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Evaluation of Anti-Tumor Property of Specific and Non-Specific BRM's in Experimental Glioma by Assessing the Microglial Cell Functional and Phenotypic Modulations

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ABSTRACT

Background: Microglial cells are considered to be the chief immunomodulatory cells of the brain. These cells play a crucial role against various neurodegenerative diseases. When modulated microglia have been shown to exert a potential anti-tumor immune response against brain neoplasms. Although several specific BRMs like IL-2, IFNγ have been shown to modulate the microglia to get an effective anti-tumor immune response, associated toxicities and detrimental side effects have posed severe limitation in their use particularly for therapeutic purposes. Objectives: In the present study, attempts have been made to elicit the modulations of microglial cell function and phenotypic expression following specific (IL-2, IFNγ) and a novel non-specific BRM (corpuscular antigen) in order to determine their anti-tumor property in an experimental glioma model. Materials and Methods: Brain tumor was experimentally induced in young Wistar rats of both sexes with N-Nethyl nitrosourea (ENU). These ENU treated animals were administrated with both specific and non-specific BRMs (IL-2, IFNγ and SRBC either singly or in combination 5 months after ENU administration and after ascertaining its degree of malignancy. Results: Microglial cells separated from different experimental groups were found to be positive for CD11b, MHC II, CD4+ and negative for CD8+ and CD16+ analysis demonstrated that different subtypes of microglial cell populations (CD25+, MHC II+ and CD25−MHC II−) in the brain tissue based on phenotypic expression, which were downregulated in the tumor bearing animals and subsequently restored with increased expression, particularly with SRBC administration. The Scanning Electron Microscopic (SEM) study also depicted modulatory cellular morphology of microglial cell predominantly with SRBC administration. Single conducted ion evaluate immunological functions at the cellular level showed increased antigen presenting capacity of microglial cell with SRBC administration, which was significantly greater than IL-2, IFNγ and combined doses (IL-2+IFNγ). However immunodulatory functions of microglial cells were not to be significantly modulated with IL-2 treatment. Conclusion: The results suggest that the non-specific BRM SRBC can exert greater modulatory effect on microglial cell function and phenotypic expression than the other BRMs used, and in doing so culminate in a potent anti-tumor immune response in experimental glioma with compatible tolerance profile.

INTRODUCTION

The CNS endogenous glial cells have been shown to take active part in initiating, regulating and maintaining an immune response. Microglia which correspond to 5–15% of total cellular composition of brain tissue are distributed throughout the CNS. Functionally, microglial (Mg) cells are considered to be the chief immunomodulatory cell of the brain. During pathological conditions, resting microglia has been shown to undergo a stereotypic activation process, and become capable of phagocytosis, antigen presentation and lymphocyte activation. The activation of microglial cells have been found in almost every CNS disease processes, including brain tumors or malignant gliomas. Although the function of the cells in CNS inflammatory processes has been studied role in glioma biology is still unclear. On one hand microglia may represent a CNS anti-tumor response, which is activated by local secretion of immunosuppressive factors by glioma cells. On the other hand this microglia have been implicates for being capable of secreting a variety of immunomodulatory cytokines. Bi-directional interaction between immune cells and neuroglial components has been shown to occur in response to infections and traumatic lesions. Recent studies have emphasized "glia-T-cell dialogue", and such interaction may interfacilitate and ampli-
fy immune effector mechanisms within CNS through cytokine secretion, resulting in an antagonistic action. T cells secreting Th1 type cytokines have been shown to provide signals for microglia to mature into functional APC, which in turn stimulate the Th1 cells, and can take part to amplify proinflammatory immune responses. On the contrary the Th2 inducing capacity of microglia has been shown to limit CNS inflammation by producing anti-inflammatory mediators.

Studies have also shown active migration of microglial cells towards gliomas, suggesting the secretion of chemotactic substances by glioma cells although the nature of this factor remains unclear, the in-vitro experiments have also confirmed infiltration of glioma cells associated microglia in rat brain tumor model perhaps to play a role in the local immune-suppressive milieu of brain tumors. Thus, further studies have been focused to look at the mechanisms by which glioma associated microglia can be activated and can be used as immunomodulators.

Several immunomodulatory BRM approaches have been attempted to modulate the microglial cell function with different cytokines. IFNγ administration has also been shown to induce elevated expression of MHC I and MHC II molecules in microglia throughout the brain and induce enhanced tumor infiltration by CD4+, CD8+ and NK cells. Synergistic administration of IFNγ and IL-2 has been shown to release NO, tetrahydrobiopterin from Microglial cells. However, the administration of such BRMs for therapeutic purposes poses severe limitation due to their detrimental side effects, associated toxicities and inconvenient dose scheduling.

Previously, we have demonstrated that sheep red blood cells, which has long been used as a classical antigen can exert potent immunomodulatory and/or anti-tumor property in experimental leukemia and fibrosarcoma. Moreover, while it was administered in experimentally induced brain tumor animals it was found to exert a profound immunostimulatory and/or immunomodulatory effect on peripheral immune system including lymphocytes, PMN and macrophages and ultimately culminating in an effective anti-tumor immune response. Importantly, such immunomodulation with SRBC was found to activate peripheral T lymphocyte and thereby facilitating infiltration of activated lymphocytes into the brain tissue across the blood brain barrier.

