Immunotherapy with T11TS / S-LFA-3 Specifically Induces Apoptosis of Brain Tumor Cells by Augmenting Intracranial Immune Status

JOYDEEP MUKHERJEE, ANIRBAN GHOSH, PALLAB SARKAR,
MALABIKA MAZUMDAR, CHANDRA BANERJEE and SWAPNA CHAUDHURI

*Cellular and Molecular Immunology Laboratory, Department of Physiology, Dr. B. C. Roy Postgraduate
Institute of Basic Medical Sciences, Institute of Post Graduate Medical Education and Research,
244 B, A.J.C.Bose Road, Kolkata - 700 020, West Bengal, India*

Abstract. *Background: Immunopotentiating agents are the best options in cancer therapeutics because they can specifically destroy tumor cells via immunocytes, which are mostly apoptotic in nature. Previously, immunotherapy with T11TS / SLFA-3 in a ethyl-nitrosourea (ENU)-induced animal model (Druckrey rats) of neural neoplasm showed a significant tumor mass destruction by augmenting the cellular immune status. Materials and Methods: The modulations of the peripheral as well as neural immune systems after T11TS administration were monitored by assessing the CD4+ and CD8+ lymphocytes, along with the cytotoxic activity of splenic and brain infiltrating lymphocytes (BIL). The rate of apoptosis of the tumor cells, microglial cells (Mg) and BIL were measured by flow cytometry-based propidium iodide analysis and TUNEL assay. Results: Cell cycle phase distribution analysis by propidium iodide –FACS and TUNEL assay revealed that T11TS administration gradually increased the number of apoptotic brain tumor cells and, at the same time, decreased the number of dividing cells. Up-regulation of the CD4+ and CD8+ lymphocytes were observed after T11TS administration in ENU - induced immunosuppressed animals. A gradual increment of cytotoxicity of splenic and BIL was also demonstrated after successive administration of T11TS.*

Correspondence to: Dr. Swapna Chaudhuri, Cellular and Molecular Immunology Laboratory, Department of Physiology, Dr. B. C. Roy Postgraduate Institute of Basic Medical Sciences, Institute of Post Graduate Medical Education and Research, 244 B, A.J.C.Bose Road, Kolkata - 700 020, West Bengal, India. Tel: +91-033-2557-3147 (R), Fax: +91-33-2435-5360, 2223-3300, e-mail: swapna_chau@rediffmail.com

Conclusion: These data strongly support the specific apoptosis-inducing role of T11TS in experimental brain tumor cells. Apoptosis of BIL and Mg, that occurred to a much lower level, can be explained in terms of changes in the neural immune system before and after T11TS application.

Brain tumors are the most difficult to treat, presumably due to the presence of the blood-brain barrier (BBB) (1). The Central Nervous System (CNS) is normally subject to immunological surveillence *via* non-antigen-dependent, non-inflammatory T lymphocyte patrolling (2, 3). The interaction between patrolling T lymphocytes and resident brain cells is of particular interest in relation to the up-regulation of molecules involved in antigen presentation, such as MHC-II (4). However, glioma-generated factors (IL-10, TGF-beta, PG-E2) minimize the immune functioning of different residual and infiltrating cells of the brain, followed by peripheral immune suppression (5). Under normal conditions, apoptotic cell death is an established mechanism for the elimination of changes associated with inflammation in the CNS (6, 7). Microglia (Mg) is the strong candidate for the clearance of cells undergoing apoptosis (8, 9).

The neoplastic changes associated with ethyl-nitrosourea (ENU) induction have been established by our group (10-13) in histological, cell kinetic and immunological levels, rendering drastic reduction of survival of the brain tumor-bearing animals. Previously, we showed that the sheep form of leucocyte function antigen-3 (SLFA-3) or T11 target structure (T11TS) is a potent immune stimulator in the brain tumor-induced, immune-suppressed animal model (11). In the present study, the hypothesis that T11TS administration in general is associated with peripheral as well as intracerebral immunopotentiation, resulting in the destruction of the tumor mass, is addressed. The greater infiltration of the CD4+ and

CD8+ lymphocytes clearly indicate that T11TS can revert back the ENU-induced immunosuppressive state caused by brain tumorigenesis. The cytotoxic activity of BIL before and after T11TS administration in ENU-induced animals completely correlated with tumor cell apoptosis. Furthermore, assessment of nectrotic cell death of all the cell types of different animal groups clearly confirms the mode of cell death. Finally, the interaction between the tumor cells, Mg and BIL before and after T11TS administration in ENU-induced animals were monitored in terms of cell protection and death.

Materials and Methods

Reagents and chemicals. RPMI 1640 medium, fetal bovine serum, streptomycin and penicillin were purchased from Gibco BRL (Gaithersburg, MD, USA). Ethyl-nitrosourea, RNase A, DEAE cellulose, collagenase I & IV, hyaluronidase, DNase and soyabean trypsin inhibitor all were purchased from Sigma (St. Louis, MO, USA). Percoll was obtained from Pharmacia Biotech. Propidium iodide was from Becton Dickinson (San Jose, CA, USA) The remaining chemicals were purchased from local firms (India) and were of highest purity grade.

Animals and animal grouping. Healthy newborn Druckrey rats, 2-3 days old, of both sexes along with the mother, were maintained in our Laboratory for the purpose of investigations. The animals (24 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 kept at ambient temperature (22˚C) in a 12-hour light / darkness cycle. The animals were grouped in batches of 24 for each experimental group, as follows: (1) Normal control (N), (2) 3- to 5-day-old neonatal animals injected with ENU intraperitoneally (*i.p.*), (3) 7-month-old ENU-treated animals injected (*i.p.*) with 1st dose of T11TS (ET1), (4) 7-month-old ENU-treated animals injected (*i.p.*) with 1st and 2nd dose of T11TS (ET2), (5) 7-month-old ENU-treated animals injected (*i.p.*) with 1st, 2nd and 3rd dose of T11TS (ET3) at an interval of 6 days for each dose. The rats were examined daily and weighed weekly throughout the experimental period. Maintenance and the animal experimental procedure strictly followed Principles of Laboratory Animal Care (NIH publication No. 85-23, revised in 1985) as well as local ethical regulations.

Induction of brain tumor with ENU. N'-N'-ethyl-nitrosourea (ENU) was freshly prepared by dissolving the compound (10 mg/mL) in sterile saline and adjusting the pH to 4.5 with crystalline ascorbic acid. ENU was injected (*i.p.*) in newborn rats (3-5 days old) with a dose of 80 mg/kg body weight (10-16).

Preparation of T11TS from sheep red blood cell (SRBC). The method followed was taken from Kitao *et al.* (17) and slightly modified Briefly, 1 mL volume of packed sheep red blood cells were trypsinised followed by non-specific protein precipitation using 25% TCA, neutralized with NaOH, and dialyzed against distilled water. The glycoprotein was separated from neutral peptides by ion exchange chromatography on a DEAE-Cellulose column (1.5x8 cm) previously equilibrated with 0.05 M-formate buffer, pH 6.8. The acidic glycopeptide was then eluted with a five-chamber gradient system containing 1 mL of either water, 0.05 M formic acid, 0.2 M

formic acid, 0.4 M formic acid or 0.4 M formic acid in 0.3 M sodium chloride corresponding to groups 1-5. Finally, the elute fraction-III was selected as the fraction containing the maximum amount of T11TS by the rosette inhibition assay (11). SDS-PAGE electrophoresis analysis of all the elute fractions obtained from ion-exchange chromatography were performed for partial characterisation (12).

Reverse phase-high performance liquid chromatography. A prepacked C_{18} column was purchased from Waters (WAT 011793) with an internal diameter of 150x3.9 mm. Two mobile phases were used to form differential gradients. Mobile phases A and B were 0.1% TFA (trifluroacetic acid) in water and 0.1% TFA in acetonitrile, respectively. The flow rate was adjusted to 18 mL/h. The column was equilibrated in 100% mobile phase A for about 50 minutes before sample administration. All chemicals used were of HPLC grade and were degassed (by sonication) and filtered (Millipore) before application. After 50 minutes of equilibration in 100% mobile phase A, 15 μl of elute fraction III obtained from ion-exchange chromatography was applied through the injector of the waters HPLC system. Fractions were collected continuously at a volume of 1 mL by a fraction collector (Waters). The fractions were analysed by ELISA using anti CD-58 monoclonal antibody to identify the fraction containing T11TS / SLFA-3. The activity peak (4 mL) appeared at an average concentration of 150 mM formate (18) (Figure 1). Reverse phase-HPLC were repeatedly performed (18) to obtain large amounts of T11TS / SLFA-3-containing fractions. These elute fractions were pooled and were concentrated using an Amicon Centricon 30 microconcentrator. These concentrated fractions were thereafter referred to as the '*T11TS fraction*' for administration to animals.

Administration of T11TS fraction. The injected dose volume of the elute fraction III was calibrated from the time-dose responsiveness of rosette inhibition assay (11). To estimate the protein content of the T11TS fraction obtained from RP-HPLC followed by concentration, Lowry's method (19) was performed and the amount determined was found to be 50 μg/mL. Consequently, the amount of T11TS administered was calculated to be 0.41 mg/kg body weight and as the average weight of the 7 to 8-month-old rats was approximately 125 grams, the required amount of T11TS was found in 1 mL. of the HPLC-purified and concentrated preparation. The 1st dose of 1 mL of T11TS fraction (*i.p.*) was followed by a 2nd booster dose on the 6th day and a 3rd booster dose on the 12th day, making a dose schedule of 1 mL, 2 mL and 3 mL to the group 3, 4 and 5 animals respectively.

