RESEARCH COMMUNICATION

Preclinical Changes in Immunoreactivity and Cellular Architecture during the Progressive Development of Intracranial Neoplasms and an Immunotherapeutic Schedule with a Novel Biological Response Modifier, the T11TS / S-LFA3.

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Abstract

Among neoplasms, brain tumors are particularly " difficult to treat" because of the partial immune privileged status of the brain and the presence of the blood brain barrier (Selmaj, 1996). Many details of progressive development of brain tumors remain unexplored and elucidation of consequent changes of the immune system with correlated cellular architecture and cell kinetics were the major objectives of the present course of investigations. Our studies have indicated that the primary resistance by the immune system to a growing tumor declines after a certain point, resulting in an immune suppressed state in a tumor bearing individual. The poor prognosis of malignant brain tumors with classical treatments like surgery, radiotherapy and chemotherapy has led to interest in immunotherapeutic protocols. In the present study, an attempt was made to determine the immunomodulatory and antitumor properties of a transmembrane glycopeptide of sheep red blood cells (SRBCs), known as S-LFA3 or T11TS. Young Druckray rat of both sexes aged 3-5 days were injected with N'-N'-ethyl nitrosourea (ENU) (i.p) to induce brain tumors and at 2,4,6,8 and 10 months of age were sacrificed for study of survival, tumor growth kinetics and immunological parameters like lymphocyte rosette formation, denoting CD2 – CD58 interactions and phagocytosis by peripheral macrophages and PMN hint at the changes during tumore development. In order to determine the immunomodulatory role of T11TS, 7 month old ENU induced animals and controls were injected with the compound (1 ml., i.p). The data obtained indicate that administration of T11TS results in increased survival of rats along with a decrease in growth kinetics of tumor cells to the normal level when compared to ENU induced animals of the same age. Pointers to mechanisms involving immunological investigations at the cellular level in these animals indicated improved lymphocyte function in terms of E-rosetting, augmented cytotoxicity and enhanced PMN and macrophage function in terms of phagocytosis. Finally histological examination showed complete reversal from the hyperplastic state to normal cellular homeostasis, indicating antitumor efficacy of T11TS, correlating very well with the data from survival and cell kinetic studies.

Key Words: Brain tumors - immunotherapy - biological response modifiers (BRMs)

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Introduction

Brain tumors comprise a heterogeneous group of neoplasms that can arise from any of the constituent elements of the CNS, including neurons, glia, endothelia and meninges, the biologic behavior of any particular lesion being dependent on its cell of origin (Bozik and Gilbert, 1997). For cancer research, experimental animal brain tumor models, which closely resemble human neoplasms histologically and biologically, are very important. The

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discovery of carcinogenic action of nitrosocompounds and their subsequent introduction into the field of experimental neuro-oncology greatly increased the possibilities for the study of pathogenesis of neural tumors (Zimmerman, 1969; Pilkington and Lantos, 1993). Two simple nitrosoamines N-methyl-N-nitrosourea (MNU) and N-ethyl-N-nitrsourea (ENU) have proved to be the most potent of neurocarcinogens, producing tumors with morphological and biological similarities with naturally occurring neural neoplasms in man and animals (Schiffer et al., 1978; Druckrey et al., 1966; Koestner et al., 1971; Lantos, 1993). Neonatal administration of a single dose of ENU produces virtually 100 percent tumor yield (Lantos, 1972).

The diffusely invasive nature of neural neoplasms in human makes the study of the early pathogenesis of brain tumors impossible (Pilkington and Lantos, 1993). Preclinical data before the onset of this deadly disease are very limited. However, immunological studies during the progression of the disease and establishment of correlations with histological findings, survival data and growth kinetics may help decipher the mode of onset of the disease.

Unfortunately, traditional treatments such as surgical tumor resection, chemotherapy and external beam irradiation have done little to alter the progression of this disease (Huncharek and Muscat, 1998; Chambertain and Kormanik, 1998; Liu and Lillehei, 2000). This lack of efficacy demonstrated by conventional therapies has prompted a search for other potentially beneficial therapies (Merchant, et al., 1997). The most promising approach for the treatment of brain tumors is the development of immunotherapy with different biological response modifiers (BRMs) (Ishizawa, 1981). Gillespie and Mahaley, 1995 have reviewed the subject exhaustively. BRMs such as microbial agents (Ishizawa, 1981; De Carvallo, et al., 1977; Berquist, et al., 1980), interferons (Endo, et al., 1986; Takiguchi, et al., 1985; Jacobs, et al., 1986; Nakagawa, et al., 1985), interleukins (Ishizawa, 1981; Berquist, et al., 1980), and SRBCs (Chaudhuri, et al., 1991; Roy, et al., 1997; Chaudhuri, et al., 1993) have been used in the treatment of brain tumors to potentiate the immune system. In experimental animal models, attempts have included combining tumor cells with nonspecific adjuvants (Kida, et al., 1983; Scheinber, et al., 1963), as well as using tumor cells transduced with specific viral allogenic MHC genes (Siesjo, et al., 1993; Yumitori, et al., 1982) or cytokine genes (Colombo, et al., 1997; Mackensen, et al., 1997; Parmiani, et al., 19960; Bubenik, et al., 1996) to augment or enhance their immunogenicity. Sheep red blood cells have long been used as classical antigen has been shown to exerting immunostimulatory and antitumor property in experimentally induced brain and other tumor models (Chaudhuri, et al., 1991; Roy, et al., 1997).

The immune potentiating activity exerted by SRBCs lies in the critical interaction that occurs between the T11 target structure (T11TS) of the SRBC membrane and T11 / CD2 molecules of the different immunocytes (Ebert, 1985; Wilkinson, et al., 1984). The membrane bound immunodominant epitope of the SRBC (T11TS / S-LFA3) has been isolated in our lab and shown to produce potent protective effects against ENU induced brain tumor in an animal model. Thus suitable stimulation of CD2 molecules with T11TS / S-LFA3 activates the different immunocytes with liberation of Interleukin – 2 (IL-2) for clonal proliferation (Fox, et al., 1985).

Though the neurotoxic role of ENU in the formation of neural tumors is well established (Schiffer et al., 1978; Lantos, 1972; Pilkington and Lantos, 1993), assessment of the gradual conversion of the normal cells to neoplasms has not yet been performed. In our present study we tried to elucidate changes chronologically from 2- 10 months after the induction of ENU in neonatal rats, with a focus on survival rate, growth kinetics, and immunological and histological parameters. In addition, the influence of T11TS as an effective immune stimulator on brain tumor regression was assessed.

