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In vitro Efficacy of Anti-glial Fibrillary Acidic Protein Monoclonal Antibodies against Human Malignant Glioma Cell Lines

Mohamed-Salah I. Abaza,[1,2,3] Raj K. Narayan[2] and M. Z. Atassi[1]

Departments of Biochemistry[1] and Neurosurgery,[2] Baylor College of Medicine, Houston, Texas, USA

Our studies have confirmed the presence of large concentrations of various intermediate filament proteins (IFPs) in glioma tissue compared to normal brain. This avenue of research was extended to assess the anti-proliferative activity of anti-intermediate filament protein monoclonal antibodies (anti-IFP mAbs) against human glioma cells. In this study, anti-proliferative activity of glial fibrillary acidic protein monoclonal antibodies (anti-GFAP mAbs) has been tested *in vitro*, using glioma cell lines prepared and established from freshly resected brain tumors. One anaplastic astrocytoma (AA), two glioblastoma multiforme (GB_1 and GB_2) cell lines and three anti-GFAP mAbs ($B_{12}C_4$, $B_{12}B_4$ and B_6C_6, all IgG_1, kappa) were used. Immunofluorescence study indicated the ability of anti-GFAP mAbs to recognize the cell surface of glioma cells and the inhibition study showed that mAb $B_{12}B_4$ inhibited the proliferation of GB_1 (96%), GB_2 (85%) and AA (93%) at a concentration of 3.2×10^{-10} *M*. mAb $B_{12}C_4$ inhibited the proliferation of GB_1 (95%), GB_2 (86%) and AA (94%) at a concentration of 3.26×10^{-10} *M* and mAb B_6C_6 inhibited the proliferation of GB_1 (75%), GB_2 (75%) and AA (91%) at a concentration of 2.074×10^{-10} *M*. Thymidine release assay demonstrated the cytolytic activities of anti-GFAP mAbs towards these glioma cell lines, and this observation was confirmed by dye exclusion, which indicated the lysis of glioma cells after anti-GFAP mAbs treatment. Anti-GFAP mAbs had little effect ($\leq$20%) on normal human lymphocyte, liver and intestine cell lines. These results look promising for radioimaging and immunotherapy of human gliomas.

Key words: Human glioma cell line — Anti-GFAP mAb — Anti-proliferative activity

Despite advances in radiotherapy and chemotherapy, there are about 16,700 new cases and 10,900 deaths due to malignant gliomas each year in the United States.[1] The 24-month survival rate for glioblastoma patients after surgical resection remains less than 15 to 20%.[2] The current treatments, including surgery, radiation therapy and systemic chemotherapy, have only a modest influence on the prognosis for patients with this disease,[3] and the limited specificity of conventional therapies for malignant cells, with resulting dose-limiting toxicity, has necessitated the development of innovative treatment modalities.

The presence of intermediate filament (IF) proteins in glioma cells has long been recognized, but in the last decade, our view of intermediate filaments has changed dramatically.[4–7] Intermediate filament proteins have the remarkable property of being expressed in a stable, a cell-type specific and differentiation-dependent manner.[8] Electron microscopic and indirect immunofluorescence studies of IFs have shown that they form complex interconnecting networks, emanating from a perinuclear ring, from which they appear to connect to the nuclear surface. IFs seem to extend throughout the cytoplasm and to terminate at the plasma membrane.[9] Such association between the plasma membrane and the nuclear surface appears to provide a continuous link that may have important implications for the organization of the cytoplasm, cellular communication, and perhaps transport into and out of the nucleus. Yet IFs are not static structures. The dynamic organization, distribution and interactions of IFs with other cellular components have been the subject of studies in many different systems, especially in cultured cells treated with drugs[10] or other chemicals,[11] injected with various anti-IF antibodies,[12] grown under varied media conditions[13] or observed during mitosis[13] and cell spreading. Many of these studies have noted that perturbation of the IF network results in the collapse of the IF onto or near the nuclear surface.