Cell isolation. a) Tumor cell isolation: Four to five consecutive 5-μm sections were cut from the tumor susceptible area of the rat brain and the central section was stained by haematoxylin and eosin. Completely neoplastic areas, as appropriate to the histopathological diagnosis and not contaminated by the normal tissue, were marked out in the stained slide by the experienced pathologist; the same area was microdissected from the other slide and the scrapings were stored (20). The microdissected tumor mass was then dissociated by enzyme digestion (Collagenase (0.03%) and DNase (0.01%) in Hank's BBS. Finally, the cell suspension was centrifuged at 80 x g for 10 minutes and thereafter redispersed in cell culture medium containing 10% FBS. A drop of cell suspension was microscopically examined to rule out the possibility

that, either because of the nature of the selected tissue or as a consequence of the isolation procedure, the sample was contaminated with normal brain cells or cellular debris (21).

b) BIL isolation: The brain infiltrating lymphocytes were isolated from all the experimental animal groups with slight modifications, as described earlier (22). The whole brain was made into a single cell suspension by teasing and then passing it through a 80-gauge wire mesh before incubation with a mixture of enzymes [collagenase Type-I (0.05 mg/mL), collagenase Type IV (0.05 mg/mL), hyaluronidase (0.025 mg/mL), DNase I (0.01 mg/mL) and soyabean trypsin inhibitor (0.2 TIU/mL)] dissolved in HBSS (22) for 15 minutes at 37˚C, twice. The digested cells were then applied over a differential Percoll density gradient of 60/50/40/30. A thin band was observed in between the interface of the 40/30 gradient of Percoll. This band was collected carefully and washed twice with PBS at 800 x g for 5 minutes. These isolated cells from all the experimental animal groups were charecterised by anti-CD3 monoclonal antibody to ascertain the purity of the lymphocytes in the isolated cell populations.

c) Microglial cell isolation: The whole brain was made into a single cell suspension by teasing and then passing it through a 80-gauge wire mesh and digesting by incubation with collagenase (250 μg/mL) and DNase (250 μg/mL) at 37˚C for 45 minutes each. The resulting cell suspensions were applied over 30/60% Percoll gradients at 1000 x g for 25 minutes (23), and brain mononuclear cells were collected from the interface. The mononuclear cell suspensions were then laid on a plastic petri dish (Corning, USA) and incubated for 30 minutes in a CO_2 incubator. The adherent cells were washed out with PBS-EDTA and laid on a Nycodenz gradient (1.068 specific gravity) (Nycomed, Oslo, Norway) for 20 minutes at 400 x g for the density gradient centrifugation. The cells were removed from the interface and then washed three times with PBS. These cells were then characterised with the help of a panel of monoclonal antibodies, *i.e.* CD11b, CD2, RTIB and GFAP (14).

Study of cell cycle phase distribution by flow cytometry. For the determination of cell cycle phase distribution, 1×10^6 cells from each group of animals were suspended in 1 mL of 2.5% paraformaldehyde for fixation. The cells were then washed with PBS and incubated with 30-50 μL/mL of DNase free RNase for 1 hour at 37˚C, followed by centrifugation. The cell pellet was resuspended in the propidium iodide (PI) using Cycle TEST PLUS DNA reagent kit, at a density of about 1×10^6 cells/mL and incubated for 1 hour at 37˚C. Cell cycle phase distribution of nuclear DNA was determined on FACS, fluorescence detector equipped with 488 nm argon laser light source and 623 nm band pass filter (linear scale) using Cell Quest software (Becton Dickinson). A total of 10,000 events were acquired and analysis of flow cytometric data was performed using ModFit software. A histogram of DNA content (X-axis, PI-fluorescence) *versus* counts (y-axis) are displayed (24, 25) (Figures 2, 3, 4).

Quantification of apoptosis by TUNEL assay. The ApoAlert DNA Fragmentation Assay Kit (Clontech Laboratories, Palo Alto, CA, USA) detects apoptosis-induced nuclear DNA fragmentation *via* a fluorescence assay (25-27). For this assay, $3-5 \times 10^6$ cells (tumor cells, microglial cells and BIL) were collected and washed twice with PBS and finally resuspended in 0.5 mL of PBS. The cells were fixed by adding prechilled 1% formaldehyde in PBS (w/v) and
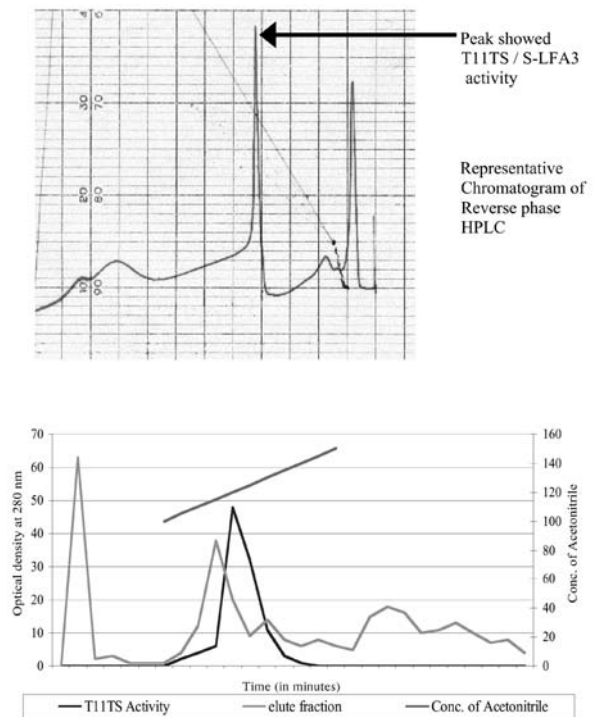

Figure 1. *Reverse-phase HPLC of the elute fraction containing T11TS activity obtained from ion-exchange chromatography.*

incubated at 40˚C for 20 minutes. The cells were again washed twice with PBS and resuspended in 0.5 mL of PBS. To permeabilize the cells, 70% ice-cold ethanol was added and incubated at –20˚C for 14 hours. Following a PBS wash, the cells were resuspended in 1 mL PBS and transfered to 1.5 mL amber-coloured microcentrifuge tube, to protect the sample from light. After centrifugation, the PBS was discarded carefully and the cells were resuspended in 80 μL of supplied Equilibration buffer (200 mM potassium cacodylate, 25 mM Tris-HCl, 0.2 mM DTT, 0.25 mg/ml BSA, 2.5 mM cobalt chloride) and incubated for 5 minutes at room temperature. The TdT incubation buffer was prepared by mixing the equilibration buffer, nucleotide mix and TdT enzyme (at a ratio of 45:5:1) and kept on ice. A TdT-minus negative control incubation buffer (replacing TdT enzyme with deionised water) was also prepared. The cells from each sample were centrifuged and resuspended in 50 μL of TdT incubation buffer. The suspension was then incubated at 37˚C in a water bath for 60 minutes with mixing by gentle pipetting at 15-minute intervals. Twenty mM of EDTA was added and mixed gently by vortexing to terminate the reaction. After centrifugation, the cells were resuspended in 1 mL of 0.1% Triton-X/BSA/PBS. This step was repeated once. Finally, the cells were centrifuged and resuspended in 0.5 mL of propidium iodide buffer (0.5 μg/mL of PI, 0.5 μg/mL of DNase-free RNase dissolved in PBS), followed by incubation in the dark at room temperature for 20 minutes. Analyses were performed in FACS. Apoptotic cells were collected based on green fluorescence at 520 nm. All cells were sorted on red fluorescence at 620nm (Figures 5, 6, 7).
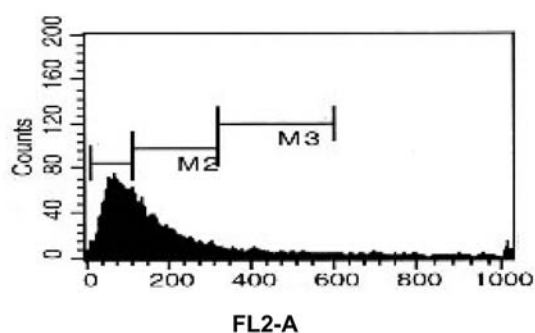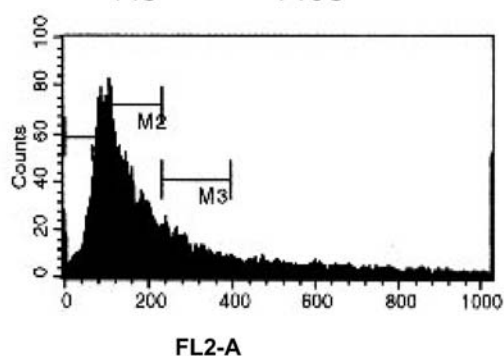
Marker	% Total	
All	88.51	
M1	42.51	**N**
M2	37.78	
M3	7.05	
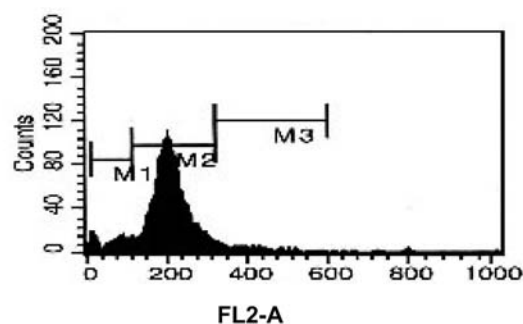
Marker	% Total	
All	83.43	
M1	6.50	**E**
M2	73.26	
M3	3.53	