Materials and Methods

Animals

Healthy newborn Druckray rats, 2-3 days old of both sexes were supplied by Central Drug Laborator, Calcutta,India was used as the experimental animals and was subsequently maintained 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 libitum and housed in a room with ambient temperature of 22^0C in 12hour light / darkness cycle. The animals were grouped in batches of 24 for each experimental group, which are as follows: - (1) Normal control (N), (2). 3-5 days old neonatal animals injected with ethyl nitrosourea (ENU) intraperitoneally (i.p), they were then divided in five groups – (a) animals sacrificed 2 month after ENU administration (E2), (b) animals sacrificed 4 month after ENU administration (E4), (c) animals sacrificed 6 month after ENU administration (E6), (d) animals sacrificed 8 month after ENU administration (E8), (e) animals sacrificed 10 month after ENU administration (E10). (3). 7 months old ENU treated animal's injected (i.p) 1st dose of T11TS (ET1), (4). 7 months old ENU treated animal's injected (i.p) with 1^{st} and 2^{nd} dose of T11TS (ET2), (5). 7 months old ENU treated animal's injected (i.p) with 1^{st}, 2^{nd} and 3^{rd} dose of T11TS (ET3). Rats were examined daily and weighed weekly throughout the experimental period. Maintenance and animal experiment procedure strictly followed "principles of Laboratory animal care. (NIH)" and also local "ethical regulations".

Induction of Brain Tumors with ENU

N'-N'-Ethyl 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 intraperitoneally (i.p.) to newborn rats of (3-5 days old) with a dose of 80mg./kg body weight (Druckray et al., 1966;

Koestner et al., 1971; Lantos, 1993).

Preparation of T11TS from Sheep Red Blood Cell (SRBC)

The method followed steps slightly modified from that of Kitao et al., 1976. Briefly, 1ml volume of packed sheep red blood cells have been incubated for one hour at 37 °C in the presence of trypsin-phosphate buffer (100mg/ml). The red tinted supernatant was removed and then treated with one-quarter volume of 25% trichloroacetic acid to precipitate the non-specific proteins. The clear supernatant was obtained by centrifugation, neutralized with NaOH, and dialyzed against distilled water. Since the glycoprotein is acidic in nature, it was separated from neutral peptides by ion exchange chromatography on a DEAE-Cellulose column (1.5 X 8 cm) previously equilibrated with 0.05M formate buffer, pH 6.8. The acidic glycopeptide was then eluted with a five chamber gradient system containing 1 ml each of (1) water, (2) 0.05M formic acid (3) 0.2M formic acid (4) 0.4M formic acid and (5) 0.4M formic acid in 0.3M sodium chloride. Fractions of each elute (3 ml) were collected and analyzed for absorbance at 280 nm as well as for sialic acid (Weiner, et al., 1973). Finally, rosette inhibition assay was performed with variable concentrations of glycopeptide. Protein estimation of the elutes were determined by Lowry's method (Lowry and Rosebrough, 1951), and also the absorbance emission of different elutes were determined at 280nm.

Administration of T11TS Fraction

The fraction of choice was that having the highest absorbance peak, and also minimum rosette forming capacity as determined by rosette inhibition assay. The dose of injected volume was calibrated from the time-dose responsiveness of rosette inhibition assay. The protein content of the elutes has been determined and also a relation with the body weight was established. The first dose 1 ml of T11TS (i.p.) from the third elute fraction (EF III) was followed by second booster dose on the sixth day and third booster dose on the 12^{th} day, making a dose schedule of 1 ml, 2 ml, and 3 ml to the group 3, 4 and 5 animals respectively.

Survival Study

Rats were examined daily and weighed weekly throughout the experimental period. 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. The survival rate of animals after 2, 4, 6, 8, and 10 month of ENU induction were also recorded. Survival rates were measured by dividing the number of alive animals with the number of total animals taken in each group and then multiplied with 100 for % presentation. Further progressive neurologic signs and weight loss were taken into account in selecting the animals for the tumor development study. Death following injection of ENU and those due to brain tumor were taken into account. Effects of T11TS on survival rate were recorded to evaluate benefit, if any.

$$\text{Survival Rate} = \frac{(\text{No. of rats} - (\text{No. of dead rats on a group}) \text{ the stipulated month})}{\text{Total no. of rats in the group}} \times 100$$

Histological Examination

Portion 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 " 5 " thickness and finally stained with routine haematoxylin / eosin.

Growth Kinetics Of Brain Tumors

Small portions of tumor susceptible areas from all the groups of rats were taken as the samples of tissue culture. Primary explant technique was employed which is briefly described as follows: chopped portions (1mm) were bathed in RPMI-1640 medium supplemented with 10% FBS (Gibco, U.S.A.) in culture dish (35mm. Corning) and incubated overnight at 37^0 C followed by increase in the volume of media 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 5×10^5 cell/ml in separate culture dishes. Cells were grown in steady subculture for obtaining a steady repopulation.

(a) Proliferation index (PI) of cultured cells:

After steady state is achieved, the growth Kinetics of cells as maintained from all above groups of animals were studied in terms of Proliferation Index (PI): 2.7×10^4 cells from each of the groups of animals were freshly inoculated in 3 ml. culture media and incubated for 24 hours at 37^0 C with 4% CO_2 and humidified atmosphere. At the end of 24 hours the cells were washed out with Trypsin-EDTA and finally washed thrice with PBS. Cells were resuspended in 1ml. media in each case and counts were taken in a cell counter. PI was calculated from the ratio of cell count at 24 hours to that at "0 hours" (Chaudhuri, et al., 2000).

(b) Fluorimetric assessment of growth kinetics:

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

Immune Parameters: Studies On Immune Functions

Spleen cells isolated from animals of different groups treated as above have been subjected for cellular immune response studies. For each study, cells from normal group of animals served as normal control.

E- rosetting

Splenic Lymphocytes were separated on a percoll density gradient elution method 0.25 ml of 3 - 4 x 10^6 lymphocytes/ml. were mixed with 0.25 ml of 1% (PCV / saline volume) sheep erythrocytes (SRBC) and incubated for 15 minutes at 37^0 C. It was then centrifuged for 5 min at 200g and was kept at 4 ^{0}C overnight. After 18 hours the pellet was gently resuspended after addition of 1% glutaraldehyde, and lymphocytes with 3 or more SRBC constellation were counted per 200 lymphocytes under a light microscope and results expressed as rosette % (Parker, et al., 1976; Boyum, et al.,1976; Ardaur, et al., 1983; Barran, et al., 1985; Plunkett, et al., 1987; Brand, et al., 1989; Raha, et al., 1990;).

