Published in final edited form as:

Cancer Genet Cytogenet. 2006 March ; 165(2): 121–134.

Isolation of immunoresistant human glioma cell clones after selection with alloreactive cytotoxic T lymphocytes: cytogenetic and molecular cytogenetic characterization

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Abstract

Intratumoral heterogeneity and genetic instability within gliomas may allow intrinsically immunoresistant (IR) cells to escape alloreactive cytotoxic T lymphocyte (aCTL) cellular immunotherapy. The potential existence of aCTL-resistant variants prompted us to investigate whether cellular immunotherapy resistant glioma models could be isolated. To generate the models, repeated intermittent or continuous selective pressure (ISP or CSP) with multiple aCTL populations was applied to a low-passage glioblastoma cell explant, 13-06-MG, obtained from a patient at initial diagnosis. IL-6 and IL-8 secretion was greater in cocultures of aCTL cells with 13-06-ISP and 13-06-CSP immunoselected cells than those with 13-06-MG parental cells. Initially, the immunoselected cells were less sensitive to aCTL lysis; however, the reduced aCTL-sensitivity was not maintained upon further selection. We therefore isolated IR clones from continuously immunoselected cells (13-06-CSP). The frequency of IR clones was 1–6 cells per 10,000 immunoselected cells. Two clones selected for further study, 13-06-IR29 and 13-06-IR30, resisted aCTL lysis in the absence of immunoselective pressure. Cytogenetic analyses revealed structural anomalies and genomic imbalances unique to the IR clones. Based on these findings, a hypothetical model is proposed that traces the origin of the IR clones to a clonal variant within the 13-06-CSP and 13-06-MG populations.

1. Introduction

Malignant gliomas are the most common of primary brain tumors in the central nervous system [1]. Of the malignant gliomas, the anaplastic astrocytomas and glioblastoma multiformes outnumber all other types. Their infiltrative nature and heterogeneity represent a significant impediment to achieving a curative intervention by conventional therapies. Even with aggressive therapeutic intervention, the median survival time for glioblastoma patients remains poor (~12–15 months) [2–4]. This dismal prognosis has compelled researchers to investigate experimental adjuvant therapies for these patients. Local adoptive cellular immunotherapy is a promising adjuvant therapy that has the potential of selective destruction of malignant cells while sparing normal brain cells [5,6].

Adjuvant cellular immunotherapy with alloreactive cytotoxic T lymphocytes (aCTL), sensitized to patient human leukocyte antigens (HLA), has shown promise in the treatment of recurrent malignant glioma patients [7,8]. Six recurrent brain tumor patients received multiple

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intermittent intracranial infusions consisting of interleukin-2 (IL-2) and aCTL derived from different donors over a 10-month period. One patient survived 40 months, and the remaining two were >11 years from the start of immune therapy and entrance into protocol. They have not required further therapy, and have no evidence of recurrent tumor on follow-up magnetic resonance imaging scans (latest follow-up June 2004). The results of the Phase I trial were encouraging; however, three of the six patients enrolled into the protocol did not respond. In order to enhance the current therapy, it is important to determine the reasons why not all brain tumor cells succumbed to treatment with aCTL. Among several plausible explanations, one could be the presence of intrinsically resistant cells to the therapy.

During application of selective pressure in situ, immunotherapy-resistant tumor cells may survive. The emergence of immunoresistant (IR) melanoma cells has been observed among patients treated with activated autologous T cells [9,10]. Reports of in vitro selection of melanoma and ovarian carcinoma cells resistant to autologous T cells or lymphokine activated killer (LAK) cells support the idea of intratumoral heterogeneity as the basis for immune therapeutic failure [11–13]. Moreover, tumor cells have been shown to employ several tactics to avoid killing by cytotoxic effector cells. Tumor cells may lose or downregulate expression of major histocompatibility complex class I antigens that can facilitate tumor peptide presentation [9,10,14,15]. Tumors may avoid immune recognition by modulating expression of tumor associated antigens [16] or adhesion molecules required for establishing cell to cell contacts with effector cells [17]. Astrocytomas expressing functional Fas ligand may counterattack effector T lymphocytes [18,19]. Secretion of immunosuppressive factors [20, 21] and upregulation of antiapoptotic proteins are also reported mechanisms providing immunoresistance [22–25].