Marker	% Total	
All	100.00	
M1	32.70	**ET1**
M2	38.05	
M3	14.79	
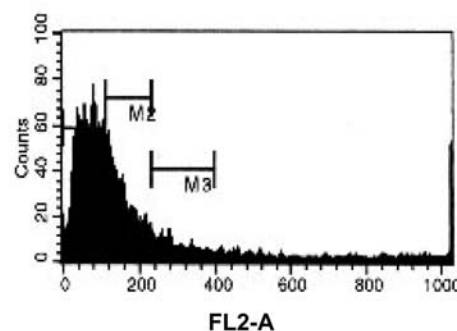
Marker	% Total	
All	100.00	
M1	45.77	**ET2**
M2	28.40	
M3	7.65	
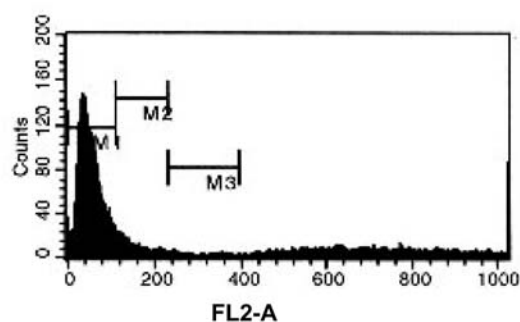
Marker	% Total	
All	100.00	
M1	66.90	**ET3**
M2	9.10	

Figure 2. *Effect of T11TS in inducing apoptosis of the neural tumor cells was determined by propidium iodide staining of the nucleus, followed by flow cytometric analysis. Maximum hypodiploid (apoptotic) tumor cells and least number of hyperdiploid (dividing) cells were found in animals that receive three booster doses of T11TS (ET3) at 6-day intervals.*
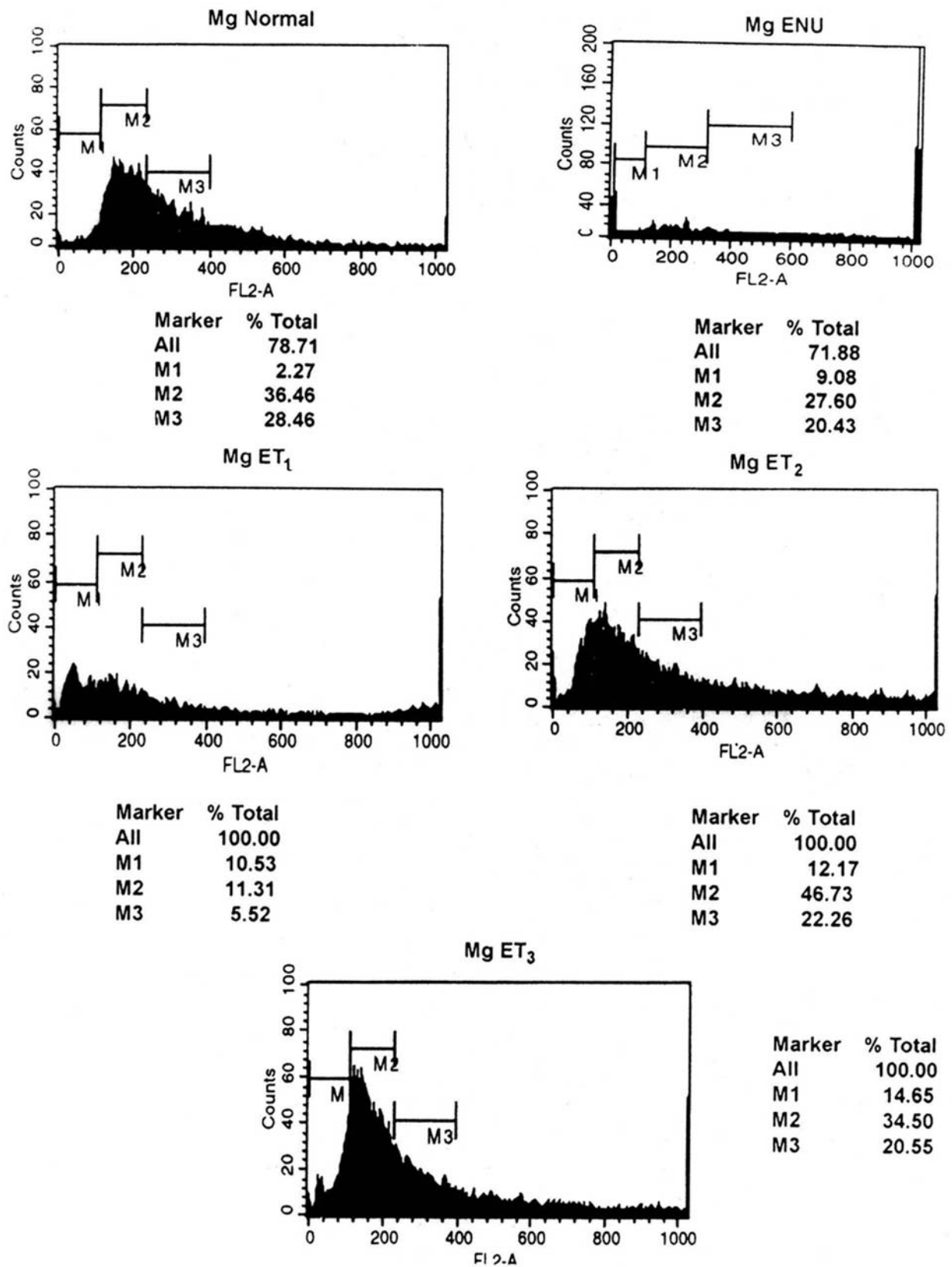
Mg Normal

Marker	% Total
All	78.71
M1	2.27
M2	36.46
M3	28.46

Mg ENU

Marker	% Total
All	71.88
M1	9.08
M2	27.60
M3	20.43

Mg ET$_1$

Marker	% Total
All	100.00
M1	10.53
M2	11.31
M3	5.52

Mg ET$_2$

Marker	% Total
All	100.00
M1	12.17
M2	46.73
M3	22.26

Mg ET$_3$

Marker	% Total
All	100.00
M1	14.65
M2	34.50
M3	20.55

Figure 3. *PI-FACS analysis revealed the fact that, in the case of microglial cells, the increase of apoptosis in the ENU group (M1-9.08) compared to the normal group (M1-2.27), remained static in ET1 (10.53) , ET2 (12.17) and ET3 (14.65), which are significantly lower than that of tumor cell counterparts.*
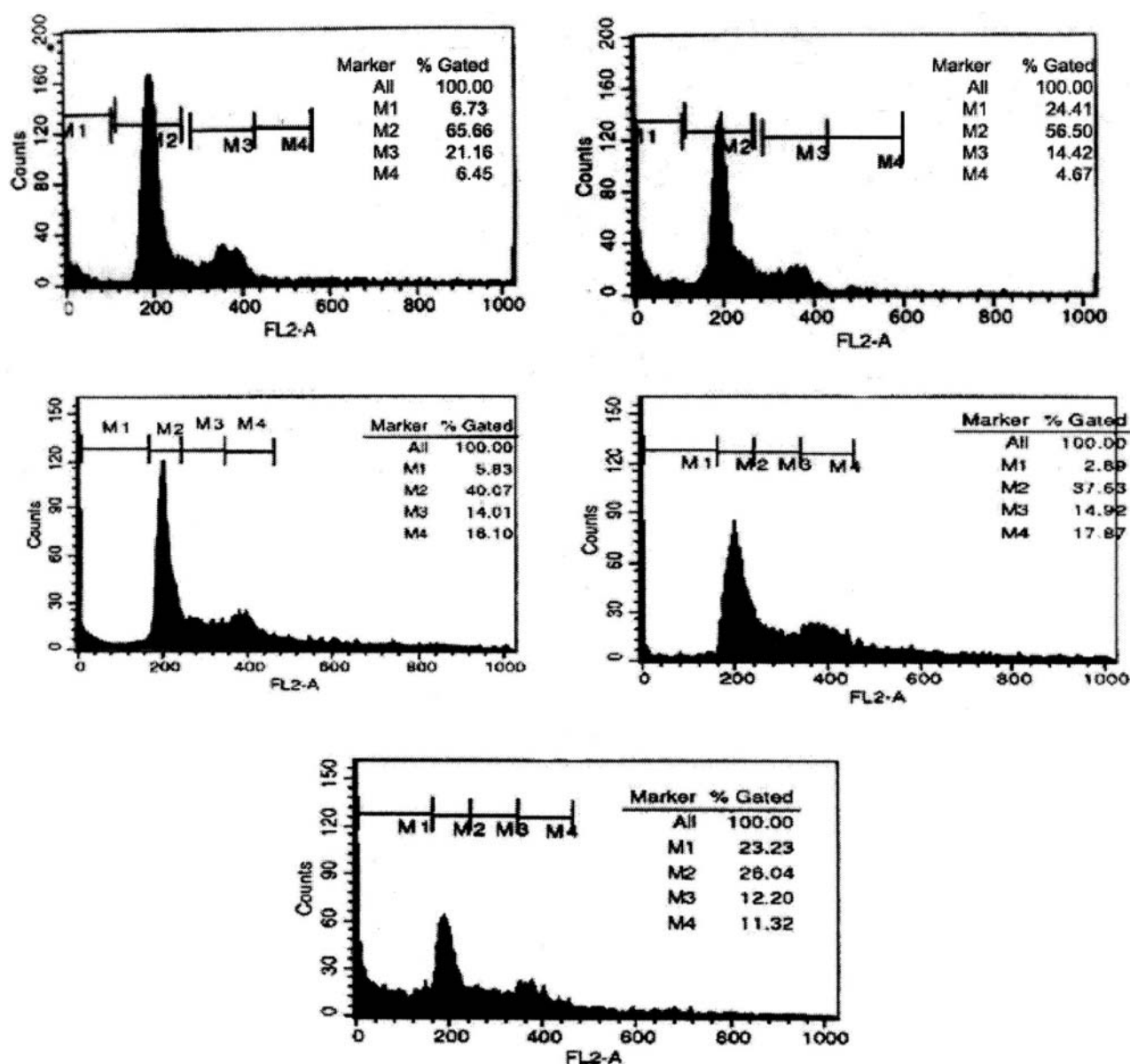
Figure 4. *PI-FACS analysis of the BIL isolated from normal animal group showed a few hypodiploid cells (1.73). BIL from the ENU group showed an increment of hypodiploid cells (apoptotic cell) compared to the normal group. The apoptosis rate of BIL increased further in the ET1 group but interestingly decreased in the ET2 group and huge apoptosis was again observed in the ET3 group.*