In the present study, attempts have been made to ascertain whether such non-specific BRM (corpuscular antigen) (SRBC) can also modulate the microglial (Mg) cell function enabling it to take part in anti-tumor immune response. In addition comparative therapeutic efficacy of Mbc has also been determined with specific BRMs like, IL-2, IFNγ, either singly or in combination.

MATERIALS AND METHODS

Animals. Healthy brown hooded mouse (120) of both sexes were originally supplied by Central Drug Laboratory, Calcutta, India and maintained subsequently in our Laboratory for the purpose of investigations. These animals formed the following groups: (1) Normal untreated controls (N), (2) ENU with inoculation of N-N’ Ethyl Nitrosourea, (III) ENU-SRBC (ES) consisted of rats injected with 7% SRBC. Group (IV), (V), (VI) comprised of ENU treated animals injected with IFNγ (Eγ), IL2 (E2) and combination dose of SRBC, IFNγ and IL2 (EγS) respectively.

The animals consisting of 24 animals in each group were wasted at 30 days of age and housed separately in isolated cages. All animals were fed ad libitum pelleted Hind-Lever pelleted diet and water ad libitum. Rats were fed weekly throughout the experimental period. Maintenance and animal experiments were strictly followed "Principles of Laboratory Animal Care" (NIH) and also local "ethical" regulations.

Induction of Brain Tumor with Ethyl Nitrosourea (ENU). Ethyl N-N’ nitrosourea (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 100 new born (3-3 days old) animals intraperitoneally (ip) with an acute dose of 80 mg/kg body weight.

Administration of Biological Response Modifiers (BRMs) Specific BRMs, Interferon-gamma (IFNγ) and Interleukin-2 (IL-2) (BD-Pharmingen, USA) were injected (ip) separately and in combination in ENU-treated animals (ENU group) after five months following ENU administration at a dose of 5 µg/kg body wt. The immunological studies were performed six days after BRMs administration in such animals.

Non Specific BRM. 7%SRBC was prepared through PCV7 YoL (saline volume) dilution. 0.5 ml of it was inoculated (ip) to the ENU treated animals five months after ENU administration. The immunological studies were performed six days after SRBC administration in such animals.

Isolation and Characterization of Microglial Cells. The culture of microglial cells was made into single cell suspension by resuspending and then passing it through a 80 µm cell strainer. Thus, approximately 30,000–50,000 cells were collected from the interface. The mononuclear cell suspensions were then laid on a 30/60% Percoll gradients at 1000 x g for 25 minutes. 2217 and brain mononuclear cells were collected from the interface. The mononuclear cell suspensions were then laid on a plastic petridish (Coring, USA), incubated for 30 minutes in a CO2 incubator. The resulting cell suspensions were layered on 30/60% Percoll gradients at 1000 x g for 25 minutes. The cells were washed out with PBS-EDTA and then washed thrice, and 2217 cell suspension was digested in collagenase (250 µg/ml) and DNase (250 µg/ml) at 37°C for 5 minutes each. The resulting cell suspensions were layered on 30/60% Percoll gradients at 1000 x g for 25 minutes. 2217 and brain mononuclear cells were collected from the interface. The mononuclear cell suspensions were then laid on a plastic petridish (Coring, USA), incubated for 30 minutes in a CO2 incubator. The resulting cell suspensions and monocytes were collected and washed thrice with PBS. These cells were characterized with the help of the following antibodies (a) anti rat CD11b, with FITC conjugate, (b) anti rat Glial fibrillary acidic protein (GFAP), (c) anti rat CD4, with FITC conjugate and (d) PE labelled anti-rat RT1B.

Survival Study. 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 was taken into account in selecting the animals for the tumor development study.

Morphometric Changes and Ultrastructure of Microglial Cell Under Scanning Electron Microscope (SEM). The Mg cells were prepared for SEM study as described below. They were fixed in 2.5% glutaraldehyde overnight at 4°C followed by washing in PBS and dehydration in graded alcohol (50-95%) for 40 min each and absolute alcohol overnight, and then washed thrice with PBS. These cells were characterized with the help of the following antibodies (a) anti rat Glial fibrillary acidic protein (GFAP), (b) anti rat CD4, with FITC conjugate and (d) PE labelled anti-rat RT1B.

SACS Analysis of Microglial Cell. The murine microglial cells were assayed by the following monoclonal antibodies. FITC labeled anti-rat CD-3 (IL-2R) and PE labelled anti-rat RT1B (Beckton Dickinson, USA). The cells were prepared and analysed on FACS caliber (Beckton Dickinson, USA) (Agen Laser, excitation at 488 nm, 515 band pass filter) as described above by using Cell Quest Software (Beckton Dickinson, USA). Adjacent isotype controls were also maintained in all the groups.