The potential existence of aCTL-resistant glioma cells prompted us to investigate whether cellular IR glioma models could be isolated. To generate the models, repeated immunoselective pressure with multiple aCTL populations was applied to 13-06-MG glioblastoma cells obtained from a patient on initial diagnosis. Immunoselected cultures initially displayed a decreased sensitivity to aCTL lysis, but this was not maintained upon further selection. Two immunoresistant glioma cell clones, 13-06-IR29 and 13-06-IR30, were isolated from continuously immunoselected cultures (13-06-CSP). We characterized 13-06-MG, 13-06-CSP, 13-06IR29, and 13-06-IR30 through molecular and classical cytogenetic techniques to identify anomalies unique to the IR cells that might be associated with other functional, cellular, or genetic characteristics that contribute to their resistant phenotype.

2. Materials and methods

2.1. Glioma cell culture

Glioma cells (13-06-MG) were isolated from a left temporal lobe surgical specimen obtained from a glioblastoma multiforme patient at initial diagnosis. The tissue was collected according to institutional regulatory body guidelines and practices. The glioma cells were established in culture as previously described [26]. The cells were maintained in RPMI 1640 (Mediatech, Herndon, VA) containing 10% fetal bovine serum (FBS) (Gemini-Bioproducts, Woodland, CA) in a 37°C humidified chamber with 5% CO₂. The cells tested negative for mycoplasma contamination by Hoechst 33342 (Sigma, St. Louis, MO) staining [27]. DBTRG-05MG glioma cells were cultured as described [28]. The human leukocyte class I antigens displayed on 13-06-MG cells were HLA-A1, A2, B44, B57, and Bw4 and the class I antigens displayed on DBTRG-05MG cells were HLA-A2, A68, B35, B38, Bw4, and Bw6 as determined by molecular typing using polymerase chain reaction sequence-specific-primers and sequence based typing (ClinImmune Labs, Aurora, CO).

2.2. Expansion of patient lymphocytes for sensitization of aCTL

Peripheral blood lymphocytes from a glioblastoma patient were isolated, expanded, and used for sensitization of precursor aCTL as previously described [29]. Briefly, heparinized blood was diluted 1:1 (v/v) with phosphate-buffered saline (PBS) and layered over a density gradient medium (Lymphocyte Separation Medium; ICN Biomedicals, Aurora, OH) and centrifuged at $1,100 \times g$ for 15 minutes. The interface between the plasma and density gradient medium containing the mononuclear cells was transferred to another centrifuge tube, diluted 1:6 with PBS, and centrifuged at $200 \times g$ for 10 minutes. The cell pellet was washed again with PBS. Mononuclear cells were suspended at 10^7 cells/mL in AIM-V synthetic medium (Invitrogen, Carlsbad, CA) containing 10% FBS, the monoclonal antibody OKT3 (Muromonab-CD3, 10 ng/mL; Ortho Pharmaceuticals, Raritan, NJ), and 240 IU/mL of human recombinant IL-2 (Chiron Cetus, Emeryville, CA) and then were placed into an artificial capillary system (CellMax Quad, Germantown, MD). Lactic acid concentration was measured daily (2300 STAT Plus; YSI, Yellow Springs, OH), and plots were made to determine the rate of lactate production; fresh medium was added every 4 to 5 days or when the lactic acid concentration was extrapolated to be >0.5 g/L. Lymphocyte blasts in medium were cryopreserved in FBS containing 15% dimethylsulfoxide (1:1 v/v) and were stored at -80°C or in liquid nitrogen until needed for sensitization of precursor aCTL.

2.3. Generation of aCTL

Propagation of aCTL was largely as previously described [29]. Under institutional regulatory body approval, precursor aCTL were obtained from either leucopaks derived from apheresis procedures, or from heparinized whole blood drawn from healthy volunteers. Patient stimulator lymphocytes from the glioblastoma patient contributing tumor 13-06-MG were irradiated at a short distance with 2,500 rads from a ^{60}Co source. In one-way mixed lymphocyte reactions (MLR), precursor aCTL donor lymphocytes were combined with patient lymphocytes at responder to stimulator ratios between 10:1 and 1:1 and were placed into RPMI-1640 containing 10% FBS and 60 IU IL-2/mL. Daily lactic acid concentrations were used to determine if replenishment with fresh culture medium was necessary. Anti-13-06 aCTL were restimulated with OKT-3 (10 ng/mL) at ~14 days after initial sensitization to keep aCTL growing. Lytic activity was always tested between 14 and 28 days of culture after the initial sensitization.