The presence of large quantities of IF proteins (IFPs), particularly glial fibrillary acidic protein (GFAP), in gliomas has been well known for years.[14] However, their presence has only been used for diagnostic purposes. In the present study the anti-proliferative activity of anti-GFAP mAbs against human glioma cells was tested. Anti-GFAP mAbs exhibited potent anti-proliferative effects against human glioma cells with little effect on normal human lymphocyte, liver and intestinal cell lines.

MATERIALS AND METHODS

Anti-glioma monoclonal antibodies (mAbs) and human cell lines Anti-glioma mAbs were prepared as described

[3] To whom correspondence should be addressed at the Department of Biochemistry, Faculty of Science, Kuwait University, P.O. Box 5969-Safat, Kuwait.

previously.[14] These mAbs showed high specificity for GFAP. They were all of IgG_1, kappa. Anti-GFAP mAbs reacted strongly with glioma extracts and showed little or no reactivity with human serum albumin, normal human serum or extracts of normal brain, liver, spleen, kidney and muscle tissues. Human glioma cell lines were prepared from freshly resected brain tumors. Glioblastoma multiforme (GB_1 and GB_2) cell lines were grown in Eagle's MEM with non-essential amino acids, sodium pyruvate (1 m*M*), Earle's BSS and 10% fetal bovine serum (FBS). Anaplastic astrocytoma (AA) cell line was grown in Leibovitz's L-15 medium with 10% FBS. Normal human liver, lymphocyte and intestine cell lines were obtained from ATCC (Rockville, MD). Normal human liver cell line was grown in Basal medium (Eagle) with Earle's BSS and 10% calf serum. Normal human lymphocytes were propagated in RPMI-1640 with 20% FBS. A normal human intestine cell line was cultivated in Dulbecco's modified Eagle's medium with non-essential amino-acids, sodium pyruvate (0.5 m*M*), oxaloacetic acid (1 m*M*), bovine insulin (0.2 μ/ml) and 10% FBS.

Recognition of human glioma cell surface by anti-GFAP mAb A single cell suspension was prepared from log phase culture of glioma cells. Cells were applied to poly-L-lysine-coated slides and air-dried at room temperature. Cells were fixed and slides were blocked with 3% bovine serum albumin in phosphate-buffered saline (BSA-PBS) for 1 h at room temperature, then washed (3×) with PBS (pH=7.2). Anti-GFAP mAb or normal mouse Ig of the same class and immunoglobulin concentration (as a negative control) was added and incubated for 1 h at 37°C in a humidified chamber. Slides were washed (3×) with PBS and stained with fluoresceinated goat anti-mouse Ig conjugate (1 : 30 dilution) for 1 h at 37°C in a humidified chamber. The cells were washed (3×) with PBS, mounted in buffered glycerol and viewed through an immunofluorescence microscope.

^{3}H-Thymidine uptake by glioma cell lines ^{3}H-Thymidine uptake was used to monitor the proliferation of the three glioma cell lines. In this experiment various cell numbers (in triplicate) of each cell line were incubated with a fixed amount of ^{3}H-thymidine (2 μCi/well) for 18 h at 37°C in a humidified 5% CO_2 atmosphere. The cells were then harvested and washed (10×) with distilled water, and the amount of radioactivity on the filter was measured with a beta counter.

Effect of radiation (1000 rad/min) on glioma cell proliferation For our study, the negative control for glioma cell lines was prepared by irradiation. In this experiment, a fixed number of glioma cells (50 μl/well, 5×10^5 cells/ml), which gave a strong ^{3}H-thymidine incorporation signal, was irradiated for various times (0–45 min). The ability of the irradiated cells to incorporate ^{3}H-thymidine was tested as mentioned in the previous section.