Functional Assays for Cellular Immune Responses

Some experiments were performed in 6 weeks old mice. These mice were separated as (described before) from normal, ENU groups with different BRMs (SRBC, IFNγ and IL2), were injected at a cell concentration of 1 x 106 cells/ml into the hind leg footpad of separate normal rats. The contralateral footpad was injected with same volume of NaCl reconstituted. Mice were maintained with the injection of Mg of the same group in contralateral footpads. After 6 days, the rats were sacrificed, popliteal lymph node was taken out and weights taken in a electronic balance and computed for antigen presenting capacity.

Histopathology. The Mg and PMN cells were prepared from splenic tissues of the above group of animals. Mg were washed from the adherent layer with PBS-EDTA while PMN were isolated from the adherent layer by a 70% Percoll density gradient centrifugation (Harbour 1989, Chandhok S 1991).
The Mg cells (separated as above) and PMN cells from normal and ENU groups with different BRMs (SRBC, IFNy and IL-2), were tested for phagocytic activity by Nitroblue Tetracolium Reduction assay (NBT).

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, USA). A probability of <0.01 was considered statistically significant.

RESULTS

The Mg Cells Are Mostly CD11b+. FACS analysis revealed that the isolated cells showed positivity towards CD-11b, CD4 and MHC-class II receptor. The positivity for CD-11b was high which distinguishes the cells from the brain Mg. However the cells did not show any positivity for GFAP thereby distinguishing them from the other glial cells.

Improvement of Survival with SRBC Administration Suggest Its Anti-Cancer Property. The average survival for the normal control animal was found to be 705 ± 35 days (n = 24). In contrast, the average survival value for the ENU treated animals was significantly (p <0.001) reduced to 190 ± 30 days. IL2 administration in ENU treated animals showed survival data of 595 ± 18 days which was slightly better than that with IFNy (345 ± 15 days). However, cocktail administration showed almost equal survival (625 ± 20 days) as with separate application of SRBC (650 ± 35 days) (Fig. 2).

SRBC Can Bring About Ultrastructural Changes of Microglia. SEM study with Mg cells with and without the application of BRMs in normal and different experimental groups revealed interesting features (Fig. 3). Scanning electron microscopic images of microglial cell showed retraction of the cytoplasmic extensions with ENU-treated group (Fig. 3B). Not much improvement was observed with IL-2 and IFNy administration in such tumor bearing animals (Fig. 3C and 3D). Reappearance of the cytoplasmic projections and Microglia was observed with SRBC administration (Fig. 3D). Combined administration of BRMs demonstrated similar results as observed with SRBC (Fig. 3F).

FACS Analysis Has Provided the Evidence for the Presence of Distinct Subsets of Microglia and Their Modulation Following BRM Administration. FACS analysis showed the expression and modulation of CD25 (IL-2R) and MHCII receptor expression on microglial cells with the administration of SRBC, IL2 and IFNy and cocktail dose (E2yS)(Fig. 4). The FACS results are indicative of the fact that various subsets of microglial cells exist according to their phenotypic distribution, in the all the groups no single positivity of MHC Class II were evident as in all PE quadrant of MHC Class II had non-significant results. But another subtype of Mg showed upregulation (15.08) of normal group of CD25 positivity was downregulated by ENU administration to 10.13 with a very slight mupregulation of ENU-IL-2 group (1.99) and further downregulation in the
Studies Conducted at the Cellular Level Show Modulation of Microglial Cell Functions

SRBC Can Modulate Antigen-Presenting Capacity of Microglia and Macrophages. The results showed a significant (p < 0.001) decrease in the weight of popliteal lymph node in ENU treated animals as compared to their normal counterparts (Fig. 5). IL-2 application showed somewhat stimulating effect on Mg and MΦ in vitro and is greater than that of IFNγ. However, greater antigen presenting capacity, as revealed by the increase in ILN weight was observed in E2 group which was even greater than the E2 group. The results also pointed to better antigen presenting capacity by the Mg cells in comparison to MΦ.

Phagocytic Capacity of Microglia, Macrophages and Polymorphonuclear Neutrophils is also Enhanced Following BRM Administration. Normal Mg was found to show low phagocytic activity (0.8 ± 0.002) when compared with MΦ and PMN. ENU treated group showed relative decrease in phagocytic activity (0.3 ± 0.02). IFNγ administration showed greater phagocytic activity than IL-2 in ENU treated animals. Administration of IL-2, IFNγ and SRBC combination in ENU group however could not improve the phagocytic capacity of Mg and was same to that achieved by separate application of SRBC and IFNγ. In fact, E2 and E group exhibited almost equal extent of mild degree of phagocytic activity (Fig. 6).