Studies on Cytolytic Efficacy of Splenic Lymphocytes by HO-33342 Release Assay

A newer approach to this method has been adopted using a fluorochrome dye Hoechst 33342 (HO-33342, Sigma, USA). In this method (Law, et al., 2001; Chaudhuri, et al., 2000) HO-33342 binds to DNA of cells irreversibly without leakage until lysed. Tumor cells (target) (a steady glial tumor line, syngenic in nature) were labeled with HO-33342 fluorochrome dye (6μ/10^6/ml) (total incorporation) for 15 minutes at 37^0 C and excess was washed off . Cytotoxicity assay was performed by maintaining an effector (splenic lymphocytes): target ratio at 10:1 through an incubation (37^0C. 4% CO_2 and humified environment) period of 18 hours. Fluorochrome released as per target lysis (experimental lysis) measured in a spectrofluorimeter (Hitachi, Tokyo) provided an index of cytotoxic efficacy of effectors. A labeled target group maintained alone for spontaneous lysis. Results were calculated as follows:

$$\frac{\text{Experimental lysis} - \text{Spontaneous Lysis}}{\text{Total Incorporation}} \times 100$$

Phagocytic capacity of polymorphonuclear neutrophils (PMN) (by tetrazolium blue reduction assay)

Splenic tissues were obtained from 1 to 5 animal groups. Single cell suspensions were prepared by teasing splenic tissues with forceps, followed by percoll density gradient centrifugation. 3 ml of cell suspensions was layered on 5 ml of percoll gradient (density 1.089) and centrifuged for 20 minutes at 800 g. The PMN layer was then removed from the interface, washed thrice with PBS and finally suspended in 1 ml of media. PMN isolated by percoll density gradient (Sp.Gravity-1.089) were allowed to phagocytose the target brain tumor cells (mixed glioma) in presence of Nitro blue tetrazolium chloride keeping the effector: target ratio as 100 : 1. Reduction of yellow NBT to blue formazan indicated the extent of phagocytic burst by effectors concerned. The preparation was incubated for 18 hours at 37^0 C in 4% CO_2 - 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. The intensity of colour assayed spectrophotometrically provided a direct measure of the extent of phagocytosis at 530 nm (Chaudhuri, et al., 1991).

Macrophage phagocytosis assay (Nitroblue tetrazolium Method):

Macrophages were separated by single cell preparations of spleen as in the following: Spleen cells were adhered in a petridish. Non-adherent cells were washed off with PBS, and adherent cells were collected by washings with PBS-EDTA and then washed with PBS, and the procedure of NBT reduction for phagocytic assay was followed as per PMN assay (Hudson and Hay, 1989).

Statistical Analysis

Statistical analysis of results was performed using student's 't' test of the standard deviation from the mean of different data. 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

1. Survival Study

The survival rates of the ENU treated animals were gradually decreased from 2 month to 10 month. An inverse correlation was found between the survival rate and time spent after neonatal ENU administration. The normal animal group showed cent percent survival rate and maximum decrease in survival rate was found in 10 month old ENU induced group (10% ± 2) (Fig – 1).

Total survival of the ENU-treated rats ranged between 96 to 330 days, the average value being 190 ± 30 days. After ENU administration the days of survival significantly ($p << 0.001$) reduced to 190 ± 30 days in comparison to the normal untreated animals (705 ± 35 days). It took about 5-7 months for the induction of brain tumors with ENU, although, in most cases significant progression of neoplastic development was observed within five months. The animals showed neurologic signs of rotatory movements and few other like occasional seizure, tremor etc. ENU administration in animals caused several manifestations including hair loss, exfoliation of the skin, stunted growth were also manifested. Before all the experiments were performed in each animal group, degree of malignancy was determined through histological examination.

Maximum decrease in survival rate was found in 10 month old ENU induced group but since after 7 month the death rate became very high and unpredictable, therapeutic protocols with T11TS were started in 7 months old ENU treated animals. Administration of 1st dose of T11TS in ENU treated 7th month old animals (ET1) showed a significant (p

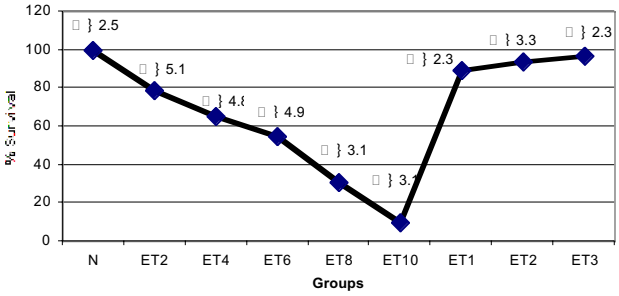

Figure 1. Survival Rate. Normal (N) and ethylnitrosourea (ENU) induced animals at 2,4,6,8 and10 months (i.e E2,E4,E6,E8, and E10) and of ENU-induced groups treated with 1st ,2nd and 3rd booster doses of T11TS (i.e ET1,ET2 and ET3) were assesssed. Maximum decrease in survival rate was apparent in 10 month old ENU induced animals. Application of T11TS however greatly increases the survival rate to the near normal value.

<<0.001) improvement of mean survival time of 610 ± 25days when compared to ENU treated groups. The 2nd booster dose showed further significant (p<< 0.001) improvement in survival (625 ± 15) whereas the maximum improvement occurs after 3rd booster dose (690 ± 18), which is almost equal to the normal value (Fig – 2).

2. Growth Kinetics

After neonatal administration of ENU in 3-5 days old rats, the proliferation index and the fluorochrome uptake study were performed at an interval of 2 month upto 10 month old ENU induced animals. The cytokinetic behavior of the ENU induced tumor cells in the rat brain following

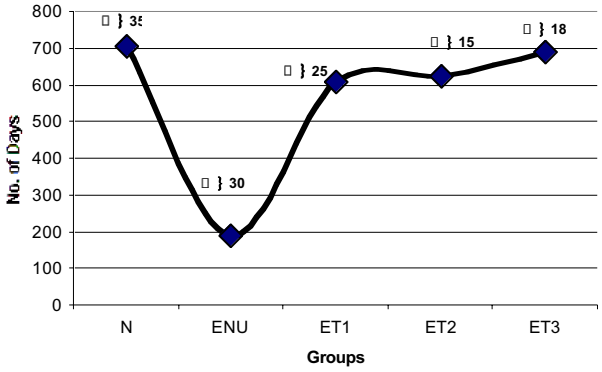

Figure 2. Survival. Mean days of survival of normal (N), ethylnitrosourea induced (ENU) and of ENU induced groups treated with 1st ,2nd and 3rd booster dose of T11TS (i.e ET1, ET2 and ET3).In the ENU induced group the data is significantly lower than the normal (N) animals where as days of survival increases to the near normal value after the 1st dose of T11TS (ET1). 3rd booster dose of T11TS application showed maximum survival in ENU induced animal group.

administration of T11TS, were also performed and expressed as above. The growth kinetics of cultured brain tumor cells in vitro are presented in Fig - 3.