2.4. Immunoselection

In order to obtain IR glioma cells, immunoselective pressure was applied either intermittently or continuously to parental glioma cells (13-06-MG) at passage 8 by coculturing them with aCTL at a ratio of 1:1. Selective pressure was always applied with aCTL between 14 and 28 days in culture after the MLR. Intermittent selection pressure (ISP) consisted of coculturing adherent glioma cells with aCTL for 1 week followed by a recovery and expansion period of 4 days prior to the addition of more aCTL from the same donor. One aCTL donor population was used for 2 weeks of selection. Continuous selection pressure (CSP) involved coculturing glioma cells with day 14 aCTL derived from one donor for 2 weeks. Day 28 aCTL from the first donor were replaced immediately with day 14 aCTL from a different donor by aspirating the aCTL in suspension and gently washing the monolayers to remove aCTL adhering to glioma cells. The aCTL derived from the second donor were then cocultured with glioma cells for another 2 weeks. This process was repeated as indicated in the results. For cytotoxicity testing, adherent immunoselected glioma cells were washed to remove aCTL and expanded 5–7 days prior to testing their susceptibility to aCTL lysis in chromium release assays.

2.5. Isolation of IR glioma clones and their cell proliferation

Because resistance to aCTL lysis was not stable in the polyclonal populations resulting from ISP or CSP, we isolated clones by limiting dilution in 96-well plates or by colony formation in 60- × 15-mm culture dishes [28]. The 13-06-ISP and 13-06-CSP immunoselected cells were cocultured with aCTL at a 1:1 glioma/aCTL ratio and allowed to recover for 4 days before plating by limiting dilution into 96-well plates. Nearly 1,800 wells were plated. Cloned cells were expanded and replicate microwell plates were made before screening them for their sensitivity to lysis. We also plated 3×10^4 13-06-CSP cells into multiple 60- × 15-mm culture dishes and cocultured them with aCTL at a 1:20 glioma/aCTL ratio. Surviving colonies were transferred into wells and expanded before assaying them for their sensitivity to lysis. The term IR was applied to those glioma cell clones that displayed a statistically significant ($P \leq 0.05$) decrease in lysis relative to parental cells at a 25:1 effector to target (E:T) ratio as determined by paired *t*-test analysis in three independent experiments. Of the isolated clones that were resistant to lysis, we chose to characterize two from CSP cultures that grew well, 13-06-IR29 and 13-06-IR30.

For growth determination, the number of viable cells was determined using hemocytometer counts at 2, 4, and 6 days from an initial plate of 2.5×10^4 cells seeded into triplicate wells of six-well culture plates. Glioma cells were collected after treatment with 0.25% trypsin and centrifugation at $200 \times g$ for 10 minutes. Cells were resuspended in RPMI 1640 containing 10% FBS and 0.02% trypan blue (Sigma) before counting. Cell doubling times were determined by linear regression analysis of the best-fit line drawn from the data points.

2.6. Cytokine secretion assays

Multiple cytokines, including those with T helper 1 type (Th1) and T helper 2 type (Th2) function, were detected in clarified supernates using a microsphere-based assay (Bio-source International, Camarillo, CA) and quantified on a Luminex 100 system at the University of Colorado Cancer Center Immunology Core Facility. Concentrations (pg/mL) of IL-1 β , -2, -4, -5, -6, -8, -10, IFN- γ , TNF- α , and GM-CSF were determined against standard curves from 50- μ L aliquots of supernates after 18-hour cocubation of aCTL with glioma cells at a ratio of 25:1. An average of 100 microspheres was acquired for each population of cytokine-specific microspheres. Because each micro-sphere is a self-contained assay, replicate test samples are unnecessary [30].

