into the immunobiology of central nervous system (CNS) and brain tumor have opened up the feasibility of applying ‘Immunotherapy’ as an alternative to the poor prognosis of malignant brain tumor with conventional therapeutic approaches. Though cytokines like IL-2 and IFN-γ used against glioma showed some favorable results by eliciting Th1 type immune response, a proper immunotherapeutic agent is still to be searched for. Sheep erythrocyte (SRBC), a corpuscular antigen showed a better therapeutic efficacy in terms of enhanced survival and augmentation of cell mediated immunity (CMI) in a glioma model developed by chemical carcinogen ethyl nitrosourea. Histological findings revealed most efficient glioma rejection in SRBC and combination biological response modifier (BRM) treated groups. Simultaneously E-rosetting, cytotoxicity of lymphocytes, phagocytosis and antigen presenting capacity of myeloid cells established the better therapeutic efficacy of SRBC alone than other BRMs viz. IL-2 and IFN-γ. Even the effect of combination therapy of different BRMs showed marginal differences in facilitating glioma reduction than the single use of SRBC. These findings emphasized the application of SRBC as an exogenous BRM having the potential as a rational therapeutic adjunct against glioma.

Keywords: Immunotherapy, IL-2, IFN-γ, Glioma, SRBC

Malignant glioma, accounting for more than half of the primary brain tumors, has been constantly offering a difficult challenge to modern medical management due to its unique anatomy and biology. Highly invasive nature of the disease, resistance due to blood brain barrier (BBB) and existence of multi drug resistance (MDR) family proteins render the conventional therapies such as surgery, radiotherapy and chemotherapy ineffective against glioma1-2. Therefore to extend the period of mean survival from 13-18 months3 to at least few years or to eradicate glioma completely, an alternative treatment modality viz. immunotherapy, has emerged. Impaired mediated immune function in glioma patients results from the tumor regulated immune dysregulation with decrease of interleukin-2 (IL-2) and/or interferon-γ (IFN-γ) production and increase in interleukins (IL) such as IL-4, IL-5, IL-6 and IL-10 production4. Release of transforming growth factor-β (TGF-β), prostaglandin E2 (PGE2), IL-2 receptor antagonist also provide an adverse microenvironment and limit the reactivity of immune systems against glioma5. Therefore different cytokines that promote the T helper 1 (Th1) type immune function have been used as immunotherapeutic agents against tumors4. Sheep red blood cells (SRBC), a corpuscular antigen that form rosette with lymphocytes5 have shown to exert immunomodulatory and antitumor property in experimental animal model and found to be effective against glioma6,7. SRBC form a ligand receptor complex with CD2 (cluster of differentiation-2) on lymphocytes by their SLFA-3T11TS (sheep form of leukocyte foreign antigen-3/T 11 target structure) membrane protein5. This interaction has a significant effect on the immune network7, which in turn fight against prevailing glioma. IL-2 and IFN-γ being two most important cytokines in the trial against glioma, in the present study immunomodulatory and tumor inhibitory property of SRBC is compared with these two cytokines.
Materials and Methods

Healthy newborn (5 days old) Druckray rats supplied by Central Drug Laboratory, Calcutta, India and maintained in the laboratory were used. Chemical carcinogen, N-N'-Ethyl nitrosourea (ENU), freshly prepared by dissolving 10 mg/ml in sterile saline and adjusting the pH to 4.5 with crystalline ascorbic acid was injected i.p to 7 days old rats in a dose of 80 mg/kg body weight\(^9\),\(^{10}\).

Endogenous biological response modifiers (BRMs) namely Interleukin-2 (IL-2) and IFN-\(\gamma\) were injected i.p separately and in combination at the dose of 5 \(\mu\)g/kg body weight in 5 months old ENU animals with the exogenous BRM, SRBC (7%, PCV/Vol in normal saline and inoculated 0.5 ml per animal). Therefore, animal groups were as follows: (i) Normal control (N), (ii) 5-6 months old animals injected with ethyl nitrosourea (ENU) (E), (iii) ENU treated animals injected (i.p.) with IL-2 (E2), (iv) with IFN-\(\gamma\) (Ey), (v) with 7%-SRBC (ES) and (vi) with combination of SRBC, IL2 and IFN-\(\gamma\) (E2yS).