Assessment of cytotoxic activity of lymphocytes. a) Splenic lymphocytes: A newer approach to this method has been adopted using a fluorochrome dye Hoechst 33342 (HO-33342, Sigma, USA). The method (11, 12) was found to be more suitable and accurate than the vital dye exclusion test or ^{51}Cr release assay, in that the former is somewhat gross and the latter involves non-specific diffusion of radio-chromium from cells spontaneously with greater masking effect, whereas HO-33342 binds to DNA of cells irreversibly without leakage until lysed. Tumor cells (target) (a steady glial tumor line, syngenic in nature) were labelled with HO-33342 fluorochrome dye (6 μg/10^6/mL) for 15 minutes at 37˚C and excess was washed off (total incorporation). The cytotoxicity assay was performed by maintaining an effector (splenic lymphocytes): target ratio at 10:1 during an incubation (37˚C, 4% CO_2 and humidified environment) period of 18 hours. Fluorochrome released as per target lysis (experimental lysis), measured in a spectrofluorimeter (Hitachi, Tokyo, Japan), provided an index of the cytotoxic efficacy of the effectors. A labelled target group was maintained alone for spontaneous lysis (Figure 8). The results were calculated as follows:

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

b)Brain infiltrating lymphocytes (BIL): A steady established ENU-induced glioma cell line served as target in this experiment. Brd-U-labelling of the target cells was performed as described above. Ona hundred mL of Brd-U-labelled target cells ($2x10^5$ cells/ml) were transferred into duplicate wells of a 96-well round-bottom microtitre plate. To these 100 μL of additional culture medium containing BIL (effector) isolated from different groups were added per well in a effector: target ratio of 100:1. Negative controls were maintained for each well. It was then incubated for 6 hours at 37˚C in a humidified atmosphere (5% CO_2). After centrifugation for 10 minutes at 250 x g, 100 μL of supernatant were taken and analysed in the ELISA procedure, as described above (28, 29) (Figure 9).

CD4-CD8 counts of the splenic and brain infiltrating lymphocytes. Splenic lymphocytes separated on a Percoll gradient (12), and the brain infiltrating lymphocytes separated as described earlier, were tagged with anti-CD4-FITC and anti-CD8 PE conjugate and isotype control IgG¡a was maintained. In total, 10,000 events were acquired and data were analysed using Cell Quest software (Beckton Dickinson). The cells were gated with the leukogate software and positive control of lymphocyte cell markers (13) (Figures 10, 11).

Statistical analysis. Statistical analysis of the results was performed using the 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, IL, USA). A probability of $p<0.01$ was considered statistically significant.

Results

Reverse phase-high performance liquid chromatography. The reverse phase-HPLC chromatogram using 0.1% TFA-water and 0.1% TFA acetonitrile gradient produced a single sharp peak, with a retention time of 36 minutes and acetonitrile gradient between 80-85%. Functional assessment along with ELISA indicated the activity of T11TS/ SLFA-3 of this peak. This was followed by a small hump at a retention time of 70 minutes onwards (Figure 1). After reverse-phase HPLC, the fractions were collected and the activity of T11TS / SLFA-3 was monitored by ELISA procedure using anti-human LFA-3. Among the fractions, 0.8 ml indicates the presence of T11TS.The identical RP-HPLC analysis was performed repeatedly, and the fraction with T11TS activity was collected (0.8 ml in each case) and pooled. In this way, 6 ml of fractions were collected and concentrated using an Amicon Centricon 30 microconcentrator: the protein content determined by Lowry's method demonstrated the presence of 50 μg of purified T11TS in this fraction.

Flow cytometric analysis of cell cycle phase distribution before and after T11TS application. The effect of the T11TS fraction on brain tumor cells, BIL and Mg cells in animals with experimentally-induced tumor (with ENU) showed very interesting results.
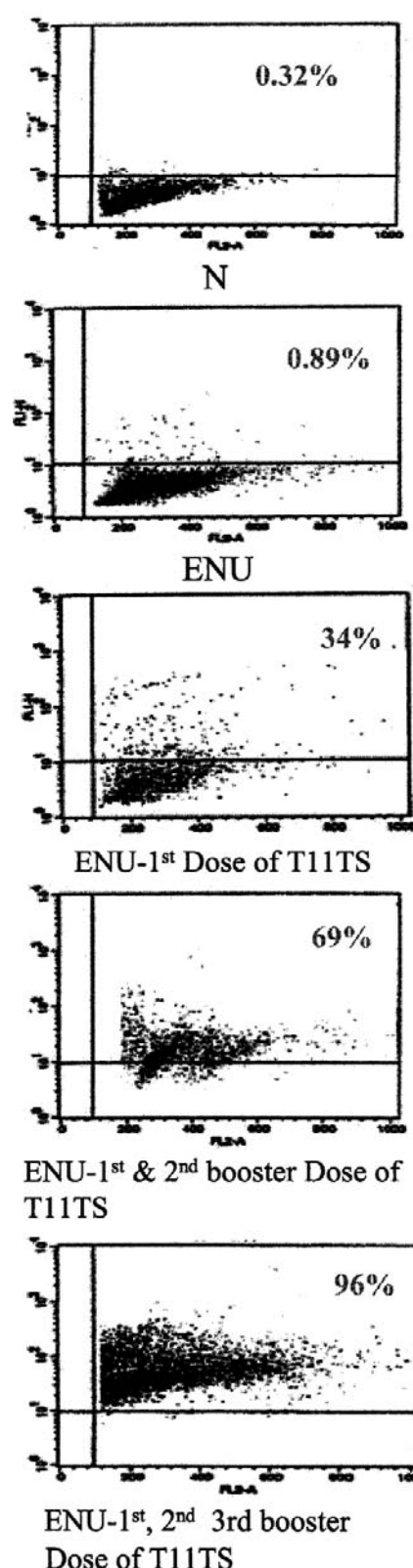

Figure 5. *Determination of percentage of apoptotic cells among the total brain tumor cells isolated from different experimental animal groups with or without T11TS application by TUNEL assay.*
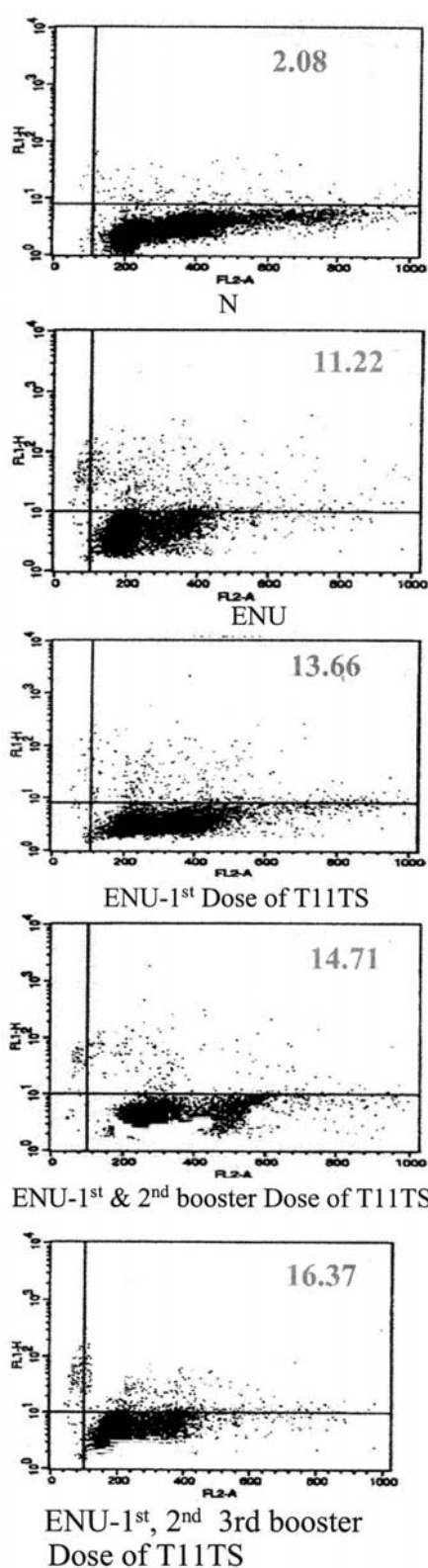
Figure 6. *Determination of percentage of apoptotic cells among the microglial cells isolated from different experimental animal groups with or without T11TS application by TUNEL assay.*
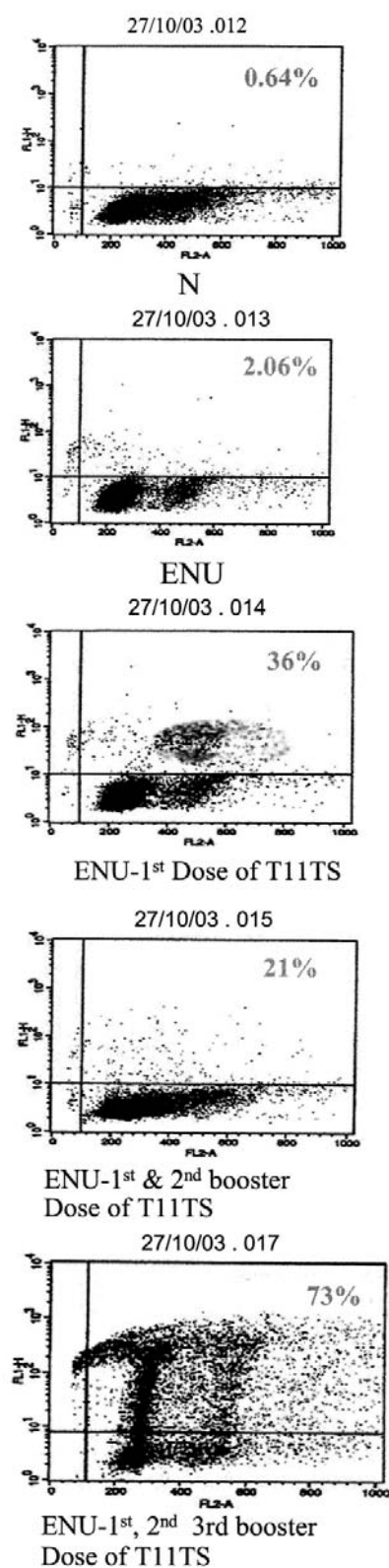
Figure 7. *Determination of percentage of apoptotic cells among the total infiltrating lymphocytes isolated from different experimental animal groups with or without T11TS application by TUNEL assay.*
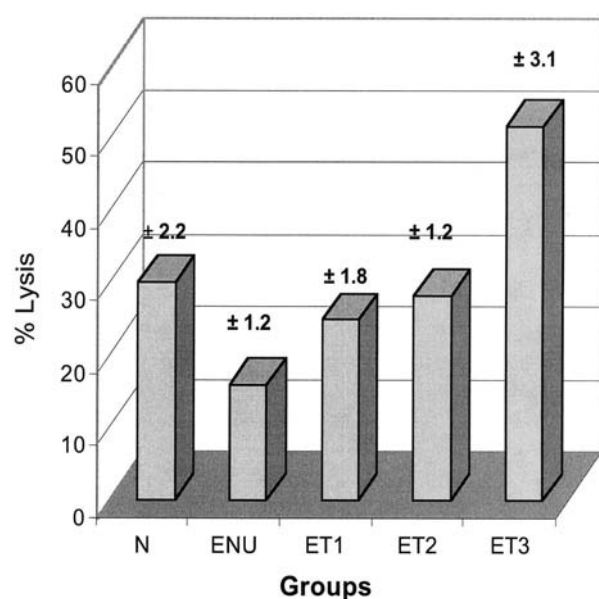
Figure 8. *Cytotoxicity assay (CTL-Assay) of splenic lymphocytes (SL) as determined through HO-33342 release in different groups of rats. Significant decrease in cytotoxic efficacy of SL is noted after ENU induction (E). The third booster dose of T11TS administration (ET3) was found to induce CTL at its maximum in the ENU-induced animals.*