Anti-GFAP mAbs inhibit the proliferation of glioma cell lines The anti-proliferative activities of three anti-GFAP mAbs towards the three glioma cell lines were determined as follows: a fixed number (50 μl/well, 5×10^5 cells/ml) of glioma cells, normal human lymphocytes, and intestinal and liver cells was seeded (in triplicate) onto 96-well plates. Various concentrations of each of the anti-GFAP mAbs and normal mouse IgG (Sigma, St. Louis, MO), as a negative control, were then added to the cells. The plates were incubated for 18 h at 37°C in a humidified 5% CO_2 atmosphere. A fixed amount of ^{3}H-thymidine (2 μCi/well) was added and the cells were further incubated for 18 h. They were then harvested and washed (10×) with distilled water, and the amount of radioactivity on the filter was measured with a beta counter.

Testing the cytolytic activity of anti-GFAP mAbs towards human glioma cell lines Cytolytic activities of anti-GFAP mAbs towards glioma cells were tested using ^{3}H-thymidine release assay. A fixed number of glioma cells (50 μl/well, 5×10^5 cells/ml) was inoculated into 96-well plates. Cells were pulsed with ^{3}H-thymidine (2 μCi/well) and incubated for 18 h at 37°C in a humidified 5% CO_2 atmosphere, then washed (5×) with the medium. Various concentrations (100 μl/well, in triplicate), of each of the anti-GFAP mAbs and normal mouse IgG (as a negative control) were added and the cells were incubated for 18 h at 37°C in a humidified 5% CO_2 atmosphere. Cell supernatants were removed and spun, and aliquots (80 μl) were monitored in a beta counter after addition of a scintillation cocktail (Scinti Verse II, Fisher Scientific).

Testing glioma cell survival after anti-GFAP mAb treatment Glioma cells, normal human lymphocytes, and intestinal and liver cells (50 μl, 5×10^5 cells/ml) were incubated with anti-GFAP mAbs (100 μl in triplicate), unrelated monoclonal antibody of the same isotype and concentration (to monitor nonspecific lysis) and tissue culture medium (to monitor spontaneous lysis) for 36 h in a humidified 5% CO_2 atmosphere. Cells were then stained with trypan blue and viewed microscopically.

RESULTS

Recognition of human glioma cell surface by anti-GFAP mAbs If anti-GFAP mAbs are to be used for radioimaging or therapy of glioma, recognition of the glioma cell surface is essential. The ability of anti-GFAP mAb to bind to the glioma cell surface was investigated by indirect immunofluorescence. Anti-GFAP mAb recognized the cell surfaces of glioma cells, where green fluorescent rings, characteristic of surface staining, were obtained (data not shown).

^{3}H-Thymidine incorporation by glioma cell lines ^{3}H-Thymidine uptake was employed to monitor the pro-

liferation of glioma cells. Various glioma cell numbers were tested for the ability to incorporate a fixed amount of ^{3}H-thymidine (Fig. 1). A cell number (5×10^5 cells/ml) which gave a strong signal was chosen for the subsequent studies.

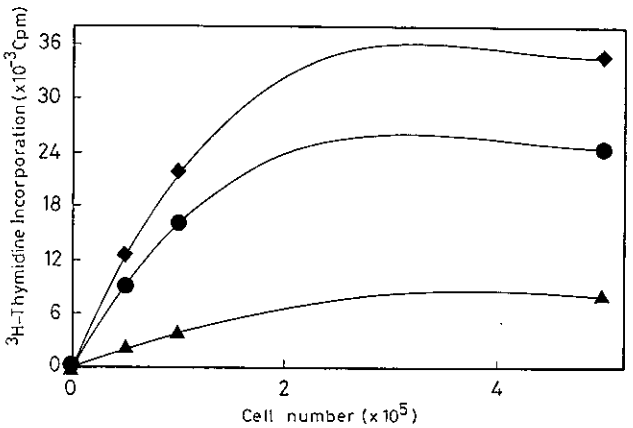

Fig. 1. ^{3}H-Thymidine incorporation by glioma cell lines. ^{3}H-Thymidine incorporation was used to monitor the proliferation of three glioma cell lines, AA (◆), GB1 (●) and GB2 (▲). Various cell numbers (in triplicate) of each cell line were incubated with a fixed amount of ^{3}H-thymidine (2 μCi/well) for 18 h at 37°C in a humidified 5% CO_2 atmosphere. The cells were then harvested and washed ($\times10$) with distilled water, and the amount of radioactivity on the filter was measured with a beta counter.