(a) Proliferation Index (P.I):

The cell proliferation index in 2 month old ENU induced (43% ± 4.2) animals showed a significant (p<<0.001) rise compared to the normal untreated control (30% ± 2.4). A steep increase of PI is observed when 2, 4, 6 months old ENU injected animal brain cells were compared (PI = 43% ± 4.2; 59% ± 5.1 ; 70% ± 3.3 respectively). But after the consistent rise upto 6 months, PI of ENU injected animal brain cells of 8 and 10 months did not vary to a great extent (PI = 70.7% ± 1.5 and 71.5% ± 1.1 respectively). ENU injected 7 months old animals were chosen for the immunotherapeutic exposure because the maximum significant increase in PI was observed at a point in between 6 and 8 months and as mentioned before after 7 months the death rate is very high and unpredictable, probably due to the advanced malignant stage. 7 month old ENU induced animals after receiving 1st dose of T11TS showed a significant decrease in PI (35% ± 2.7), compared to 7 months old ENU induced animals. The 2nd and 3rd booster doses did not exhibited such pronounced effects toward cell kinetics of the tumor cells. PI = 33% ± 3.1 and 31% ± 1.8 respectively. Infact, cells from such tumor induced animals

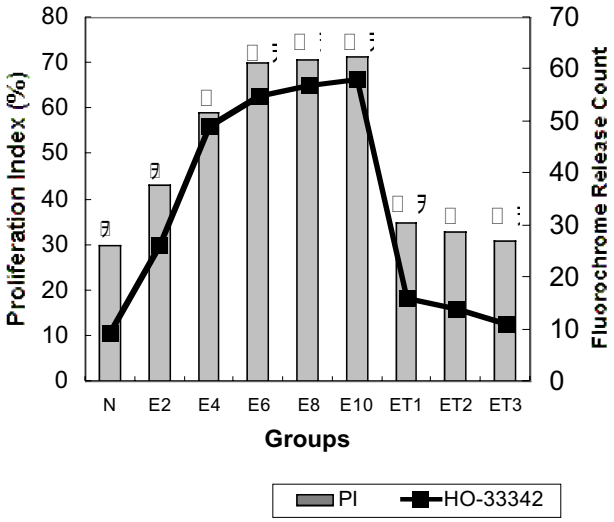

Figure 3. Cell Proliferation Index (PI) of Glial cells. Assessment was before and after induction of tumors and following application of T11TS. The figure shows high counts in tumor induced animals (E6, E8) and near normal counts in T11TS treated tumor groups.

Cytokinetic Index of Glial cells Fluorescent spectra of HO-33342 uptake by glial cells under variable conditions (see Text). The spectra show a significantly higher dye uptake in tumor induced glial cells and near normal values following T11TS treatment in tumor groups.

showed significant (p <<0.001) recovery following T11TS administration when compared 7 – 10 months old ENU induced groups.

(b) Fluorochrome uptake study:

Compared to the normal healthy control, the fluorescent spectra increased gradually from 2 month to 10 month. Maximum fluorescent spectra i.e. six fold increase (compared to the normal untreated control) was found in 6 months old ENU induced animals, which represent a hyperkinetic malignant feature of concerned brain cells but from 6 – 10 months, the fluorochrome uptake showed a near plateau feature as seen in Fig-3. Administration of 1st dose of T11TS was able to limit the cytokinetic process of the ENU treated brain cells. Whereas the 2nd and 3rd booster dose effectively neutralized the hyperplasticity of brain cells and bring back the normal physiology.

3. Immunological Parameters

(a) Spontaneous E – rosetting:

The number of rosette forming lymphocytes significantly (p << 0.001) reduced in ENU treated animals in comparison to the normal control group (N = 18% ± 1.2). Two months after ENU administration the rosetting capacity diminishes to 11% ± 0.8 and maximum decrease of rosetting capacity was observed at tenth month (E10 =2% ± 0.5). Administration of isolated immunodominant group of SRBC i.e. T11TS showed greater improvement in the number of rosetting lymphocytes (20 % ± 1.8), which is significantly (p<< 0.001) higher than the normal value. The second booster dose of T11TS showed maximum rosetting capacity (42.75% ± 2.5). Although the number of E-rosettes was elevated with 1st dose of T11TS administration (20% ± 1.8), maximum improvement was observed with the 2nd booster dose (42.75 ± 2.5) in 7th month old ENU induced animals. ENU administration showed gradual decrease in SRBC constellations of the individual lymphocytes as well as decrease in the total rosette forming lymphocytes. SRBC or T11TS administration to such animals not only increased the total count of rosette forming lymphocytes but also the capacity of individual lymphocytes to form super rosettes with greater SRBC attachment (Fig – 4).

(b) Macrophage mediated phagocytosis:

Phagocytic activity of macrophages in brain tumor immunity has been found to be modulated with the application of ENU. Macrophage mediated phagocytosis was reduced to 0.005 ± 0.001(O.D at 530 nm) in two months after ENU administration and was significantly (p<< 0.001) lower than the normal untreated control (N = 0.037± 0.002) (O.D at 530 nm). Later in the subsequent months after ENU administration the phagocytic activity gradually decreases & maximum decrease occur in the fourth month (0.012 ± 0.001) (O.D at 530 nm). Application of isolated immunodominant epitope of SRBC, the glycopeptide T11TS showed maximum efficacy of the macrophages (0.055 ± 0.004) (O.D at 530 nm) after 1st dose (Fig – 5).

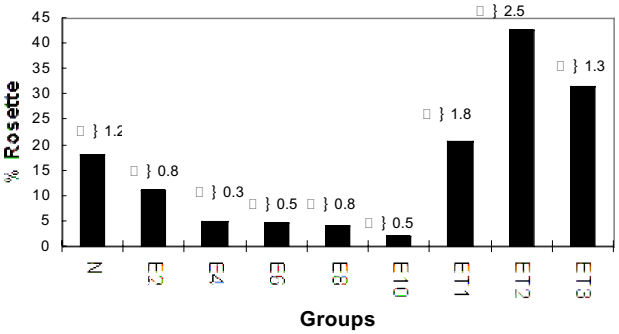

Figure 4. E-Rosette Formation. Rosetting forming capacity of the lymphocytes was drastically reduced in ENU groups (E2 to E10), 1st dose of T11TS administration was found to be effective in bringing back the value to normal level . Second booster dose (ET2) produced maximum rosette forming efficacy in splenic lymphocytes of ENU treated animals.

(c) Cytotoxic efficacy of the splenic lymphocytes:

The lymphocyte mediated cytotoxicity as determined by percent lysis was 30% ± 2.2 in the normal animals which was increased subsequently up to the sixth months (41% ± 3.2) after ENU administration. But sharp decrease in cytotoxic efficacy occurs in the 8th months after ENU administration (16.1% ± 1.4) and maximum decrease was observed at 10th month (14.2% ± 2.1). Significant increase in cytotoxic efficacy (24.83% ± 1.8) was observed when 1st dose of isolated immunodominant group of SRBC i.e. TIITS was applied in ENU induced tumor bearing animals. However, the most significant (P<< 0.001) improvement in cytotoxic activity of the lymphocytes was observed with 3rd dose of TIITS application (51.5% ± 3.1) (Fig – 6).