2.7. Cytotoxicity assays

The ^{51}Cr release assay was used to determine the lytic activity when effector cells were cocubated with glioma cells [31]. Briefly, 5×10^6 glioma cells suspended in 0.1 mL of their growth medium were labeled with 100 μCi of $\text{Na}_2^{51}\text{CrO}_4$ (Amersham, Park Ridge, IL) for 90 minutes at 37°C . Cells were washed twice with PBS and suspended in RPMI growth medium. In a final volume of 0.2 mL, 1×10^4 tumor target cells were placed into 96-well round bottom microtiter plate wells that contained various effector cell numbers to reach specific E:T ratios. The plates were centrifuged at $200 \times g$ for 5 minutes and incubated for 4 hours at 37°C , in a humidified 5% CO_2 atmosphere. Following centrifugation at $200 \times g$ for 5 minutes, 50% of the well volume was harvested and counted. Maximal release was produced by incubation of the targets with 2% Triton X-100 (Sigma). Spontaneous release was the counts per minute (cpm) of targets in assay medium alone. The percentage of specific release was calculated by the formula $[(\text{cpm}_{\text{experimental}} - \text{cpm}_{\text{spontaneous}})/(\text{cpm}_{\text{maximal}} - \text{cpm}_{\text{spontaneous}})] \times 100\%$. Values were reported as the mean percentage of lysis calculated from triplicate wells with standard error of the mean.

2.8. G-banding and spectral karyotyping analysis

Mitotic arrest of glioma cells was induced with colcemid (0.05 µg/mL) for 2.5 hours, and chromosome elongation was achieved using ethidium bromide (5 µg/mL) for 1 hour before harvesting. Hypotonization was performed with 0.075 M KCl and 1% sodium citrate at 37°C for 12 minutes. The cell suspensions were dropped onto microscope slides and incubated overnight at 53°C. GTL bands were visible after the slides were exposed to 0.6% trypsin for 20–25 seconds and then stained with Leishman's stain for 2 minutes. G-banding karyotyping was performed with a computer imaging system (CytoVision; Applied Imaging, Santa Clara, CA) with 10–14 metaphases from each cell population. Because there were many unidentified rearranged and translocated chromosomes in the specimens after G-banding karyotyping, they were subjected to spectral karyotyping (SKY) analysis using a SKY probe cocktail (human spectral karyotyping reagent; Applied Spectral Imaging, Vista, CA) as previously described [32]. In addition to those metaphases analyzed by GTL banding, another 13 to 16 metaphases were analyzed by SKY for each of the related glioma cell preparations. Chromosomal designation followed ISCN 1995 [33]. When the parental cells were analyzed on two separate occasions, they did not vary by more than five passage numbers (P_n) from the immunoselected cells or the clones (13-06-MG, P8, P20; 13-06-ISP and 13-06-CSP, P10; 13-06-IR29, P25; 13-06-IR30, P20). The presence of structural or numerical anomalies in 3 or more cells defined those cells as being a cytogenetic clone. Anomalies observed frequently (i.e., in >60% of the analyzed cells) are marked as such in the tables.

3. Results

3.1. Resistance of immunoselected and unrelated glioma cells to lysis by aCTL

We monitored human parental (13-06-MG), intermittently and continuously immunoselected glioma cells (13-06-ISP and 13-06-CSP) of similar passage number for their susceptibility to anti-13-06 aCTL lysis in 4-hour ^{51}Cr release assays. As an example, data are shown when the latter two had received selective pressure from 10 different aCTL populations (Fig. 1). Although statistical significance was not reached at all the E:T ratios tested, the 13-06-CSP and 13-06-ISP cells resisted aCTL-mediated cytolysis more than the parental cells, and the resistance to lysis was generally greater with the 13-06-CSP glioma cells. In parallel, the cytolysis of an unrelated glioma tumor target, DBTRG-05MG [28] was measured that displayed class I HLA antigens different from the 13-06-MG cells, with the exception of the A2 and Bw4 alleles. Consistent with minimal overlap to target antigens, the anti-13-06 aCTL caused measurable but low cytolysis of DBTRG-05MG. The substantial lysis of the 13-06-MG tumor cells further suggests the specificity of the cells within the aCTL preparation were largely directed to the HLA antigens of that tumor.