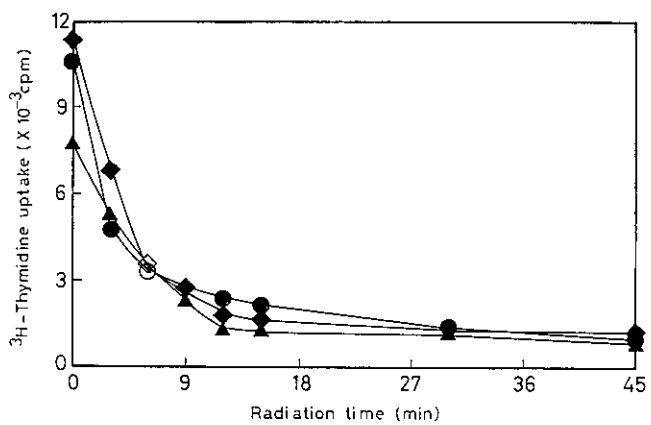

Fig. 2. Effect of radiation (1000 rad/min) on glioma cell proliferation. Negative controls for this study were prepared by employing radiation. A fixed number of glioma cell lines (50 μl of 5×10^5 cells/ml), which gave a strong ^{3}H-thymidine uptake signal, was exposed to radiation for various times (0–45 min) and the ability of the irradiated cells to take up ^{3}H-thymidine was tested as mentioned above. The three cell lines [AA (◆), GB_1 (●), GB_2 (▲)] tested lost their ability to take up thymidine after 15 min of irradiation.