MΦ-mediated phagocytic activity was found to be modulated with the different BRMs. Phagocytosis was reduced to 1.2 ± 0.21 in the ENU group compared to normal control (2 ± 0.8). IFNγ improved the activity (2.5 ± 0.33) and was more pronounced than IL-2 (1.8 ± 0.16). E2 and E2yS group showed almost equal level of activity (2.7 ± 0.31 and 2.6 ± 0.32 respectively) as exhibited by the E group (Fig. 6). The cocktail administration of SRBC, IL-2 and IFNγ in ENU group showed highly significant increase in phagocytic activity of PMN when compared with the normal (1.7 ± 0.32) and the ENU group (1.1 ± 0.31). The depression was combated somewhat with IFNγ administration (2 ± 0.39) and IL-2 administration(1.6 ± 0.20). However, SRBC administration in ENU group showed significant improvement (p<0.001) in the phagocytic activity of the PMN (2.9 ± 0.21) (Fig. 6).
separately or in combination showed the immunomodulatory as well as anti-cancer property in ENU induced tumor in rat, the most significant efficacy has been observed with SRBC application, which has been evidenced by survival, and immunological findings. This non-specific BRM, has long been found to exhibit tumor-inhibiting effects, and has been explained in terms of immunomodulation. Indeed it has been shown to exert a direct stimulatory effect on the systemic immune system and thereby activating the total lymphokine network. Results suggest that among the BRMs used, the corpuscular antigen SRBC can exert better immunomodulatory and anti-cancer property particularly on microglial cells within the brain tissue and thereby help to generate a potent anti-tumor immune response against brain tumor.

The immuno-regulatory property of CNS resident cells, particularly of microglia (Mg), has been found to play a crucial role in brain tumor immunity. When modulated, this cell can facilitate and amplify immune effector mechanisms in CNS, resulting in an effective anti-cancer/anti-tumor immune response. Several investigators have reported role of microglial cells in tumor regression. That activated microglia can secrete number of cytokines having anti-tumor property and capable of secreting nitric oxide, free radicals suggests its potent role in anti-cancer immune response against brain neoplasm. Administration of SRBC in ENU induced tumor bearing animals has demonstrated significant modulation of Mg cell function culminating in an effective anti-cancer immune response. Anti-tumor properties as determined through the immunomodulatory therapeutic efficacy of BRMs is the modulation of microglial cells and consequently the abrogation or inhibition of brain tumor in rats, resulting in increase in survival of ENU-induced tumor-bearing animals. This has long been proved that N\'N\' ethyl nitrosourea (ENU) is the most potent of neurocarcinogens and produce tumors with morphological and biological similarities to naturally occurring neural neoplasms in man and animals. In the present study average survival of animals has been found to be decreased with such carcinogen induced tumor-bearing animals. Interestingly, total survival period has increased significantly with administration of SRBC in ENU treated animals. The result is better than the combined action of the other BRMs. Thus SRBC can be considered as a formidable agent against experimental brain tumor showing anti-cancer property.

It has been previously been found that SRBC when administrated in ENU-induced brain tumor bearing animals (ENU-animals) can potentiate cell mediated immune (CMI) response in the peripheral immune system, including enhanced lymphocyte functions. Most importantly, activation of CD2 lymphocytes has been shown to occur with SRBC administration. Since only activated lymphocytes can infiltrate across the blood brain barrier (BBB) activation or priming of lymphocytes with SRBC at the peripheral immune system thus provides an effective means to recruit activated lymphocytes into the brain compartment in order to carry out immune reactivity, thereby facilitating interaction of glial cells with the activated lymphocytes particularly with microglia (Mg).
ANTI-TUMOR PROPERTY OF SPECIFIC AND NON-SPECIFIC BRMs IN EXPERIMENTAL GLIOMA

Figure 5. A comparative analysis of the PIN assay of microglia and macrophage cells showing microglial cells have a higher antigen-presenting capacity than the macrophages in the ENU and the combination group which were much greater than the normal. Values were highest in the E-SRBC groups, where both cell types showing same capacity as was also evidenced in the ENU12 and a depressed capacity in ENU12N group.

Therapeutic efficacy of SRBC can be attributed to the modulation of microglial cells with specific non-specific BRM. The present study has clearly demonstrated the modulation of brain microglial cell with BRM administration. Scanning electron microscopic (SEM) images of microglial cell have provided evidences for the alteration of cellular ultrastructural topography. Such as, the retraction of the cytoplasmic extension from microglial cells in ENU-induced tumor bearing animals show the gross changes in cellular morphology when compared to normal, suggesting modulation of cellular plasticity during tumor pathogenesis. Significant modulation of such microglial cells following BRM administration depicts the plasticity of the microglial cells. Importantly, reemergence of cytoplasmic projections and filopodia from the cell surface with SRBC administration further strengthens the assumption that SRBC could be a potent modulator for microglial cell function. Such modulations of the cellular morphology with SRBC presumably hint at the possible expression and/or up regulation of cell surface molecules. Although, IL-2 and IFN gamma has also been shown to modulate the cellular morphology of microglial cells but is not attiring as SRBC.

In the present study, IL-2 and IFN gamma used were taken as an indices of most commonly used cytokines in brain tumor therapy and consequentlty SRBC was used as an comparative immunotherapeutic agent for brain tumor. SRBC acts by stimulating the CD2 receptors present on many immunocytes like lymphocytes, NK cells, PMN and macrophages and also microglias (data in press) through its SLFA-3/TL11T5 ligand.