Figure 9. *Cytotoxicity assay of the brain infiltrating lymphocytes, as assessed by ELISA assay kit also demonstrated increased cytotoxic activity of the infiltrating lymphocytes after T11TS administration.*

a) Brain tumor cell: The percentage of glial cells in G0/G1-phase (diploid *i.e.* normal cell) and S-phase (hyperdiploid *i.e.* dividing cells) were 73.26 and 3.53, respectively in the normal animal group (Gr-1). The percentage of tumor cells in S-phase markedly increased (M3-14.79) in the ENU-induced group along with the decrease in the G0/G1-phase percentage (M2-38.05). With the application of the immunotherapeutic dose of T11TS, there was a steep increase in the apoptotic rates from 42.51 in ET1 to 66.90 in the ET3 group, indicated by the sharp and significant increase of percentage of hypodiploid cells. Correspondingly, in the M3 (cells in S-phase) quadrant the hyperdiploidy nature or newer DNA synthesis sharply decreased from the ENU group (14.79) to the ET3 group (2.08), which was even lower than that of the normal group (Figure 2).

b) Microglial cells: The increase of apoptosis in the ENU group (M1-9.08) compared to the normal group (M1-2.27) remained static in ET1 (10.53) , ET2 (12.17) and ET3 (14.65), which were significantly lower than that of the tumor cell counterparts (Figure 3).

c) Brain infiltrating lymphocytes: The percentage of G0/G1-phase cells was 65.56 in BIL isolated from normal animal group with very few hypodiploid cells (1.73). However, BIL from the ENU group showed a slight increase of hypodiploid cells (apoptotic cell) and fewer cells in G0/G1-phase (M2-56.5) and S-phase (M3-14.42) compared to the normal
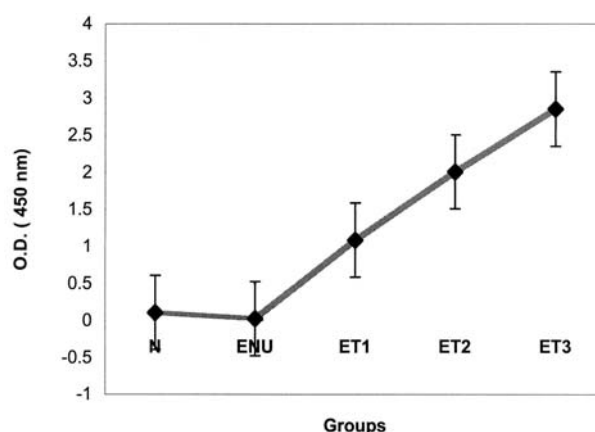
group. After T11TS application, BIL apoptosis in ET1 (35.83) increased significantly, but is interestingly reduced in ET2 (22.89), with a sharp rise in ET3 (69.231), indicating the homeostatic killing of the higher numbers of activated infiltrated cells. A slight decrease in hyperdiploidy from the normal group (21.16) was maintained more or less at a steady state in all the other groups (Figure 4).

TUNEL assay. The results obtained from the TUNEL assay (using ApoAlert DNA fragmentation assay kit, Clontech, Palo Alto, CA, USA) exactly quantify the apoptotic cells in different cell populations isolated from different experimental animal groups. In the case of tumor cells isolated from ENU-induced groups, a slight increase of apoptosis (0.89%) was observed compared to the normal control (0.32%). T11TS / SLFA-3 administration in ENU-induced animal presented a drastic increase of apoptosis of the brain tumor cells in a dose-dependent manner, *i.e.* ET1 (34%) and in ET2 (69%). The maximum apoptosis was observed in the ENU-induced groups that received three consequtive booster doses of T11TS. The major proportion (96%) of the isolated tumor cells was apoptosis-positive in the ET3 group (Figure 5).

For microglial cells, a slight increase of apoptosis in the ENU-induced group (11.22%), compared to the normal (2.08%) could be observed. Whereas, after three successive booster doses of T11TS administration, the apoptosis rate of the microglial cells slightly increased in each respective dose (ET1-13.66; ET2-14.71 and ET3-16.37) compared to the ENU-induced state (E-11.22) (Figure 6).

The apoptotic infiltrating lymphocytes were 2.06% in ENU-induced animals, which increased after the 1st dose of T11TS administration (36%). Interestingly, reduction of apoptotic BIL decreases after the 2nd dose of T11TS (21%), whereas after the 3rd booster dose a huge apoptosis
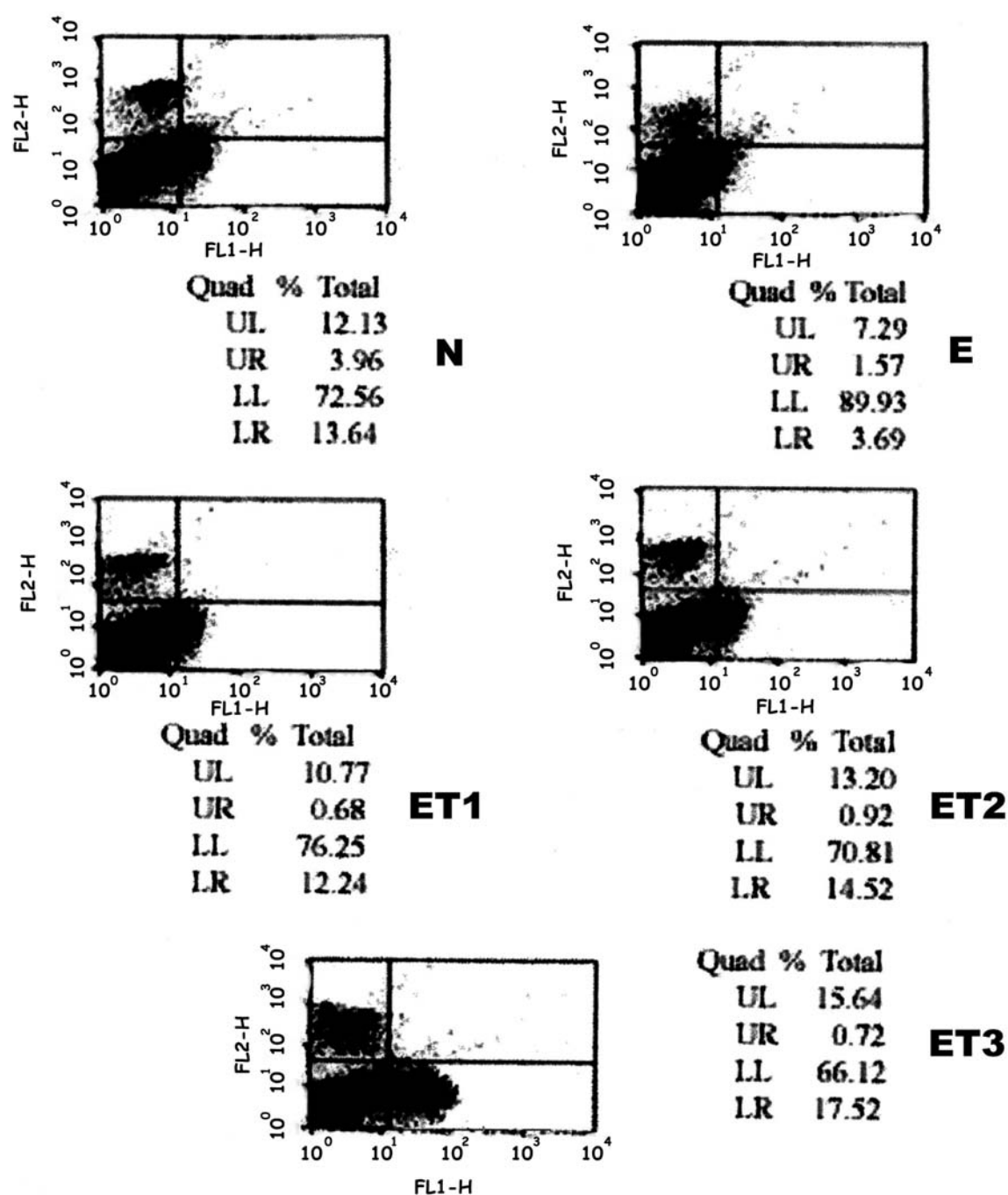
Figure 10. *Effects of T11TS administration (ET1-ET3) on CD4/CD8 expression on splenic lymphocytes (10a:N, 10b:E, 10c:ET1, 10d:ET2, 10e:ET3): Lymphocytes from the ET2 animals showed maximum CD2 expression as revealed by flow cytometric analysis (FACS-Calibur, Beckton Dickinson).*

(73%) of BIL was observed, indicating that it is a regulatory dose following an effective immune response (Figure 7).