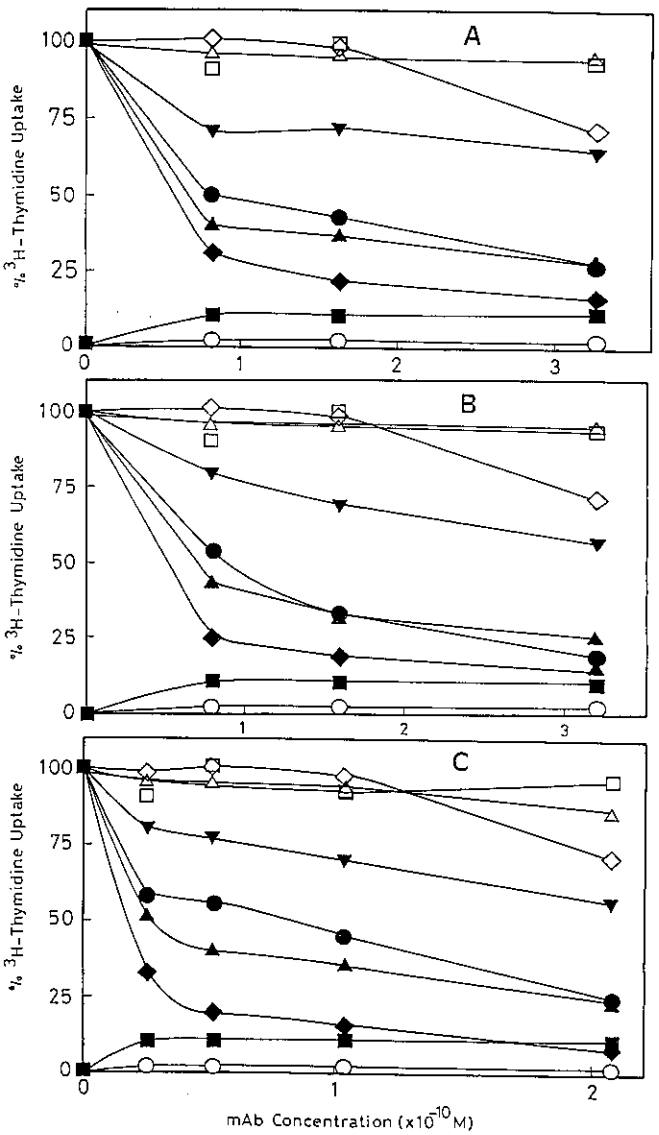

Fig. 3. *In vitro* efficacy of anti-GFAP mAbs against human glioma cell line. The anti-proliferative activities of anti-GFAP mAbs [$B_{12}B_4$ (Fig. 3A), $B_{12}C_4$ (Fig. 3B) and B_6C_6 (Fig. 3C)] towards three glioma cell lines, AA (◆) GB_1 (●) and GB_2 (▲), were determined. A fixed number (50 μl/well, 5×10^5/ml) of glioma cells, normal human lymphocytes (△), and intestinal (◇) and liver (▽) cells was incubated (in triplicate) in 96-well plates. Various concentrations of each of the anti-GFAP mAbs and normal mouse IgG (□) as a negative control were then added to the cells. The plates were incubated for 18 h at 37°C in a humidified 5% CO_2 atmosphere. A fixed amount of ^{3}H-thymidine (2 μCi/well) was added and the cells were further incubated for 18 h. They were harvested and washed ($10\times$) with distilled water, and the amount of radioactivity on the filter was measured with a beta counter. (■) represents the % of ^{3}H-thymidine uptake by irradiated AA and GB_1 cells while (○) represents the % of ^{3}H-thymidine uptake by irradiated GB_2 cells (negative controls).

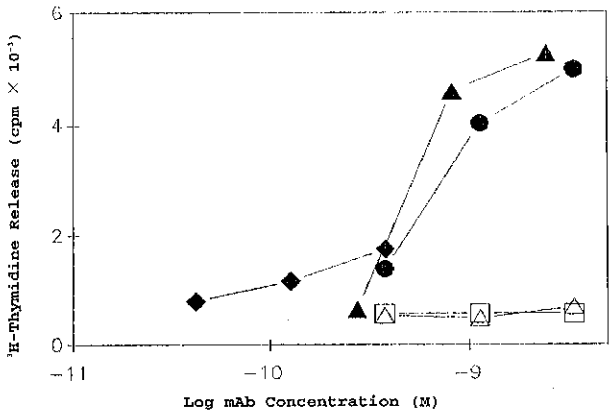

Fig. 4. Cytolytic activity of anti-GFAP mAbs towards human glioma cells. The cytolytic activities of anti-GFAP mAbs towards glioma cells were tested by ^{3}H-thymidine release assay. In this experiment, a fixed number of glioma cells (50 μl/well, 5×10^5 cells/ml) was inoculated into 96-well plates. Cells were pulsed with 2 μCi/well of ^{3}H-thymidine and incubated for 18 h at 37°C in a humidified 5% CO_2 atmosphere. Cells were washed (5×) with the medium. Various concentrations (100 μl/well in triplicate) of each of the anti-GFAP mAbs [$B_{12}B_4$ (▲), $B_{12}C_4$ (●) and B_6C_6 (◆)] and normal mouse IgG (□) as a negative control were added to the cells and incubated for 36 h at 37°C in a humidified 5% CO_2 atmosphere. Cell supernatants were removed and spun (17,000×30) and aliquots of these supernatants (80 μl) were monitored in a beta counter. Spontaneous ^{3}H-thymidine release was determined using the medium (△).