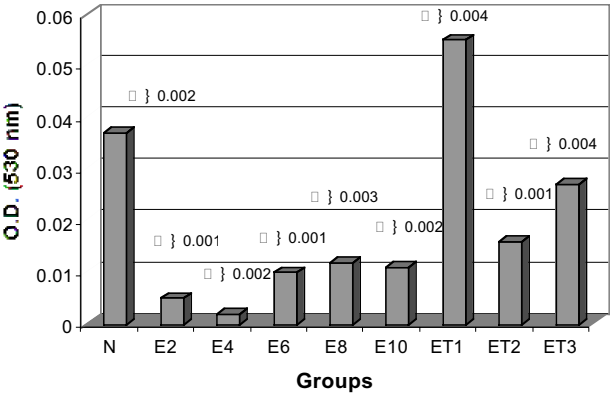

Figure 5. Phagocytic Activity of Macrophages. Values determined by NBT reduction assay showed initial drastic decrease in the first two ENU induced groups (E2 & E4). The next three ENU induced groups showed slight increase in the phagocytic activity (E6, E8 & E10).The 1st dose of T11TS showed the most significant stimulation of macrophages for tumor phagocytosis.

(d) Phagocytic activity of the PMN:

Polymorphonuclear neutrophils (PMN) mediated phagocytosis was assayed by the reduction of yellow nitro blue tetrazolium chloride to blue formazan indicating the extent of phagocytic burst (O.D at 530nm) by the effectors concerned. The phagocytic activity gradually depressed chronologically from 2 months to 10 months after induction of ENU. The lowest phagocytic activity (0.013 ± 0.002) (O.D at 530nm) was observed in the 10 months old ENU induced animals. The ENU treated group receiving first dose of T11TS showed a significant ($p << 0.001$) elevation of phagocytic activity (0.033 ± 0.004) (O.D at 530nm)which is near to the normal level. Further, it was significantly increased with the injection of second booster dose of T11Ts fraction (0.065 ± 0.002) (O.D at 530nm) ($P<<0.001$). No further improvement of phagocytic activity was observed with third booster dose of T11Ts fraction (Fig – 7).

4. Histological Study

Histological studies of the normal animal brain show normal glial cell populations with few astrocytes, oligodendrocytes and a few neurons (Fig - 8a). ENU were applied in neonatal animals and after two months of induction, the histological finding showed hyperplastic oligodendroglial cell populations (Fig - 8b). In fourth month after induction the slide showed gametocytes, acidophilic cytoplasm i.e. eosinophilic, reactive astrocytes, grade-1 astrocytoma (Fig – 8c). A complete reversion of cellular architecture occurred when compared to the normal . The effect of ENU after 6th month of induction showed grade-IV oligodendroglioma with mitotic figure, giant cells and absence of intercellular spacing (Fig - 8d). In 10th month after ENU administration, the slide showed string of closely packed dividing cells, degenerative fibrils,

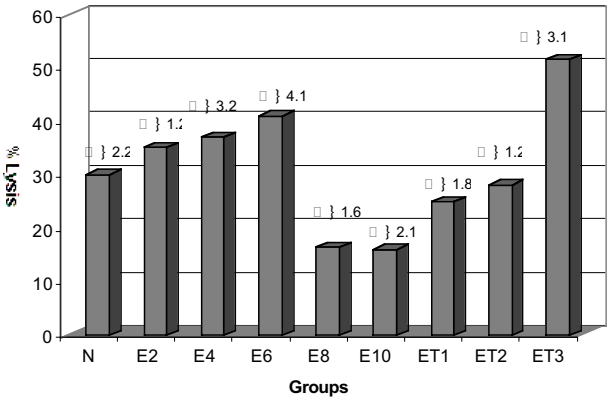

Figure 6. Cytotoxicity Assay Results. CTL-Assays of splenic lymphocytes (SL) were conducted for HO-33342 release in different groups of rats. Significant decrease in cytotoxic efficacy of SL is noted from 8 month after ENU induction (E8 & E10). Third booster dose of T11TS administration (ET3) was found to induce CTL at its maximum in the ENU induced animals.

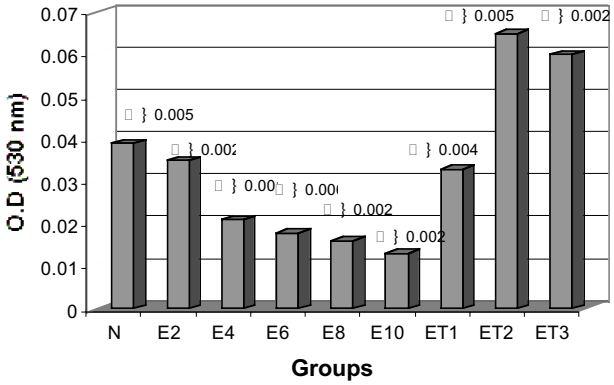

Figure 7. Phagocytic Activity of PMN. NBT reduction assays were conducted for different groups of animals. Gradual suppression of activity is noted from 2 – 10 month after administration of ENU in neonatal rats. 1st dose of T11TS administration restored the activity . The most significant effect was observed with ET2 with highest phagocytic burst by PMN in tumor bearing animals.

oligodendroglioma grade-4, mixed glioma and without any intercellular space (Fig – 8f). Effect of 1st dose of T11TS fraction showed reduced glial population with enlarged nuclei due to increased permeability of nuclear membrane to water. Most importantly the reduction in the number of oligodendroglioma cells with spongiosis and apoptotic figures, lymphocytic infiltration and margination have been observed with this dose (Fig – 8g). The hypocellularity with oedema and degenerative changes and presence of lymphocytes have been observed following administration of 2nd dose of T11TS fraction (Fig – 8h). The third dose of T11TS fraction showed reversion of neoplastic glial features to normal glial features with gliosis. Evidence of cell death may be due to apoptosis and calcification is noted. (Fig – 8i). Finally the effect of T11TS has been observed with the reduction in hypercellularity and reestablishment of normal cellular homeostasis (Fig 8g – 8i).

Discussion

The details of progressive development of brain tumor to a full blown clinical tumor, remains unexplored and the changes of the immune reactivity during the progressive tumor development (preclinical development) in the present course of investigations indicates how the primary resistance offered by the immune system to an ongoing tumor gives vent after a certain point. The intricate mechanisms of cellular immunity following N'-N' ethylnitrosourea (ENU) induced brain tumor development in rats were used to decipher the preclinical status. The important cellular components operative under the instance, namely, the lymphocytes forming the spontaneous E-rosette demonstrating the CD2 receptor modulation, cytotoxic

Figure 8(a) - 8(i). Results of Histological Assessment

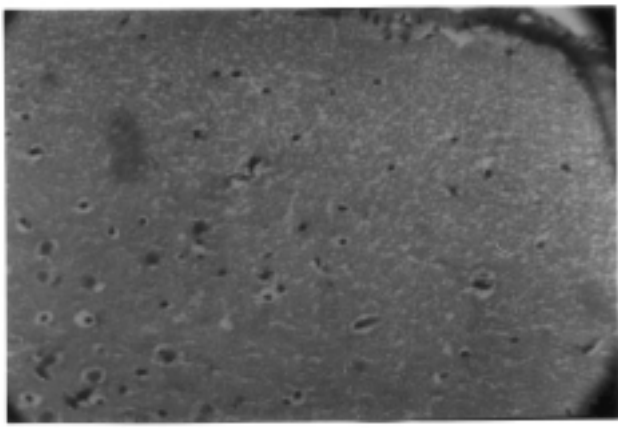

Figure 8(a). Normal Brain Tissue with the Presence of Few Astrocytes, Oligodendrocytes and Few Neurons.