Cytotoxic activity of the lymphocytes increases after T11TS administration. a)Splenic lymphocytes: The lymphocyte-mediated cytotoxicity, as determined by percent lysis, was 30%±2.2% in the normal animals. However, a sharp decrease in cytotoxic efficacy occured in the 6th month after ENU administration (14.2%±2.1%). A significant increase in cytotoxic efficacy (24.83%±1.8%) was observed when the 1st
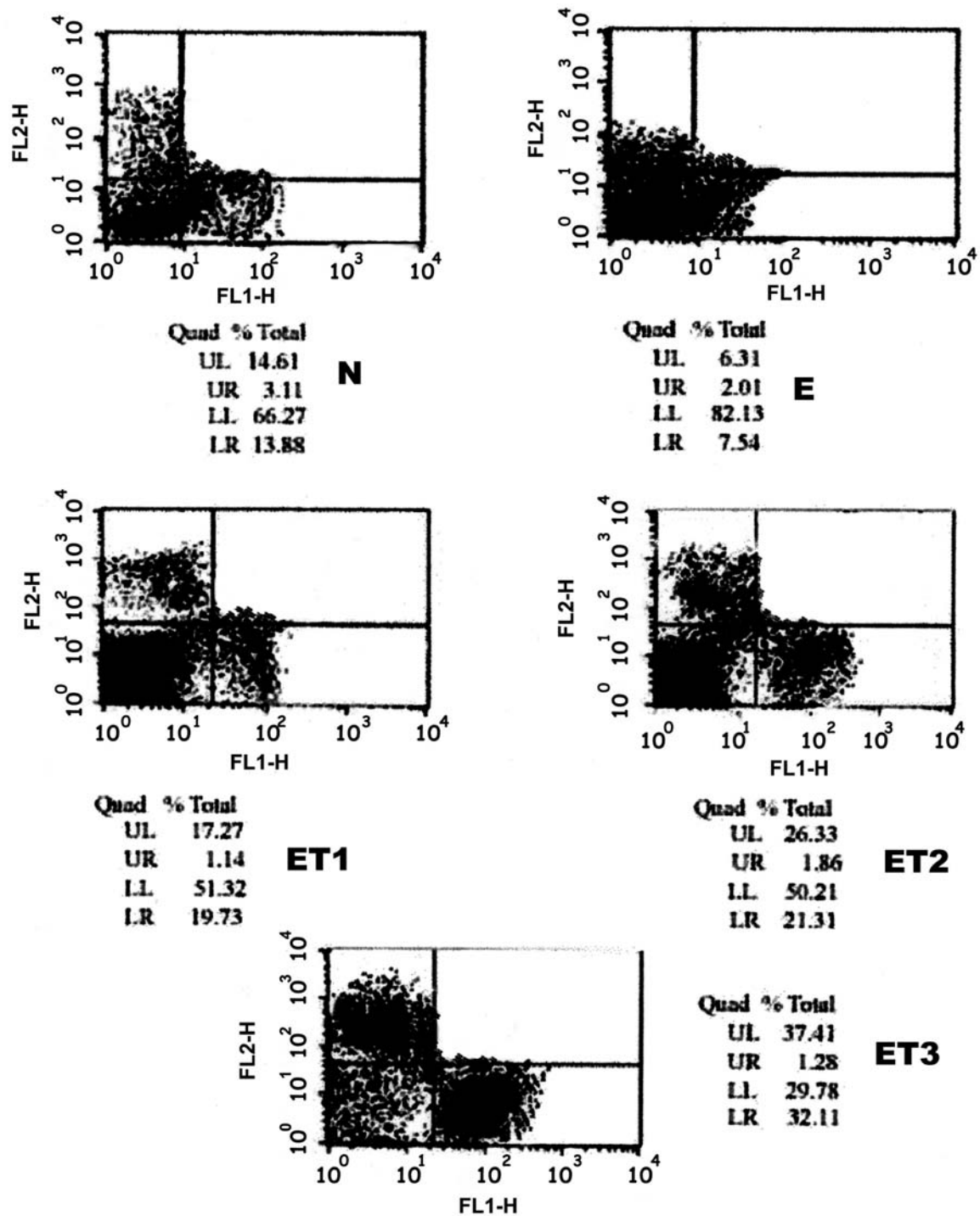
Figure 11. *Effects of T11TS administration (ET1-ET3) on CD4/CD8 expression on brain infiltrating lymphocytes (11a:N, 11b:E, 11c:ET1, 11d:ET2, 11e:ET3): Lymphocytes from the ET2 animals showed maximum CD2 expression as revealed by flow cytometric analysis (FACS-Calibur, Beckton Dickinson).*

dose of isolated immunodominant group of SRBC, *i.e.* T11TS, was administered in ENU-induced, tumor-bearing animals. However, the most significant ($p<<0.001$)

improvement in cytotoxic activity of the lymphocytes was observed with the 3rd dose of T11TS application (51.5%±3.1%) (Figure 8).

b)Brain infiltrating lymphocytes (BIL): A sharp decrease in cytotoxic activity of the BIL was observed in ENU-induced group (E-0.028) compared to the normal group (N-0.112). The cytotoxic activity of BIL increased in a dose-dependent manner after the application of T11TS in the ENU-induced animals (ET1-1.089; ET2-2.003). However, the most significant increase was observed in ET3 BIL (ET3-2.857), which surpassed the normal value (Figure 9).

CD4-CD8 counts of the splenic and brain infiltrating lymphocytes. Flow cytometric analysis quantified the presence of CD4+ and CD8+ lymphocytes in the brain infiltrating lymphocytes isolated from normal, ENU-induced, as well as T11TS-treated animals. Compared to the normal splenic CD4+ (13.64) and CD8+ (12.13) cells, after ENU induction a drastic decrease of CD4+ (3.69) and CD8+ (7.29) cells was observed (Figure 10). The CD4+/CD8+ cell count from the brain infiltrating lymphocytes also showed the same type of results, suggesting a leukopenic state after ENU administration. However, after three successive doses of T11TS administration in these leukopenic animals, a reversal of the immunosuppressed leukopenic state to an immune potentiated state was observed. This was best observed after the third booster dose of T11TS, in which the CD4+ brain infiltrating cells were 32.11% and the CD8+ cells were 37.41%, indicating a strong antitumor immune response within the cranium (Figure 11). The peripheral immune system also correlated strongly with the intracranial findings after the third dose of T11TS.

Discussion

In the present study, the CD4+-CD8+ lymphocyte infiltration along with the cytotoxic activity of the splenic and brain infiltrating lymphocytes was greatly enhanced after T11TS administration, indicating potentiation of intracranial immune responses. In our earlier studies, we reported that T11TS could elicit an effective antitumor response by immune potentiation in an animal model of brain tumor. T11TS administration in ENU-induced brain tumor showed significant prolongation of survival with histological demonstration of reversal of hyperplasia (characteristic feature of neoplasia in ENU-induced animals) to the normal cellular state (in ENU-induced T11TS-administered group) (10, 12). Among the resident brain cells, microglia (Mg) is the chief immunomodulatory cell (30). Previously, we also showed (14) the activation and functional compertmentalisation of Mg cells after T11TS administration in tumor-bearing animals. The present study indicated a more or less steady apoptosis of Mg cells in the ENU-induced animals, with and without T11TS administration. The results suggest that T11TS did not generate any cytotoxicity to the resident brain cells, as evidenced from its effect on Mg cells. It specifically destroyed the tumor cells by inducing apoptosis and increased the cytotoxic activity of the infiltrating lymphocytes at the same time, suggesting the immunocyte-mediated apoptotic death of tumor cells.

Characterising the splenic CD4+ and CD8+ lymphocytes revealed that tumorigenesis resulted in a decrease of both the CD4+ and CD8+ positive cell population in the peripheral compartment (Figure 10). Lymphocytes isolated from the intracranial part also showed decreased number of both the subtypes (CD4+ and CD8+) in the ENU-induced tumor-bearing animals (Figure 11). Previous studies from other groups (31) also showed total T-cell lymphopenia as a hallmark of patients harbouring glioma. The responsible factor for the decrease in T cell number was possibly the Fas/Apo-1 and Fas ligand-mediated apoptosis of T cells in the brain. As it has been shown (32-34) that glioma cells can also express FasL and its counterpart, Fas/ Apo-1, it is possible that T cells could be signaled to die by direct contact with the glioma cells in the brain (35). Tumor-T cell contact may not be required for T cell apoptosis in peripherally circulating cells, as recent observations suggest that soluble factors and /or cytokine dysregulation generated by peripheral blood-derived monocytes may influence T cell function and could potentially facilitate T cell apoptosis (36). T11TS administration in such T cell lymphopenic animals demonstrated a dramatic increase of CD4+ and CD8+ cell count, both in the peripheral as well as intracranial parts. This indicated the restoration of the T cell proliferative potentials along with lesser T cell apoptosis *via* Fas-FasL interaction with the tumor cells (within the cranium) and by the soluble factors and cytokine dysregulation (in the peripheral part). This study revealed the fact that T11TS not only activates and restores the proliferative potentials of the lymphocytes, but in turn it also renders protection against the possible routes to apoptotic death.