Results

Survival — The spans of survival of all age-matched animals in every group (24 in each) were recorded and survival was plotted using a Kaplan-Mayer survival curve. The survival benefit of different BRMs could be obtained by comparing different groups\(^{10}\). For survival study, the day of ENU induction (i.e., day 7 from birth) was considered as ‘day 1’ for all groups including the normal control. Of the 24 animals of untreated tumor control group, 85%-90% survived upto 219 days and the rest died within first 10 days due to the acute effect of the chemical carcinogen ENU induced in neonatal rats. Around 8%-12.5% (2-3 animals out of 24) died due to secondary infection after ENU induction within 60 days. The remaining animals, which survived among all groups then developed ENU induced glioma. Full-grown glioma was found within 5-6 months after ENU induction. Therefore the treatment with different BRMs was scheduled at the 150\(^{th}\) day. After ENU challenge (untreated control) 16 out of 24 rats (i.e., 66.67%) were long-term survivors with a median survival of 192 days, which was increased significantly with the IL-2 treatment (median survival of 299 days, \(P<0.001\)). However with IFN-\(\gamma\) application survival increased to 333 days showing the better efficiency of IFN-\(\gamma\) than IL-2. But a significant improvement of survival was found in the groups treated with SRBC and combination dose of different BRMs (IL-2, IFN-\(\gamma\) and SRBC) in comparison to the untreated control \((P<0.001)\). The
median survival was 629 days in SRBC treated glioma animals whereas in the combination dose treated group it was 626 days having no significant difference between these two groups. Therefore, in comparison to the normal control where the median survival of long term survivors was 691 days, the best options to combat glioma were the last two treatment groups i.e., SRBC treated (ES) and combination dose treated (E2yS) groups (Fig. 1).

Histological evidences — Portions of brain tissues from respective group of animals were subjected to routine histological preparation through histokinate processing. Tissue sections were cut at 5μm thickness and finally stained with routine haematoxyline/eosin. Histological section of normal rat brain showed normal glial cell population with astrocytes, oligodendrocytes and few neurons (Fig. 2a). ENU administered animals at the 6th month of age showed Grade IV oligodendroglioma with mitotic figures, giant cells and absence of intracellular edema, degenerative changes of the cell surface (Fig. 2b), hyperproliferation of oligodendrogial cells were observed. IFN-γ treated group showed reduced cellularity with some lymphocyte margination and infiltration within brain tissue (Fig. 2c). In IL-2 treated animals though cell number was reduced to significant amount than the ENU animals, the presence of oligodendrocytes in higher number than the normal was observed (Fig. 2d). The combination dose of IL-2, IFN-γ and SRBC on the ENU animals showed hypocellularity with edema, degenerative changes of the oligodendroglioma nuclei, deformed cellular architectures indicating the apoptotic nature of cellular destruction and presence of lymphocytes within the brain parenchyma (Fig. 2e). But SRBC alone had shown remarkable effect showing reversion of neoplastic glial features to near normal feature after 6 days of SRBC administration. A number of astrocytes and oligodendrocytes were found to be scattered within brain parenchyma with a few neurons (Fig. 2f). These observations indicate the potential of rejection of glioma by a non-specific BRM-SRBC, in comparison with the specific BRMs like IL-2 and IFN-γ.

Immunological parameters — Spontaneous E-rosetting: Lymphocytes separated from spleen cell suspension on a percoll density gradient (0.25 ml of 3-4 x 10⁹ cells) were incubated with 0.25 ml of 1% (PCV/saline volume) sheep erythrocytes (SRBC) and kept at 4°C overnight. Number of rosettes formed was expressed as rosette (%)¹, which was significantly (P<0.001) reduced in ENU treated animals (2±1.03%) compared to normal control group. E-rosette formation was elevated with IL-2 administration (13±2.4%) in tumor bearing animals and was greater than that was observed with IFN-γ administration (9±4.0%). Administration of SRBC in tumor bearing animals (ES) however showed greater improvement in the number of rosetting lymphocyte (18±2.22%) than IL-2 (E2) and IFN-γ (Ey). Although a combined administration of IL-2, IFN-γ and SRBC (E2yS) in tumor bearing animals showed increase in the number of rosetting lymphocytes (19.5±1.6%), no significant difference (P<0.001) was observed with SRBC administration (ES) (Fig. 3). E-rosette formation between lymphocytes and SRBC occurs due to the specific ligand-receptor interaction between CD2 on lymphocytes and T11T/SLF/3 on SRBC membrane². The upregulation of CD2 receptors that play crucial role in co-stimulation and immunological synapse formation in immunocytes is indicated by the qualitative improvement of the rosette forming capacity² and the results of both ES and E2γS groups are very important in this respect.