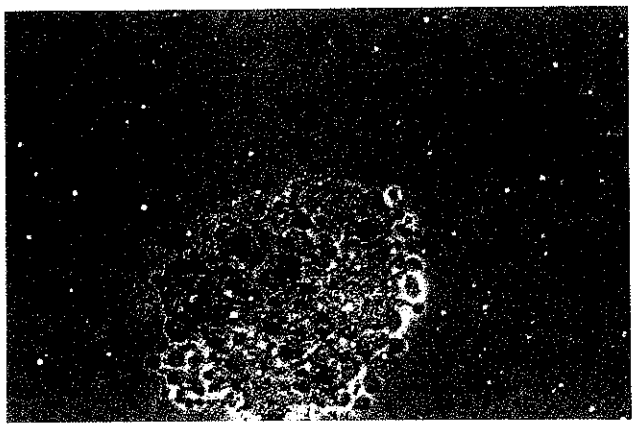

Fig. 5. Viability of glioma cells. Glioma cells (50 μl, 5×10^5 cells/ml) were incubated with $B_{12}B_4$ mAb (100 μl, in triplicate) for 36 h in a humidified 5% CO_2 atmosphere. Cells were then stained with trypan blue and viewed microscopically. Most of the glioma cells retained the dye, indicating their death.

Effect of radiation on glioma cell lines proliferation The negative control was prepared by exposing the different glioma cells to radiation. The ability of these cells to proliferate was completely destroyed by 20 min radiation, i.e. 20,000 rad (Fig. 2). However, it was decided to use a longer radiation time (45 min, 45,000 rad) to prepare irradiated glioma cells (negative control).

***In vitro* efficacy of anti-GFAP mAbs against human glioma cell lines** The anti-proliferative activities of our anti-GFAP mAbs were tested *in vitro*, using glioma cell lines recently prepared and established in our laboratory from freshly resected brain tumors. In this study, three glioma cell lines; one AA and two glioblastomas (GB_1 and GB_2) were used, and three anti-GFAP mAbs ($B_{12}B_4$, $B_{12}C_4$ and B_6C_6, all IgG_1, kappa), were tested. The mAb $B_{12}B_4$ inhibited the proliferation of GB_1 (96%), GB_2 (85%) and AA (93%) at a concentration of 3.2×10^{-10} *M* (Fig. 3A). mAb $B_{12}C_4$ inhibited the proliferation of GB_1 (95%), GB_2 (86%) and AA (94%) at a concentration of 3.26×10^{-10} *M* (Fig. 3B). mAb B_6C_6 inhibited the proliferation of GB_1 (75%), GB_2 (75%) and AA (91%) at a concentration of 2.074×10^{-10} *M* (Fig. 3C). These anti-GFAP mAbs showed little effect (≤20%) on normal human lymphocytes, and liver and intestinal cell lines.

Cytolytic activity of anti-GFAP mAbs towards human glioma cell lines ^{3}H-Thymidine release assay was used to test whether the anti-GFAP mAbs were cytotoxic to glioma cells or just interfered with ^{3}H-thymidine uptake by glioma cells. Fig. 4 showed that $B_{12}B_4$ and $B_{12}C_4$ have greater cytolytic activities towards glioma cells than mAb B_6C_6. The cytolytic activity of anti-GFAP mAbs on glioma cells was confirmed by examining cell viability after incubation with anti-GFAP mAbs. Most of the glioma cells retained trypan blue, indicating their death.

Glioma cell survival after anti-GFAP mAb treatment In the survival experiment, glioma cells, normal human lymphocytes, and intestinal and liver cells were treated with anti-GFAP mAbs, or unrelated mAb of the same isotype and Ig concentration (to monitor non-specific lysis) or tissue culture medium (to monitor spontaneous lysis). Anti-GFAP mAbs lysed most of the glioma cells, as indicated by retention of trypan blue (Fig. 5), but had no effect on normal human lymphocytes and very little effect on normal human liver cells. Unrelated mAb and tissue culture medium had no effect on any of the tested cell lines (data not shown).