Glioma-bearing patients exhibited deficient *in vivo* T cell immune reactivity, resulting in cell-mediated immune suppression (37). Moreover glioma-generated factors such as TGF-β, PG-E2 *etc.,* that are known to suppress T cell responses, alter the cytokine profile of monocytic APC that, in turn, inhibit T cell functions (38). Our findings also support the fact that tumor development is associated with rapid down-regulation of immune functions, as evidenced from the cytotoxic activity of the splenic as well as infiltrating lymphocytes in the tumor-bearing group (Figures 8, 9). The diminished cellular immunity may be due to cytotoxic dysfunction with decrease in IL-2 and / or IFN-γ (Th1) production and increases in IL-4, IL-5 and / or IL-10 (Th2) production and ultimately contribute to tumor-associated immune dysregulation (38-41). T11TS / SLFA-3 exerts a direct stimulatory effect on the peripheral immune

system and thereby activates the total lymphokine network. Current data suggest that the activation of the splenic lymphocytes play an integral role in the T11TS-mediated response. The dose-dependent increment of the cytotoxic activity of the splenic as well as BIL was observed after 3 successive booster doses of T11TS / SLFA-3, in ENU-induced immunosuppressed animals, suggesting peripheral immune potentiation followed by intracranial immune potentiation (Figures 8, 9). These findings strongly support the fact that T11TS administration can revert the glioma-induced suppression of lymphocytes back above its normal active state. Several researchers (42, 43) have also reported that cytotoxic lymphocytes destroy the virus-infected cells or tumor cells *via* caspase-independent apoptosis machinery. In detail, cytotoxic lymphocytes secrete certain protease-like perforin and granzyme. Perforin makes holes in the membrane of the target cells, thus facilitating the entry of granzyme into the cell. Following entry, granzyme-B cleaves and thereby activates caspase-3 (44, 45). The activation of caspase-3 has been linked with the onset of hallmark apoptotic events such as DNA fragmentation and membrane alterations through the cleavage of cellular substrate, such as the inhibitor of caspase-activated deoxynuclease (ICAD) (DNA fragmentation factor-45) (46) and gelsolin (47), as well as numerous others.

Propidium-iodide FACS analysis demonstrated the fact that hyperdiploidy of tumor cells was arrested by T11TS administration. Moreover, hypodiploid cells (*i.e.* apoptotic tumor cells) increased in number after each successive dose of T11TS administration, indicating the fact that tumor cells were induced to undergo apoptosis by the immunocytes that were potentiated after T11TS administration (Figure 2). From the TUNEL assay, it was clear that a negligible amount (0.32%) of apoptotic-positive cells were demonstrated in the glial cells isolated from the normal animal groups. Although there was a slight increase in the rate of apoptosis in the ENU-induced group (0.89%) compared to the normal, the increment was not so prominent. The slight increase of apoptotic-positive cells in the ENU-induced animals could be explained by the fact that some apoptosis is always associated with tumorigenesis. Immunotherapy with T11TS in ENU-induced animals showed a drastic increase of apoptosis in a dose-dependent manner and apoptosis-positive cells showed a maximum in the animal group that received three successive booster doses of T11TS (Figure 5). These findings clearly indicated that T11TS induced (directly or indirectly) the tumor cells to enter the apoptotic cascade, thereby leading to tumor mass destruction.

The underlying molecular interactions of T11TS action lie in the fact that it acts as a ligand for the CD2 / T11 molecules present on different immunocytes (T cell, NK cell, PMN, macrophages and microglia) (13, 48-50). Several studies showed that CD2-CD58 interaction between T cells and their cognate partners facilitated the T cell recognition process and subsequent T cell activation (48). Furthermore, the CD2 interaction with CD58 regulates the response of activated T cells to IL-12 (51, 52). Hence both IL-12-stimulated T cell proliferation and IFN-γ production are markedly augmented by CD2 ligation. Sedwick *et al.* (4) reported that most of the brain infiltrating lymphocytes were CD2+, αβ+ type, strongly suggesting T11TS-mediated triggering followed by activation and infiltration of CD2+ positive cells into the brain case. Although the immunomolecular background of CD58 and its CD2-mediated signal transduction procedure were described earlier, we are the first to report a unique antitumor immune potentiating role of the CD2-T11TS interaction.

As the apoptosis-inducing role of T11TS was found in case of isolated tumor cells, the effect of T11TS on the microglial cells was monitored. T11TS administration activates the splenic and infiltrating lymphocytes, resulting in intracranial immune potentiation. Microglial response in such immune-modulation is an important factor in controlling the proper functioning of the brain immune system. Here, we used propidium iodide-DNA staining to identify apoptotic cells and cells at different stages of the cell cycle. We report that, full grown tumor in CNS (Gr-2) (E) leading to increased apoptotic death of Mg cells may be due to immuno-suppressive factors generated by the tumor cells. Application of T11TS in such ENU-induced tumor-bearing animals showed little increase of apoptosis of Mg in three successive booster doses. The number of diploid cells (*i.e.* 2n) (M-2) and hyperdiploid (dividing) cells (M3) increased in the ET1 and ET2 groups indicating the restoration of the proliferative potentials of Mg cells after T11TS administration (Figure 3). Data obtained from the TUNEL assay showed a low level of apoptosis of Mg cells in normal condition, indicating that there must be a renewal of Mg cells occurring in normal course. In tumorigenic condition (ENU group), the apoptosis of Mg cells increased (11.22%) and this might be attributed to the changes in the intracranial microenvironment after tumor formation. These changes include the decrease of supply of nutrients and growth factors, change in the neural immune system due to the secretion of different suppressive factors by the tumor cells, and the Fas (CD 95) / FasL (CD95L)-mediated killing of the Mg cells. Application of T11TS in such ENU-induced animals showed a very small increase in apoptosis in three successive doses (ET1-13.66; ET2-14.71; ET3-16.37) (Figure 6).

Brain infiltrating lymphocytes play a distinct role in brain inflammation. PI-FACS analysis revealed that the percentage of hypodiploid BIL (apoptotic BIL) slightly increased in ENU-induced tumor-bearing animals, which may have lost the activity due to secretions of the suppressive factors from the tumor cells. After the 1st dose of T11TS, the rate of apoptosis increased (35.83%), whereas in the ET2 group, the apoptosis rate was drastically reduced

(22.89%), indicating T11TS-mediated protection of the infiltrating lymphocytes. Interestingly, the hypodiploid cell (apoptotic cell) percentage again increased after the 3rd booster dose, indicating that a regulatory dose maintained the intracranial cellular homeostasis (Figure 3). The results obtained by the flow cytometry-based TUNEL assay completely correlated with the results obtained from the PI-FACS analysis (Figure 7). It was clear that infiltrating lymphocytes underwent a greater degree of apoptotic death in the ET1 group than the ET2 group, whereas in the 3rd dose the apoptotic rate increased sharply.

All these observations establish T11TS / SLFA-3 as a specific apoptosis inducer of brain tumor cells by potentiating the intracerebral immune status. Apoptosis of Mg and brain infiltrating lymphocytes can be explained in terms of changes in the neural immune system before and after T11TS administration. Moreover, increase of apoptosis of Mg cells was observed after ENU administration, which slightly increased further after T11TS administration along with increased proliferative potentials, indicating renewal of the Mg population of the brain. To conclude, these results imply a specific tumor cell destruction capability of T11TS / SLFA-3 by modulating the interaction between the infiltrating lymphocytes and microglia.

Acknowledgements

References

1 Greene GM, Hitchon PW, Schelper RL, Yuh W and Dyste GN: Diagnostic yield in CT-guided sterotactic biopsy of glioma. J Neurosurg 71: 494-497,1989.

2 Wekerle HC, Limington H, Lassmann H and Pleyermann R: Cellular immune reactivity within the CNS. Trends Neurosci 9: 271-279, 1986.

3 Hickey WF, Hsu BL and Kimura H: T-lymphocytes entry into the central nervous system. J Neurosci Res 28: 254-262, 1991.

4 Sedgwick JD, Ford AL, Foulcher E and Airriess R: Central nervous system microglial cell activation and proliferation follows direct interactions with tissue infiltrating T-cell blasts. J Immunol 160: 5320-5330, 1998.

5 Dix AR, Brooks WH, Roszman TL and Morford LA: Immune defects observed in patients with primary malignant brain tumors. J Neuroimmunol 100: 216-232, 1999.

6 Gold R, Hartung HP and Lassmann H: T-cell apoptosis in autoimmune diseases: termination of inflammation in the nervous system and other sites with immune defense mechanisms. Trends Neurosci 20: 399-404, 1997.

7 Bauer J, Bradl M, Hickley WF, Fross-Petter S, Breitschopf H, Linington C, Wekerie H and Lassmann H: T cell apoptosis in inflammatory brain lesions: destruction of T cell does not depend on antigen recognition. Am J Pathol 153: 715-718, 1998.

8 Savill J, Dransfield I, Gregory C and Haslett C: A blast from the past: clearance of apoptotic cells regulates immune responses. Nature Rev Immunol 2: 965-975, 2002.

9 Becher B, Prat A and Antel JP: Brain immune connection: immuno regulatory properties of CNS- resident cells. Glia 29: 293-304, 2000.

10 Mukherjee J, Dutta S, Sarkar S, Begum Z, Ghosh A, Chaudhuri S and Chaudhuri Swapna: Preclinical changes in immunoreactivity and cellular architecture during the progressive development of intracranial neoplasms and an immuno- therapeutic schedule with a novel biological response modifier, the T11TS / S-LFA-3. Asian Pacific J Cancer Prev 3: 325-337, 2002.