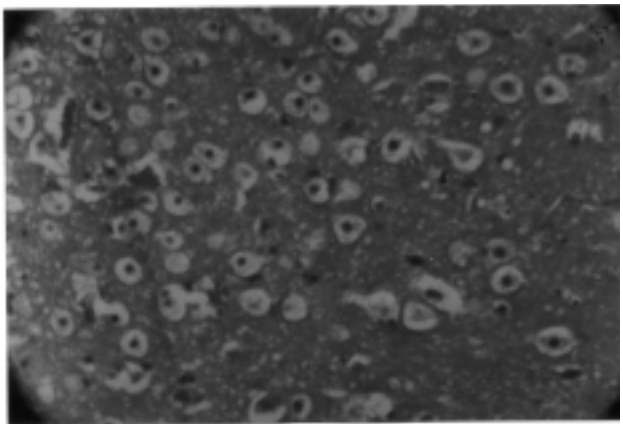

Figure 8(d). Numerous Oligodendroglial cells in Division.

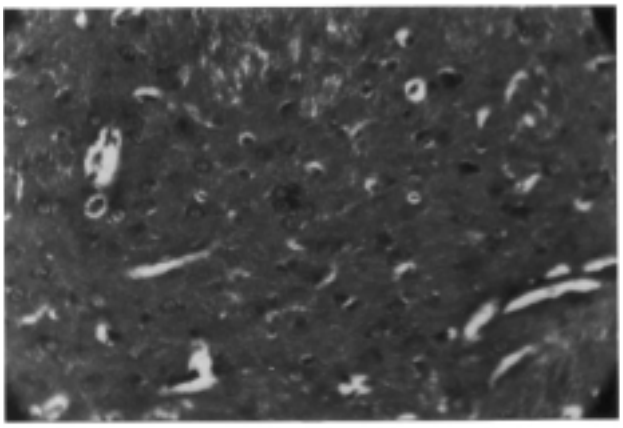

Figure 8(b). Showing Hyperplasia of Astrocytes and Oligodendrocytes with a Clear Nexus of Dividing Oligodendrocytes Seen in the Centre.

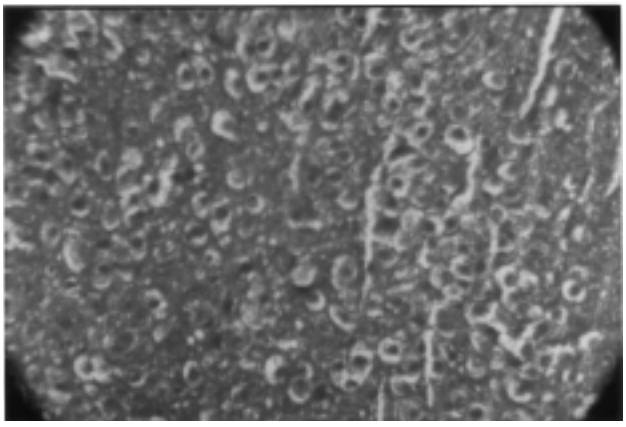

Figure 8(e). Typical Honeycomb like Appearance with Oligodendroglioma.

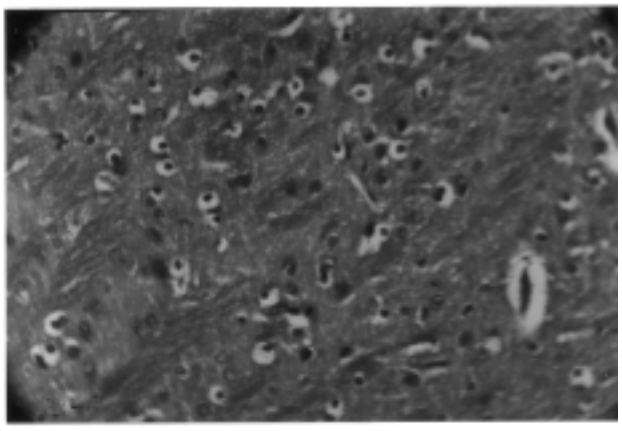

Figure8(c). Increase in the Frequency of Oligodendrocytes with Evidence of Angiogenesis.

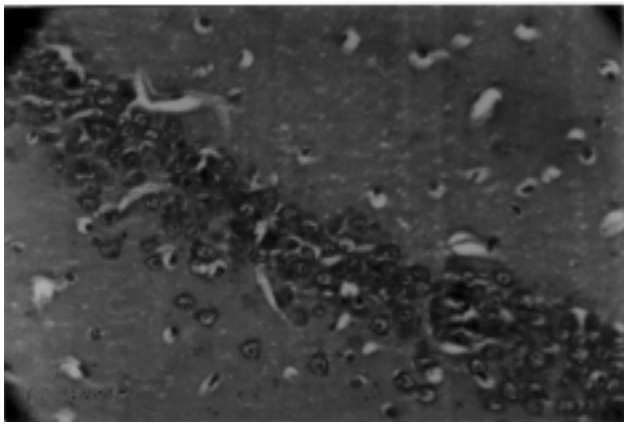

Figure 8(f). A String Closely Packed of Dividing Oligodendroglial Cells with Intervening angiogenesis with Clotted Blood.

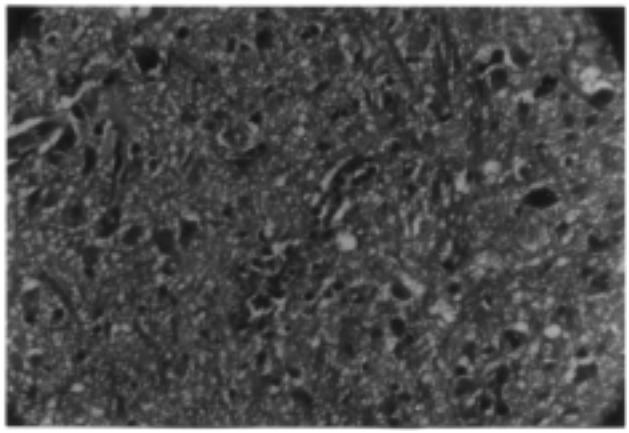

Figure 8(g). Reduced Glial Cell Population with Enlarged Nuclei and Lymphocytic Infiltration and Margination.