3.2. Cytokine secretion differences when aCTL are incubated with parental, immunoselected, and unrelated glioma cells

In parallel to the cytotoxicity assay just mentioned, 13-06-MG parental, 13-06-ISP, and 13-06-CSP immunoselected, and unrelated control DBTRG-05MG glioma cells were coincubated with anti-13-06 aCTL at a 25:1 E:T ratio for 18 hours. The supernates of the individual and coincubated cell populations were screened for a panel of cytokines that can be grossly grouped into Th1 and Th2 categories (Table 1). Relatively unremarkable differences in the GM-CSF, IFN γ , TNF α , IL-1 β , and IL-4 cytokines secreted within the various coincubates containing relevant (i.e., related) gliomas and the irrelevant (unrelated) glioma were obtained. Not unexpectedly, aCTL produced the T helper (Th) 1 cytokine, IFN- γ , and secretion was slightly upregulated upon their coincubation with all the glioma cell populations tested. Similarly, the IL-5 and IL-10 Th2 cytokines were secreted by the aCTL and upregulation of them occurred upon their coincubation with the gliomas, with the exception of 13-06-CSP.

More remarkable distinctions were obtained with the IL-6 and IL-8 cytokines. IL-6 (Th2) was minimally produced by the aCTL and the irrelevant DBTRG-05MG tumor target cells. Furthermore, the amount produced was slightly more than additive upon their coincubation. IL-6 was abundantly secreted individually by the 13-06-MG glioma cells, but the amounts secreted by the 13-06-CSP and 13-06-ISP cells were 7.2-fold and 2-fold higher, respectively. We noted that the IL-6 cytokine was greatly upregulated upon coincubation of the aCTL with the three relevant tumor types, and more dramatically so with the coincubates involving the immunoselected glioma cells. Similar findings were obtained for the IL-8 chemokine. All cell types produced it, but upregulation of IL-8 occurred more so in coincubates containing aCTL with the immunoselected 13-06-CSP and 13-06-ISP cells. Thus, different patterns of cytokine secretion resulted from coincubation of the anti-13-06 aCTL with immunoselected gliomas relative to the parental tumor. Cumulatively, the data imply that the recognition–response interactions between the aCTL effectors with the unrelated, irrelevant targets (DBTRG-05MG) were different than those with the relevant targets (13-06-MG glioma cells and variants); also, the interactions between aCTL effectors with the relevant parental cells (13-06-MG) differed from those with the relevant immunoselected variants (13-06-ISP and 13-06-CSP).

3.3. aCTL immunoselected glioma populations display a reduced sensitivity to lysis that is not stable upon continued application of selective pressure

To monitor for the stability of resistance, we periodically examined the aCTL sensitivity of intermittently and continuously immunoselected glioma cells (13-06-ISP and 13-06-CSP) relative to the 13-06-MG parental cells (Fig. 2). We tested the lysis after immunoselection with 10, 12, 13, and 15 anti-13-06 aCTL preparations made from different donors. At multiple E:T ratios in 4-hour chromium release assays, intermittently selected cells (13-06-ISP) were less susceptible to lysis after selection with 10 and 12 aCTL populations (Figs. 2a and 2b) relative to the parental cells. However, a reduced sensitivity to aCTL lysis was not evident after selection with 13 and 15 aCTL populations (Figs. 2c and 2d). Continuously selected cells (13-06-CSP) were less susceptible to lysis after selection with 10, 12, and 13 aCTL populations (Figs. 2e, 2f, and 2g) but not after 15 (Fig. 2h). These data show variable lysis of 13-06-MG from the multiple aCTL preparations. When using different donors as the source of the aCTL, variability is expected because (a) lytic events are driven by the differences in Class I antigens between the donors and the patient and (b) the CTL precursor frequency can vary as well as the natural killer cell activity from one donor cell pool to the next.