11 Sarkar S, Begum Z, Dutta S, Chaudhuri S and Chaudhuri Swapna: Sheep form of leucocyte function antigen-3 (T11TS) exerts immunostimulatory and antitumor activity against experimental brain tumor: a new approach to biological response modifier therapy. J Clin Expt Can Res 21(1): 95-106, 2002.

12 Mukherjee J, Sarkar S, Ghosh A, Duttagupta AK, Chaudhuri S and Chaudhuri Swapna: Immunotherapeutic effects of T11TS / SLFA-3 against nitrosocompound mediated neural genotoxicity. Tox Lett 150: 239-257, 2004.

13 Sarkar S, Ghosh A, Mukherjee J, Chaudhuri S and Chaudhuri Swapna: CD2-SLFA-3/T11TS interaction facilitates immune activation and glioma regression by T11TS. Canc Biol Ther 3(11): 1121-1128, 2004.

14 Begum Z, Ghosh A Sarkar S, Mukherjee J. Mazumdar M, Sarkar P and Chaudhuri Swapna: Documentation of immune profile of microglia through cell surface marker study in glioma model primed by a novel cell surface glycopeptide T11TS/SLFA-3. Glycoconj J 20: 515-523, 2004.

15 Druckray H, Ivancovic S and Preussman XX: Tetragenic and carcinogenic effects in the offspring after single injection of ethylnitrosourea to pregnant rats. Nature 210: 1378-1379, 1966.

16 Lantos PL: Chemical induction of tumors in nervous system: In: (Thomas GT, ed.) NeuroOncology. Curchill Livingstone, New York, London, pp. 85-107, 1993.

17 Kitao T, Takeshita M and Hattori K: Studies on glycopeptide released by trypsin from sheep erythrocytes. J Immunol 117: 310-312, 1976.

18 Hearn MTW (ed.): HPLC of Proteins, Peptides and Polynucleotides. VCH Publishers Inc., NY, 1991.

19 Lowry OH, Rosebrough NJ, Farr AL and Randell RJ: Protein measurement with the folin-phenol reagent. J Biol Chem 193: 265-275, 1951.

20 Chattopadhyay P, Rathore A, Mathur M, Sarkar C, Mahapatra AK and Sinha S: Loss of heterozygosity of a locus on 17p13.3, independent of p53, is associated with higher grades of astrocytic tumors. Oncogene 15(7): 871-874, 1997.

21 Westphal M, Giese A, Meissner H and Zirkel D: Culture of cells from human tumors of the nervous system on an extracellular matrix derived from bovine corneal endothelial cells. In: Pollard JW and Walker JM (eds.): Basic Cell Cuture Protocols. Humana Press, Totowa, New Jersey, 2nd Edition, pp. 185-207, 2002.

22 Ando H, Saio M, One N, Tamakawa N et al: B7.1 immunogene therapy effectively activates CD4^{+} tumor-infiltrating lymphocytes in the central nervous system in comparison with B7.2 gene therapy. Int J Oncol 20: 807-812, 2002.

23 Fischer H and Reichmann G: Brain dendritic cells and macrophage / microglia in central nervous system inflammation. J Immunol 166: 2717-2726, 2001.

24 Nicoletti I, Migliorati G, Pagliacci MC, Grignani F and Riccardi C: A rapid and simple method for measuring thymocytes apoptosis by propidium iodide staining and flowcytometry. J Immunol Meth *139*: 271-279, 1991.

25 Darzynkiewicz Z, Bruno S , Del Bino G , Gorczyca W , Hotz MA, Lassota P and Traganos F: Feature of apoptotic cells measured by flowcytometry. Cytometry *13*: 795-808, 1992.

26 Piqueras B, Autran B, Derbe P and Gorochov G: Detection of apoptosis at the single cell level by direct incorporation of fluorescein-dUTP in DNA strand breaks. BioTechniques *20*: 634-640, 1996.

27 Gavrieli Y, Sherman Y and Ben-Sasson SA: Identification of programmed cell death *in situ via* specific labeling of nuclear DNA fragmentation. J Cell Biol *119*: 493-501, 1992.

28 Berke G: Debate: the mechanism of lymphocyte-mediated killing. Lymphocyte-triggered internal target disintegration. Immunol Today *12*: 396-399, 1991.

29 Curnow SJ: The role of apoptosis in antibody-dependent cellular cytotoxicity. Cancer Immunol Immunother *36*: 149-154, 1993.

30 Aloisi F: Immune function of microglia. Glia *36*: 165-179, 2001.

31 Brooks WH, Markesbery WR, Gupta GD and Rozman TL: Relationship of lymphocyte invasion and survival of brain tumor patients. Ann Neurol *4*: 219-224, 1978.

32 Weller M, Malipiero U, Rensing-Ehl A, Barr PJ and Fontana A: *Fas/APO-1* gene transfer for human malignant glioma. Cancer Res *55*: 2936-2944, 1995.

33 Weller M, Frei K, Groscurth P, Krammer PH, Yonekawa Y and Fontana A: Anti-Fas/APO-1 antibody-mediated apoptosis of cultured human glioma cells. Induction and modulation of sensitivity by cytokines. J Clin Invest *94*: 954-964, 1994.

34 Weller M, Weinstock C, Will C and Wagenkneckt B: CD95-dependent T-cell killing by glioma cells expressing CD95 ligand – more on tumor immune escape, the CD95 counterattack, and the immune privilege of the brain. Cell Phys Biochem *7*: 282-288, 1997.

35 Saas P, Walker PR, Hahne M, Quiquerez AL, Schnuriger V and Perrin G: Fas ligand expression by astrocytoma *in vivo*; maintaining immune privilege in the brain? J Clin Invest *99*: 1173-1178, 1997.

36 Elgert KD, Alleva DG and Mullins DW: Tumor-induced immune dysfunction: the macrophage connection. J Leukoc Biol *64*: 275-290, 1998.

37 Zou J, Morford AL, Chougnet C, Dix AR, Brooks AG, Torres N, Shuman JD, Coligan JE, Brooks WH, Roszmann TL and Shearer GM: Human glioma induced immune suppression involves soluble factor(s) that alters monocyte cytokine profile and surface markers. J Immunol *162*: 4882-4892, 1999.

38 Roszmann TL, Brooks WH and Elliot LH: Inhibition of lymphocyte responsiveness by glial tumor cell derived suppressive factors. J Neurosurg *67*: 874-879, 1987.

39 Urbani F, Maleci A, LaSala A, Lande R and Ausiello CM: Defective expression of interferon-γ, granulocyte-macrophage-colony-stimulating factor, tumor necrosis factor-α and interleukin-6 in activated peripheral blood lymphocytes from glioma patients. J Interferon Cytokine Res *15*: 421-429, 1995.

40 Brooks WH, Latta RB, Mahaley MS, Roszmann TL, Dudka L and Skaggs C: Immunology of primary intracranial tumors. Part V. Correlation of a lymphocytic index and clinical status. J Neurosurg *54*: 331-337, 1981.

41 Huettner C, Paulus W and Roggendorf W: Messenger RNA expression of the immunesuppressive cytokine IL-10 in human gliomas. Am J Pathol *146*: 317-322, 1995.

42 Sarin A, Williams MS, Alexander-Miller MA, Berzofsky JA, Zacharachuk CM and Henkart PA: Target cell lysis by CTL granule exocytosis is independent of ICE / Ced-3 family proteases. Immunity *6*: 209-215, 1997.

43 Heibein JA, Barry M, Matyka B and Bleackley RC: Granzyme-B induced mitochondrial inner membrane potential (Dym) and cytochrome-c are caspase independent. J Immunol *163*: 4683-4693, 1999.

44 Alimonti JB, Shi L, Balaji PK and Greenberg AH: Granzyme B induces BID mediated cytochrome c release and mitochondrial permeability transition. J Biol Chem *276*: 6974-6982, 2001.

45 Trapani JA and Smyth MJ: Functional significance of the perforin/granzyme cell death pathway. Nature Rev Immunol *2*: 735-747, 2002.

46 Rogalinska M: Alterations in cell nuclei during apoptosis. Cell Mol Biol Lett *7*: 995-1018, 2002.

47 Kothakota S, Azuma T, Reinhard C, Klippel A, Tang J, Chu K, Mcgarry T, Kirchuer M, Koths K, Kwiatkowsi D and Williams L: Caspase-3 generated fragment of gelsolin: effector of morphological changes in apoptosis. Science *278*: 294-298, 1997.

48 Siliciano RF, Pratt JC and Schmidt RE: Activation of cytolytic T lymphocyte and natural killer cell function through the T11 sheep erythrocyte binding protein. Nature *317*: 428-430, 1985.

49 Niiazova OP, Osipova SO, Badalova NS and Dekhkan-khodzhaeva NA: Early and late E-rosetting forming neutrophils in persistent lambiasis. Med Parasitol (Mosk) *1*: 43-47, 1995.

50 Gutierrez M, Froster FI, Mc Conell SA, Cassidy JP, Pollock JM and Bryson DG: The detection CD4+, CD8+, and WC1+ T lymphocytes, B cells and macrophages in fixed and paraffin embedded brain tissue using a range of antigen recovery and amplification techniques. Vet Immunol Immunopathol *71(3-4)*: 321-334, 1999.

51 Moingeon P, Chang HC, Wallner BP, Stebbins C, Frey AZ and Reinherz EL: CD2-mediated adhesion facilitates T lymphocyte antigen recognition function. Nature *339*: 312-314, 1989.

52 Koyasu S, Lawton T, Novick D *et al*: Role of interaction of CD2 molecule with lymphocyte function - associated antigen3 in T-cell recognition of normal antigen. Proc Natl Acad Sci USA *87*: 2603-2607, 1990.