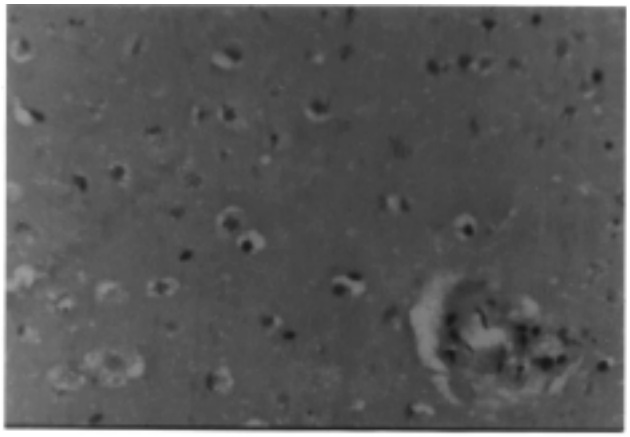

Figure 8(h). Hypocellularity with Oedema and Degenerative Changes and Presence of Lymphocytes.

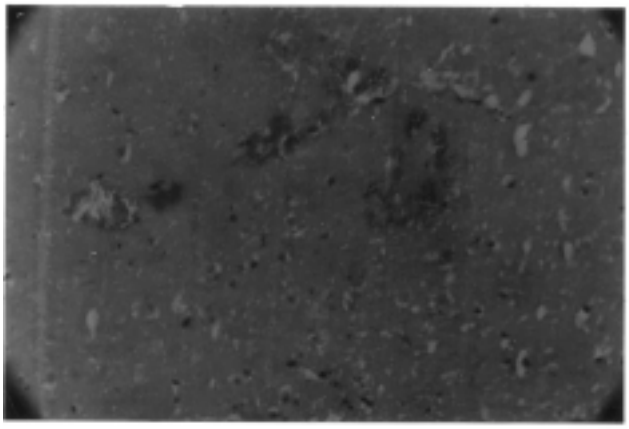

Figure 8 (i). Reversion of Neoplastic Glial Features to Normal Glial Feature with Gliosis and Evidence of Cell Death and Calcification.

efficacy of lymphocytes, phagocytic activity of the polymorphonuclear cells & macrophages in terms of ENU induction and immunomodulation have been documented. Data of the present work showed that tumor progression occurs gradually from 2 months following ENU administration. Chronological assessment of cellular architecture after ENU administration showed the gradual changes from normal to neoplastic stage. The histological sections two months after ENU administration in rats, showed hyperplasia, which indicates the beginning of neoplastic processes. After four months of ENU induction the progressive increase in oligodendroglial cell population indicate initiation of uncontrolled cell division and sixth month onwards, the histological slide clearly showed the specific characteristic features of neoplasia, such as mitotic figures, giant cells and absence of intracellular spacing, degenerative fibrils. Clear evidence of establishment of intracranial neoplasm after ENU administration was found in histological studies (Fig 8a – 8e).

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) and percent fluorescence uptake (HO-33342) of cells at 24 hrs of culture. The uptake of HO-33342 by cells in culture has been introduced on the basis of the binding capacity of the fluorescent material with nuclear DNA which is irreversible and non-diffusible from the cells in culture when administered in optimal concentrations (Chaudhuri, 2000). The method was found to be suitable for studying growth kinetics of cells in culture as it corresponded well with the cell counting index (Fig. - 3). The degree of malignancy as determined by the above method also corresponded well with the higher morbidity rate in the group of animals concerned. Administration of T11TS (1ml i.p.) in 7 month old ENU treated animals revealed some interesting results: total survival period significantly increased, histological studies indicates reversal of normal cellular architecture from the hyperplastic malignant form and also the cell proliferation rate decreases to the near normal value.

The mechanism of ENU action had been worked out previously by several workers (Lantos, et al., 2000; Russel and Rubinstein, 1990; Swenberg et al.,1972). Malignant transformation by alkylating agent is the mode of interaction of ENU on cellular DNA. Oxygen centered base alkylation adducts are directly mutagenic and most current evidence suggests that the most abundant of these, O^6-e^G plays a major role in the mutagenesis and carcinogenesis by ENU (Swenberg et al., 1972; Gowth and Rajewsky, 1974; Margison and Kleihues, 1975; Kleihues et al., 1979). The major O-alkylated base is O^6 -alkylguanine which during DNA replication mispairs with deoxythymidine causing G:C → A:T transition mutation. O^6-ethylgnanine is repaired by O^6 -alkylguanine DNA alkyltransferase (AGAT) & this occurs less efficiently in the brain. This deficiency of the

CNS was considered to be the mechanisms of preferential induction of brain tumor by ENU application (Beranek, 1990)

In the present study significant correlation has been found between progressive tumor development (2-10 months) and immune responses. Compared to the normal untreated group E-rosette formation in different ENU group shows sharp decrease progressively upto 10th months. In rosette forming lymphocytes the "the critical interaction " occurs between T11 target structure /sheep form of LFA-3 (a glycoprotein molecule present on the surface of SRBC) with CD2 / T11 (Pan T- cell marker) molecule (Hunig et al., 1987; Hunig, 1985; Giegerich et al., 1989; Springer et al., 1987). The growing tumor burden down regulates the CD2 molecules of the lymphocytes resulting in decrease in rosetting capacity of lymphocytes in ENU induced brain tumor condition. Gradual decrease of rosette formation from 2 months onward is due to the progressive down regulation of CD2 molecules both per individual lymphocytes and also decrease in the total number of CD2 bearing lymphocytes resulting in decreased lymphocyte count along with decreased rosette count.

The cells of the myeloid series namely macrophages showed altered phagocytic behavior in different ENU groups. After ENU administration the phagocytic activity of the macrophages decrease upto 4th month and this may be due to the decrease in reactive oxygen production. Enhanced phagocytic activity occurs from 6th month onward hinting at the probable arming of the macrophages against the ongoing tumor (Fig – 5). The phagocytic activity of PMN was gradually reduced as age proceed from 2 to 10 months old animals. Maximum reduction in phagocytic activity occurs in 10 months old ENU induced tumor group (E10), as revealed from reduced colour density of the phagosomes (0.013 ± 0.001) (Fig – 7).

The extent of cytotoxic efficacy of lymphocytes, obtained through HO-33342 assay, indicates an alliance between the size of tumor and the secreting inhibitory cytokines (Fig – 6). The cytotoxic efficacy of the lymphocytes takes a progressive upper hand after the administration of ENU in neonatal rats upto 4th month and is sharply depressed 6th month onwards . This may be possible due to the action of the inhibitory cytokine feed back mechanisms exerted by the full grown tumor mass (IL-10, TGF-β, PGE-2) (Bodmer and Strommer, 1989; Hishhi et al., 1995; Kuppner et al., 1990).