DISCUSSION

In the last decade, our view of intermediate filaments has changed dramatically.[4-7] Once thought of as static structures with ill-defined functions, it is now clear that IFs are dynamic and undergo dramatic changes in re-

sponse to cell cycle signals, cellular differentiation, and pathogenic events. Diagnosis of many diseases, particularly cancers, has relied on the presence of IFs as cell type-specific markers in discerning the etiology of an unknown tissue.[15, 16] However, the functional role(s) of IFs is still unclear, particularly with respect to tumorigenesis.[17]

The presence of large quantities of IFPs, particularly GFAP, in glioma has so far been used only for diagnostic purposes. In a previous study we reported that xenogeneic immunization of freshly prepared human glioma extracts into goats yielded a polyclonal antiserum which, after multiple absorptions, specifically identified antigenic entities only in glioma extracts and not in appropriate controls. These results were confirmed by the generation of a panel of murine mAbs (anti-GFAP) which recognize a subset of the same antigen with no apparent cross-reactivities to normal brain, serum, liver, muscle, kidney, spleen or melanoma tissues.[14] In the present study, the ability of anti-GFAP mAbs to control the proliferation of glioma cells was tested *in vitro*, using three anti-GFAP mAbs ($B_{12}B_4$, $B_{12}C_4$ and B_6C_6, all IgG_1, kappa) and three glioma cell lines; one AA and two glioblastoma multiforme (GB_1 and GB_2). Recognition of glioma cell surfaces by anti-GFAP mAbs is a prerequisite for their potential application in glioma radio-imaging and immunotherapy. Indirect immunofluorescence staining gave green fluorescent rings characteristic of surface staining (data not shown) and clearly demonstrated the ability of anti-GFAP mAbs to recognize the surfaces of glioma cells. The preparation of glioma cells for surface staining involved the fixation of cells with paraformaldehyde without permeabilization before treatment with anti-GFAP mAbs, a procedure which precludes the possibility of cytoplasmic staining, because under these experimental conditions anti-GFAP mAbs would not be able to penetrate the cell membrane. Inhibition of glioma cell proliferation by mAb $B_{12}B_4$ was in the range of 85–96.5% at 3.2×10^{-10} *M* (Fig. 3A), that by mAb $B_{12}C_4$ was in the range of 86.7–95.5% at 3.26×10^{-10} *M* (Fig. 3B), and that by mAb B_6C_6 was in the range of 75.6–91.6% at 2.074×10^{-10} *M* (Fig. 3C). Anti-GFAP mAbs lysed glioma cells, as shown by ^{3}H-thymidine release assay (Fig. 4) and dye exclusion (Fig. 5). The anti-proliferative activity of anti-GFAP mAbs towards human malignant glioma cell lines is encouraging. The bio-distribution of ^{125}I-GFAP mAbs in rats bearing brain tumors should be examined to evaluate the efficacy of anti-GFAP mAbs for radioimaging and immunotherapy of glioma. Anti-GFAP mAbs could be conjugated to cytotoxic drugs to generate immunotoxins. Such conjugation is expected to increase the potency of anti-GFAP mAbs.

A possible interpretation of the *in vitro* inhibition of glioma cell growth by anti-GFAP mAbs is that binding of anti-GFAP mAbs to the intermediate filament network may lead to its collapse, causing cell lysis, because without a proper IF network, cells become fragile and prone to breakage.[18] IF or IF-like proteins form a skeletal scaffold, a continuous network from the plasma membrane to the nuclear matrix. This scaffold includes cell surface-associated proteins, such as desmoplakin[19] and ankyrin,[20] cytoplasmic IF proteins and IFAPs[21] and the nuclear lamins.[22] According to this scheme, the nuclear and cytoplasmic IF systems act in concert to convey mechanical and molecular information from the cell surface to the nucleus and/or from the nucleus to the cell surface through the various cellular compartments.[22] The IF network may represent a tracking system for targeting specific mRNAs to specific intracellular compartments.[23] The attachment of some mRNA molecules to the IF system may be important not only for determining their intracytoplasmic location, but also ultimately for their translation. For example, there is evidence suggesting that the attachment of mRNA to cytoskeletal sites is necessary for protein synthesis.[24] IF proteins are excellent substrates for several signal transduction effectors such as protein kinase C, cAMP-dependent protein kinase, CaM kinase II and the cdc2 kinase.[6] Thus, the effects of anti-GFAP mAbs observed in glioma cells probably reflect damage to many structural and biochemical connections mediated throughout the IF network.

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