Flowcytometric analysis has revealed some interesting results, showing modulation of cell surface molecules on microglial cells with BRM administration. Moreover, FACS analysis has identified distinct subsets of Mg cells in the brain compartment, based on their phenotypic expression. The current data suggest that no single subtype of Mg cells is involved with BRM administration further strengthen the assumption that SRBC could be a potent modulator for microglial cell function. Significant modulation of such microglial cells following BRM administration depicts the plasticity of the microglial cells. Importantly, reemergence of cytoplasmic projections and filopodia from the cell surface with SRBC administration further strengthens the assumption that SRBC could be a potent modulator for microglial cell function. Such modulations of the cellular morphology with SRBC presumably hint at the possible expression and/or up regulation of cell surface molecules. Although, IL-2 and IFN gamma has also been shown to modulate the cellular morphology of microglial cells but is not attiring as SRBC.

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This has been observed from the PLN-assay, which in fact reflects the cellular/particular antigen immune responsiveness and exhibit a direct co-relation between the MHCI class II expression and PLN weight. The MHCI context and antigen presentation are indeed parallel events for most immunocompetent cells. While determining antigen-presenting capacity through PLN-assay, combined doses of IL-2, IFNγ and SRBC (E2yS) has shown to modulate antigen-presenting capacity of microglia, which is significantly greater than on macrophages. However, the best modulatory effect on APC capacity has been shown to occur with SRBC administration. This demonstrates a positive co-relation between the MHCI expression along with CD25, as evidenced through FACS analysis. It can be presumed that IFNγ secreted from 'SRBC-activated lymphocytes' specifically Th1 type lymphocytes and NK cells, can strongly modulate antigen presenting capacity of microglia and to express MHCI class II molecules, thereby reflecting antigen presentation.

This study demonstrates that effect of SRBC on APC function is significantly greater on microglia than on macrophages particularly in experimental brain tumor.

Studies conducted on the phagocytic activity of the cells of the myeloid series namely Mφ and PMN in the peripheral system and Mφ in the intracranial system showed altered phagocytic activity with the administration of the different BRMs. It has been found that Mφ has very low phagocytic activity when compared with Mφ and PMN and application of BRMs does not significantly modulate the phagocytic activity of the Mφ cells. The phagocytic activity of PMN showed improvement with IFNγ. Moreover, the enhancement of phagocytic activity of PMN seemed to occur by SRBC activated CD4+ lymphocytes indirectly through cytokine network (neutrophil activating factors). Also our previous study has shown that tumoral phagocytic activity is increased in SRBC treated animals.

Moreover, direct activation of PMN phagocytic activity with SRBC is also possible, through CD2 receptor reported to be present on its surface. 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.

Among the different BRMs used, SRBC can exert better anti-cancer property than others by modulating the microglial cell surface receptor expression and its effector function. Moreover, administration of 0.5 ml (p) of SRBC has not produced any unoward reactions or toxicity. In addition, the study has also indicated the presence of receptor for SRBC on microglial cell surface. However such speculation needs further investigations.

In conclusion, it can be assumed from the present study that SRBC as whole cell was used as an effective non-specific biomodulator when injected in animal models induced with brain tumors and its efficacy was better than IL-2 and IFNγ. However in case of human application, the injection of whole SRBC is not suggested, but the immunomodulatory epitope of SRBC (i.e. T11TS / SLFA) which is responsible for such immunomodulatory effect have been isolated in our lab, with better therapeutic efficacy than SRBC and can be injected in human patients. This will minimize the chance of rejection because T11TS or SLFA-3 has a 50% homology with human LPA-3 which is present in different antigen presenting cells (APC) and endothelial cells.

References


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Dear Swapna:

Hereewith I am sending the original corrected copy (red-inked) of your article (GJICSM#1, "Documentation of Immune Profile of Microglia through Cell Surface Marker Study in Glioma Model primed by a Novel Cell Surface Glycopeptide T11TS/SLFA-3") to the Journal office today and the copy of that (black-inked) to you also. Because of our time shortage the Journal office promised to me that they would incorporate all the corrections in your electronic copy and would send you by e-mail for your final approval. In the meantime you will get my hand corrected copy also by Global express mail.

I am sorry for this unusual delay in the publication of these three volumes for the 6th ICSM Proceedings. Personally, I was quite occupied in editing the three volumes of the "Glycoconjugates and Cell Signaling" for last one year. The first part came out last month and the other two parts are coming out of press in next two months. However, with our present effort we are expecting to publish the 6th ICSM Proceedings as a series, "Glycoconjugates and Drug Research" in three parts within May-June of this year. By the end of this month I will be able to send to the Journal Office the corrected articles GJICSM#1 to GJICSM#6 to be printed in the first part of this Proceedings.