Studies on cytolytic efficacy of splenic lymphocytes by HO-33342 release assay: As described previously, HO-33342 (DNA binding fluorochrome dye from Sigma, USA) labeled tumor cells (target) were incubated with splenic lymphocytes (effector) at 10:1 ratio and measured in a spectrofluorimeter (Hitachi, Tokyo). This provided an index of cytotoxic efficacy of effectors of different groups³. Cytotoxic efficacy of lymphocytes (Fig. 4) was significantly (P<0.001) decreased in ENU induced tumor bearing animals (E) compared to normal control animals. Application of IL-2 in ENU group (E2) showed improved cytotoxic activity, which was significantly greater than the ENU group (E). However, IFN-γ could not show any significant changes in the cytotoxic activity of lymphocytes when compared to IL-2 (E2). Significant increase in cytotoxic efficacy (47±3.4%) was observed with a combination dose of IL-2, IFN-γ and SRBC (E2yS) administered to ENU group of animals and was found to be nearly equal to that observed with single administration of SRBC (ES) (49±4.4%). No significant difference was observed within group E2γS and ES (Fig. 4). Effector CD8⁺ T cells or
Fig. 2 — The representative histological features of the brain parenchyma of normal (a), disease control (b) and different treatment groups (c,d,e,f) showed the tumorogenic state and efficiency of tumor rejection by different treatments. In SRBC treated group (f) the reversion of neoplastic glial feature to normal glial feature is prominent (magnification 400X).
cytotoxic T-lymphocytes (CTL) play a crucial role in immune response against tumor cells through different effector mechanisms. Cytotoxicity of lymphocytes is remarkably low in glioma condition than the normal counterparts due to suppressive microenvironment of tumor bearing condition mediated by TGF-β, PGE₂, gangliosides etc. IL-2 and IFN-γ improved the cytotoxic efficacy, but single dose of SRBC greatly enhanced the cytotoxicity of lymphocyte population, even much higher than the normal, showing its efficacy of modulating cytotoxic activity. The combined dose of BRMs also effectively enhanced the cytotoxic capacity, but slightly lower than the single SRBC treatment.

Discussion
The present results indicated the comparative tumor regression efficacy of two endogenous BRMs viz. IL-2 and IFNγ along with an exogenous BRM viz. SRBC. Though all three BRMs applied either separately or in combination contributed to improve the cell mediated immune (CMI) status and subsequent rejection of glioma, separate dose of SRBC and also the combination dose of three BRMs proved themselves as the two best options against glioma. The Kaplan-Mayer survival curve of different groups clearly indicated the more or less similar efficiency of SRBC treatment and combination BRM therapy against glioma extending the survival period of tumor bearing animals at least to the near normal level. Even single dose of SRBC sometimes exceed the combined effects of BRMs and histological findings also support the notion that sheep erythrocyte (SRBC) singly regress glioma most efficiently than IL-2 and IFNγ. This particular BRM-SRBC therapy is found to stimulate most of the major cellular events of the “Immunological Orchestra” and this property is unique in intracranial immunotherapy.

SRBC had been established as a potent immunostimulatory and anti-neoplastic agent but to prove its efficacy in the field of immunotherapeutics, it was essential to compare it with the immunotherapeutic agents IL-2 and IFN-γ which had been used both experimentally and clinically for brain tumor patients. The results revealed SRBC as a superior immunostimulant/antineoplastic agent than these two endogenous cytokines, since SRBC primarily stimulated the Th1 type cytokines which essentially secret IL-2 and IFN-γ and it also stimulates the cytokine network with the release of IL-12 and IL-15 which further strengthen the action of IL-2.

The cells of myeloid lineage in peripheral system viz. macrophage along with the intracranial microglia are the important cells in phagocytosing defective self cells and foreign pathogens, presenting their antigen. Application of different BRMs showed their altered phagocytic activity in ENU induced glioma animals. Begum et. al.¹⁴ found that microglial phagocytic activity was low compared to the PMN and macrophage and was modulated less efficiently by the application of BRMs. However phagocytosis of macrophages and PMNs were modulated significantly with different BRMs than the depressed ENU condition. Here again SRBC administration and cocktail administration of IL-2, IFNγ and SRBC both have demonstrated almost equal level of activation, slightly lower than the individual IL-2 and IFNγ
treatment. Even the antigen presentation of intracranial microglia and peripheral macrophage when compared by poplitial lymph node (PLN) assay among different groups, SRBC treated group showed the most promising result.

These data indicating “a real medical problem” provoked the thought of isolating the immunodominant glycopeptide from the SRBC membrane. Thus the present study was the basis of choosing and more precisely isolating the immunodominant epitope of the SRBC cell membrane i.e T11TS (responsible for E-rosetting) and employing it as the immunotherapeutic adjunct against brain tumor.

Acknowledgement

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

Reference