Conventional treatment of brain tumor further suppresses the immune system (Diengdoh and Booth, 1976; Nakagaki et al., 1976) which is already suppressed by the factors secreted by the developing tumor. Immunotherapy with different endogenous biological response modifiers (BRMs) have been reported earlier but significant prognosis without side effects were not found. In the present course of investigation, administration of a transmembrane glycopeptide (T11TS / S-LFA3) derived from sheep red blood cell showed a resultant therapeutic effect on ENU induced rat brain tumor. A single administration of T11TS showed interesting improvement of cellular immune status (CMI), compared to the depressed neoplastic condition.

Administration of T11TS has been found to modulate the activity of lymphocytes. Compared to the ENU induced untreated group, E-rosette formation in tumor bearing animals that received a single dose of 1ml of T11TS fraction demonstrated greater rosetting capacity ($20.83\% \pm 1.8$) indicating significant lymphocyte proliferation and activation ($p<0.001$). Upregulation of CD2 molecules in individual lymphocytes indicated by super rosetting and also quantitative lymphocyte proliferation was indicated by significant increase in rosette forming cells. The rosetting capacity of lymphocytes further increased to $42.75\% \pm 2.5$ ($p<<0.001$) with the second booster dose but the third dose ($31.5\% \pm 1.3$), ($p<<0.001$) could not increase the rosetting capacity possibly due to the saturation of the CD2 molecules (Fig – 4).

T cell growth and clonal expansion is a tightly regulated process requiring the growth factor IL-2, and hence the extent of the T cell proliferative response is determined by the concentration of IL-2 available to the cell and the level of IL-2R (CD25) expression on the cell (Hatakeyama, et al., 1991). Fox et al., (1985) demonstrated that activation of human thymocytes via the 50 Kd T11 sheep erythrocytes binding protein induces the expression of IL-2R both on $T3^+$ and $T3^-$ populations. Furthermore, Meuer et al., (1984) hinted at an alternative pathway of T-cell activation through 50 Kd T11 sheep erythrocyte receptor protein. It is possible that a single booster dose of T11TS fraction may generate a strong mitogenic stimulus through T11 (CD2) that closely simulates the view of Meuer and co-workers. It can further be pointed out that the specific interaction between the CD2 (T11) and T11TS (sheep form of LFA3 or sheep CD58) play important role via CD2 mediated co-stimulatory pathway during T-cell activation and proliferation (Koyasu. et al., 1990; Krensky. et al.,1984). Further, it can reverse T cell anergy (Boussiotis. et al., 1994) caused by tumorigenic inhibition. Hatikeyama et al (1991) and Bell et al (1992) demonstrated that such a strong mitogenic stimulus through T11 (CD2) associate src-like protein tyrosin kinase from the surface of the T lymphocytes.

A linear relationship with the dose of T11TS fraction to the cytotoxic efficacy of lymphocytes has been obtained through HO – 33342 incorporation assay. Since T11 (CD2) is a pan Tcell marker. The administration of T11TS invivo may involve the CD4+, CD8+ and NK cells (Semnanirt, et al., 1994). The activation of CD4+ T cells through T11TS provide means to recruit the other effector cells such as macrophage and iMf, PMN. Activation of CTL and NK function through the T11 sheep erythrocyte binding protein have also been demonstrated by other workers (Dustin et al.,1985; Moingeon. et al., 1989; Siliciano et al., 1985). Data of the present attempt strongly indicate the linear increase of cytotoxic efficacy of lymphocytes in tumor bearing animals with 1st (24.83 ± 1.8) ($p<<.001$), 2nd (28.1 ± 1.2)($p<<.05$) and 3rd booster dose of T11TS (51.5 ± 3.1) ($p<<.001$) (Fig – 6).

Macrophage mediated phagocytosis was found to be activated with the application of T11 target structure. Significantly higher ($p<0.001$) & optimum activation (0.005 ± 0.003) was observed with the first booster does of T11TS fraction in tumor bearing animals (fig 5). These data suggest lymphokine-mediated activation of macrophaghes that might occur in a dose dependent manner. Activation of $CD4^+$ cell may recruit the other effector cells as well as macrophage, and PMN (Gutierrez et al.,1999).

Results of phagocytic activity of polymorphonuclear neutrophil has been expressed in a dose-dependent manner. The phagocytic activity of PMN with the first dose of 1 ml of T11TS fraction (ET1)reaches the normal level, the second booster dose (ET2) showed the highest stimulation (0.065 ± 0.002) ($p<<.001$) and the third dose keeps a near parity with the second dose (Fig.-7). It is possible that activated lymphocytes in the T11TS treated animal recruited PMNs indirectly through the cytokine network (neutrophil activitating factor). Our previous study (Chaudhuri et al, 1993) has shown that tumoricidal phagocytic activity of PMN is increased in SRBC treated animals.

Immunological findings have been strongly corroborated with the histological findings. Administration of T11TS fraction in ENU treated animals demonstrated the anti-tumor activity in a dose dependent manner as evidenced through the reversion of neoplastic glial features to normal glial features. Infiltration of lymphocytes and margination of the endothelial lining in the brain is the hallmark of the 1st dose of T11TS administration in ENU animals. The hypocellularity observed in the first dose indicates the death of neoplastic cell population, may be by apoptotic machinery. The distorted nuclei observed after the 2nd dose give a strong evidence that maximum of the neoplastic cells are on the process of death. Finally the third dose leads to total clearence of the total tumor mass and a distinct calcification (Fig - 8f – 8g).

The present work delineates the preclinical changes occurring during the development of intracranial neoplasm. Immunological parameters indicated that rosetting capacity of lymphocytes progressively decrease during tumor development, whereas the phagocytic activity of the macrophages decreased upto 4th month but increased from 6th to 10th month. The initial decrease of phagocytic activity of macrophage may be due to the time taken by the macrophage to arm itself against the tumor cells. But the cytotoxicity of the lymphocytes decrease gradually during brain tumor development showing an effective relationship between intracranial immunity and peripheral immune system, where any change in intracranial immune reactivity shows its reflection on the peripheral counterpart, probably by intervention of blood brain barrier. The therapeutic effectiveness of the immunodominant epitope of SRBC i.e. T11TS or SLFA-3 as isolated, exhibited potentiating role on experimentally induced brain tumors in rats by way of augmenting the CMI function. The interaction of CD2/T11 with T11TS/SLFA3 may play an important role in T-cell activation and proliferation and thereby inducing activation of other immune cells like macrophages and PMN through the cytokine network. The administration of T11TS (i.p) was found to produce no adverse side effects and / or toxicity in the animals concerned.

The observation of the present work provided us the impetus to study the mechanism of action of the T11TS in detail. Thus our ongoing work aimed at the purification & characterization of T11TS for its future application in human.

The subjects of the above findings are covered under pending Indian Patent Application.

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