On behalf of the Glycoconjugate Journal let me congratulate you for writing a beautiful article and it will definitely enrich the field with hard work of you and your collaborators. With my best wishes,
Cordially,

Subhash Basu, Ph.D., D.Sc.
Editor, Glyco. J. -Special issues

PS- Please see the attached list of all the papers to be published in the three parts of this, “Glycoconjugates and Drug Research”

GJICSM#1-SC-040804
Research Paper

Differential Regulation of the Protein Tyrosine Kinase Activity Following Interleukin-2 (IL-2), Interferron Gamma (IFN-γ) and SRBC Administration in Brain Tumor-Induced Conditions

SRBC Acting as a Dual Potentiator in Regulating the Cytokine Profile

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ABSTRACT

Protein tyrosine kinases (PTKs) act as an important class of signal transducer in cytokine-mediated signaling. Defects in phosphorylation of tyrosine residues of intracellular substrates of the immunocytes are a noted phenomenon in glioma induced immune suppression. Administration of BRMs like Interleukin-2 (IL-2), Interferon (IFN-γ) and SRBC in glioma induced experimental models, improved their survival status. In the present study focuses on the differential modulation of the protein tyrosine kinase activity in lymphocytes and microglia following the administration of the 3 BRMs. Our findings indicate that PTKs actively transduce signals on administration of exogenous IL-2. But exogenous IFN-γ administration fails to elicit the enzyme activity. With SRBC administration a differential PTK activity modulation was observed in the two Immunocytes. SRBC not only shifted the cytokine profile to Th1 subset of lymphocytes but also simultaneously upregulated the expression of the activation marker IL-2Rα/CD25 thereby resulting in auto-activation of the hosts immunocytes.

INTRODUCTION

T lymphocyte receptor interaction between the T cell receptor and the Peptide-Major Histocompatibility Complex (TGR-pMHC) forms the hallmark of T cell activation. The TCR-pMHC engagement initiates the tyrosine phosphorylation cascade. Tyrosine phosphorylation of intracellular substrates is amongst the earliest of events playing part in the activation of immunocytes. Defects in the phosphorylation of the intracellular substrates in pathologically diseased conditions bring abnormalities, in the immune cell-to-cell interactions within the host system. In fact when, purified T cells obtained from glioma patients were studied, numerous defects in the early transmembrane signaling were observed. The weak interaction of immune related cells in glioma may be due to multifactorial reasons like altered substrate tyrosine phosphorylation patterns within immunocytes, reduction in protein tyrosine kinase levels, inability to acquire and respond to cytokines (like IL-2) and/or may be due to altered cytokine receptor (Interleukin2R, IL-2Rγ) expression downregulation. Hence, deregulation of the cytokine signaling as observed in glioma conditions provides a platform to readily biological response modifiers (BRMs) (which include cytokines like Interleukin and Interferon) as a modality of treatment. In augmenting the immune response in glioma induced immune suppression. Previously it has been reported by us, that a single dose administration of IL-2, IFN-γ and SRBC in glioma induced experimental models, improved their survival status. SRBC was shown to exert maximal immune boosting both at peripheral and intracranial level. The up-regulation of the functional activity of the intracranial immune components, the microglia, the most important immunomodulatory cell of the brain, along with the peripheral immune components, the lymphocytes, clearly points out to a cytokine cross talk between the two immune components that play an orchestrated role in tumor cell clearance. However, all cytokine signaling processes do not involve the protein tyrosine kinase activity. In the present course of study attempts have been made to elucidate the modulation of protein tyrosine kinase activity in cytosolic and membrane components of microglia and lymphocytes during brain tumor growth and after the application of different BRMs (IL-2, IFN-γ and SRBC) in animal models with reference to age matched healthy animals serving as controls. In the above context it was also checked whether the above BRMs traverse through the PTK pathway for the signal transduction effecting in appendage of tumor cells.

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**MATERIALS AND METHODS**

**Animals**. Male Swiss Webster mice, 9 weeks old were supplied by Central Drug Laboratory, Kerala, India were used as experimental animals and subsequently maintained in the laboratory. They were housed in groups of 5 mice in each cage and were fed ad libitum on a control diet. The animals were housed in climate-controlled rooms with ambient temperature of 22°C. In the laboratory, Kolkara, India were used as experimental animals. The experiments were carried out in accordance with Institutional Animal Ethical Committee. The animals were treated with a single IP injection with 0.5 mL saline and the membrane (0.07 ± 0.007) fraction of microglia compared to KNU.

**Preparation of Membrane Fraction**. After the removal of the supernatant (cytosolic fraction), the pellets were dissolved in hypotonic brain buffer, and the suspension was homogenized in a Dounce homogenizer. The homogenate was centrifuged at 10,000 × g for 30 minutes at 4°C. The pellet collected and resuspended in hypotonic brain buffer and the result was confirmed as the membrane fraction.

**Protein Tyrosine Kinase Activity of ENU Induced Animals Administered With Specific Ilrinos (IL-2 and IL-4). With IL-2 administration a significant increase in the PK activity was observed in both the cytosolic (0.33 ± 0.07) and membrane (0.18 ± 0.02) fraction of microglia compared to ENU induced animals. Lymphocytes of specific origin (0.17 ± 0.03) and the membrane (0.93 ± 0.03) activity was observed to be 10,000 × g for 10 minutes at 4°C. The homogenate obtained was centrifuged at 10,000 × g for 10 minutes at 4°C. The cytosolic supernatant fraction was carefully collected.

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After obtaining the cytosolic and membrane fractions, the assay procedure was initiated by adding the substrate (protein tyrosine kinase, 50 µL) to each fraction separately. Reaction was initiated by adding 10 ng/ml of 4-nitro phenyl phosphate (4NPP) to each fraction, and the reaction progress was monitored at 365 nm. The absorbance was read at 365 nm, and the absorbance was corrected for background.

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Figure 2 (Above). PIK activity of the membrane and cytosolic fraction of lymphocytes.

Figure 3 (Above). Comparison of the cytosolic fraction of microglia and lymphocytes.

(0.06 ± 0.007) also showed a significant (p < 0.001) hyperstimulation of the enzyme activity from their ENU counterparts. In both cases recovery did not reach the normal levels. In contrast, with ENU administration no recovery was observed when compared with their normal counterparts. A significant (p < 0.01) decrease in the PTK activity was observed in case of both microglial membrane fraction (0.03 ± 0.004) and cytosolic (0.06 ± 0.007) fraction but in case of lymphocytes the membrane fraction (0.04 ± 0.007) showed a significant decrease in the PTK activity while the cytosolic (0.07 ± 0.01) fraction showed a significant decrease in the enzyme activity, when the values were compared with that of ENU induced animals, though all values were significantly larger than normal counterparts.

Protein Tyrosine Kinase Activity of ENU Induced Animals Administered with Non-Specific BRM (SRBC). Following SRBC administration, the microglial cytosolic PTK activity (0.11 ± 0.009) increased significantly (p < 0.002) as compared to ENU induced conditions while the membrane enzyme activity (0.07 ± 0.01) was not significant when compared to that of ENU animals. In case of lymphocytes the cytosolic (0.03 ± 0.000) PTK activity increased but the membrane enzyme activity (0.06 ± 0.01) increased significantly (p < 0.005) when all the results were compared with the corresponding ENU values. The recovery rates were however significantly lower than normal levels.

DISCUSSION

In the present study, we have tried to adjudge the signal transduction pathways operative at the intracranial and peripheral levels by quantifying the protein tyrosine kinase activity, in experimentally induced glioma models and their normal counterparts following the administration of the three BRMs—IL-2, IFN-γ and SRBC. A significant decrease in PTK activity in both cytosolic and membrane fraction could be observed in ENU induced conditions in both microglia (intracranial immune component) and lymphocytes (peripheral immune component) with respect to their normal counterparts. The result is in accordance with the fact that glioma patients exhibit a broad suppression of the cell-mediated immunity. Immuno-suppression in glioma patients is a multi-factorial event. Glioma cells are known to secrete multiple factors such as TGFβ1,17-23 PGF2α,18 [IL-2]-26-29 and gangliosides (GANGs—sialic acid-containing glycosphingolipids)30-32 that can modulate lymphocyte responsiveness. GANGs are also known to suppress Ca2+ influx in T cells,33 a defect noted in T cells obtained from patients with gliomas. In our study, the reduction of PTK activity in lymphocytes as well as microglia corroborates to the fact, that the consequence of reduction of PTK activity, is the reduced tyrosine phosphorylation of the substrates within the said immunocytes. Hence the alteration in the expression of cellular protein tyrosine kinases will ultimately lead to the abnormal pattern of basal tyrosine phosphorylation. Therefore the expression of the important tyrosine kinase signal transducers such as p56lck and p59fyn,2 two Src family of protein tyrosine kinase will also be altered in both the immunocytes.3

Prior studies had suggested a critical role of lymphocyte specific protein tyrosine kinase p56lck in early T cell activation events triggered via the TCR including CD3 chain ITAM motif phosphorylation and subsequent activation of the tandem SH2 containing ZAP-70 kinase in a serial engagement process.34,35 A second non-receptor protein tyrosine kinase p59fyn also has been shown to participate in TCR signal transduction.36 Earlier studies had pointed out the presence of Src kinase in the perinuclear region of microglia.37 The reduction in the PTK activity in microglial cytosolic fraction thus points out that the Src kinases are not phosphorylated in ENU induced conditions. The reduction in the membrane PTK activity of microglia can be attributed to the fact that the phosphorylation of receptor tyrosine kinase of the membrane as well as the intrinsic tyrosine moieties present in other microglial receptors do not occur.

Membrane PTK activity of lymphocytes has been significantly (p < 0.001) lowered in ENU conditions. In glioma induced immune suppression, CD3 chain of TCR-CD3 complex cannot be detected,2 indicating that with the disappearance of the ITAMs of the CD3 chain, the phosphorylation of the receptor is impossible and hence the significant decrease in the needed PTK activity. The cytosolic PTK activity accordingly is also reduced but very insignificant amount of the enzyme activity might be operative obtaining cues from other receptor-induced signals.

Immunostimulatory agents like IL-2 have been used in the treatment of brain tumors to arouse or heighten the immune response. IL-2 induced effects are mediated by heterodimerisation of two related transmembrane proteins of the hematopoietin receptor family that are designated as IL-2 receptors β and δ (IL-2RB and IL-2RD). In addition, a third nonconforming protein with short cysteplastic
domain (TCR or IL-2Rα) represents an accessory receptor subunit that can serve as a positive affinity modulator through its regulated expression. In glioma conditions, immunocytes fail to express normal levels of the IL-2Rα/CD25 because of an inability to express the α chain on its surface. Hence the absence of IL-2-IL-2Rα/CD25 interaction results in dysregulation of IL-2 production leading to immunocytes unresponsiveness. IL-10 liberated by gliomas has also been shown to reduce the antigen presenting capacity of monocytes by down modulating MHC Class II expression, thus hindering antigen stimulated proliferation of T cells. IL-10 mediated suppression of human T cell proliferation could be overcome with exogenous IL-2. Our observation also showed that with exogenous IL-2 application in gliomas-induced conditions, significant increase (p < 0.001) in the PTK activity was observed in cytosolic and membrane fraction of lymphocytes and in the membrane fraction of microglia membrane. Inact the PTK activity was restored to near normal level in Microglia. Thus Microglia was able to regain the functional activity of IL-2Rα/CD25 subunit and the intrinsic IL-2 receptor associated tyrosine moieties were phosphorylated. Our observations thus are in accordance with the previous findings of Taga and Tosoto (1992) and hence in murine conditions exogenous IL-2 can also restore the activation of T cells by regulating the expression of IL-2Rα receptors. Cytosolic fraction of lymphocyte showed significant rise of PTK activity (p < 0.001) as compared to the ENU induced conditions but the insignificant recovery was just significant (p < 0.02) in the case of microglia. SRBC, a long known classical antigen forms ligand receptor complex with CD2 via CD58/CD48/SLFA-3 present on its membrane. This interaction results not only in the initiation of the signaling repertoire within immunocytes but also results in the activation of the concerned immunocytes by regulating the cytokine network. In addition to augmenting adhesions between T cells and their cognate APCs and facilitating TCR-triggered activation, CD2-CD48/CD58 interaction uniquely optimizes T cell response to IL-12. The varied immuno-modulatory effects of IL-12 includes the stimulation of proliferation and production of IFN-γ by T cells and it also has a central role in the development of Th1 type of immune phenotype. Previously it had been reported by us that SRBC (as whole cells) when used as a nonspecific biocompound in animals induced with brain tumors, its efficacy was better than IL-2 and IFN-γ. The immune functional parameters were extremely boosted up with SRBC application. The membrane fraction of lymphocytes did exhibit significant PTK activity but membrane fraction of Microglia and cytosolic fraction of lymphocytes showed insignificant recovery as compared to ENU induced conditions. Signaling through human CD2 is dependent on its 117 amino acid cytoplasmic tail of the CD2 receptor. The membrane linked receptor's cytoplasmic domain has no intrinsic tyrosine kinase (PTK) activity and lacks any tyrosine residues able to serve as docking sites for SH2 domains on phosphorylation. Recent studies have also indicated that the CD2 receptor's extracellular and intracellular interactions are crucial in mediating cellular responses.
domains are differentially involved in regulating T cell activation through interaction with tyrosine phosphatase CD45 and the zeta chain of TCR/CD3/ζ complex. Now CD2-mediated signaling involves the ITAM motifs of the TCR/CD3/ζ complex. ITAMs includes intrinsic tyrosine residues that are phosphorylated on CD2-mediated activation of TCR/CD3/ζ complex and thus accounts for significant membrane PTK activity of lymphocytes. As Microglia lacks the TCR/CD3/ζ complex, we get insignificant membrane PTK activity of Mg. The increase in membrane PTK activity of lymphocytes can then be attributed to the increase in the two factors occurring side by side. SRBC on one hand up-regulates IL-2R/CD25 and simultaneously occurs the T cells to manifest Th1 type cytokines via IL-12 (IL-2 and IFN-γ). A positive feedback loop results from IL-2R/CD25. IL-2 interaction and IL-2-mediated signaling starts involving the intrinsic membrane IL2 receptors (IL2R) associated tyrosine moieties which in the process gets phosphorylated. IFN-γ administered singly was not effective in antigenum activity. Though IL-2 has been reported as a single agent for malignant glioma therapy, but one of the major side effects of IL-2 administration has been a "capillary leak" syndrome leading to anasarca and multiple organ system dysfunction, most notably intestinal pulmonary oedema. In contrast SRBC administration showed maximum immune boosting and also devoid of any side effects in glioma induced conditions.1

Thus the dual potential role of SRBC, where on one hand it stimulates IL-2 production orienting the lymphocytes to a Th1 type cytokine profile and concurrently on the other hand results in immune boosting and also devoid of any side effects in glioma induced conditions.1

1. References