3.4. Isolation of immunoselected clones and their proliferation

Because we observed a decreased sensitivity to aCTL lysis that was not sustained in the 13-06-ISP and 13-06-CSP immunoselected glioma cells, we concluded that we were working with heterogeneous, polyclonal populations of glioma cells. To help control for glioma cell heterogeneity within the immunoselected glioma cell preparations, we proceeded to isolate aCTL-resistant clones from them by limiting dilution or by clonogenic assay. By both methods, the frequency similarly approximated 1 to 6 out of every 10,000 cells (0.01–0.056%). Interestingly, stably IR clones were isolated only from populations receiving CSP. We chose to characterize two clones, 13-06-IR29 and 13-06-IR30. Relative to the parental cells, both IR clones resisted lysis when tested in three independent assays by the aCTL in 4-hour ⁵¹Cr-release assays at multiple E:T ratios. Representative data with *P*-values at ≤ 0.01 by paired *t*-test are shown (Fig. 3). The clones, unlike the parental cells, did not exhibit a dose-response to effector cell numbers that would be expected going from high to low E:T ratios. The cell doubling times of the clones were determined from hemocytometer counts of cells that excluded trypan blue dye. The doubling time of the 13-06-MG, 13-06-IR29, and 13-06-IR30 cells was 2.6, 2.3, and 2.7 days, respectively.

3.5. Maintenance of resistance of aCTL lysis by immunoselected clones upon removal of immunoselective pressure

Following removal of the aCTL immunoselective pressure from the clones, we examined the stability of the resistance as a function of number of cell doublings, because genetic changes occur when cells divide. Sensitivity to lysis was examined periodically over a 20 week period in 4-hour cytotoxicity assays at a 25:1 E:T ratio. Variations in cytolysis of the same tumor target by different donor aCTL would be expected. To normalize data between experiments, the mean percent lysis of parental cells was adjusted to 100% and the lysis of the clones expressed as a percent of parental cell lysis. For discussion purposes, we arbitrarily defined the resistant phenotype as maintained if lysis was <60% of parental cell lysis. For clone 13-06-IR29, resistance was maintained for at least 40 cell doublings, because the lysis ranged from 25 to 54% of the parental during that time, but exceeded the cutoff limit by 61 cell doublings, which occurred at 20 weeks of culture (Fig. 4a). In the same time period, resistance was maintained for clone 13-06-IR30 for at least 13 cell doublings (15–60% of the parental lysis), but lysis exceeded the cutoff limit by 34 cell doublings (Fig. 4b).

3.6. G-banding and SKY analysis of IR clones, parental and immunoselected glioma cells

To discern if there were any cytogenetic commonalities among the 13-06-IR29 and 13-06-IR30 IR clones, we cytogenetically analyzed ~30 metaphases from each of them by classic G-banded karyotyping and SKY and compared them to those of the 13-06-MG parental and 13-06-CSP immunoselected glioma cells. Composite karyotypes derived from these analyses are given in Table 2. Our analyses were based on triploid as being the reference ploidy, and as such, because the majority of glioma cells analyzed had chromosomal contents ranging from hypertriploid to hypotetraploid (i.e., chromosome number range 66–126), the imbalances detected were more commonly gains than losses.

Typical of other high-grade gliomas that we have characterized [28,34–36], the glioma cells displayed complex karyotypes (Table 2) involving many genomic imbalances (Table 3) and structural rearrangements (derivatives, translocations, duplications, isochromosomes, and deletions) (Table 4). The 13-06-MG parental cells were hypertriploid to hypotetraploid, with 72–86 chromosomes per cell. Two cytogenetic clonal variants were identified within the 13-06-MG parental population (Fig. 5). Clone 1 represented 67% of the cells and clone 2 represented the remaining 33%. These cytogenetic clones shared some abnormalities, such as del(17)(q12q21), i(17)(q10), and del(19)(p13), but each also displayed specific changes (Table 4). For instance, clone 1 showed der(1)t(1;15), i(1)(p10), i(1)(q10), der(7)t(7;10), and der(9)t(9;10) but clone 2 showed der(4)t(4;7), der(4)t(4;9), rea(7), i(14)(q10), der(16)t(3;16), and der(20)t(7,20). The 13-06-CSP immunoselected population was also hypertriploid to hypotetraploid (72–87 chromosomes) and contained two cytogenetic clonal variants, 1 and 2, similar to those observed in the parental population, and at similar frequencies (clone 1 = 78%, clone 2 = 22%) (Table 4; Fig. 5). Overall, the IR 13-06-IR29 and 13-06-IR30 clones displayed a slightly reduced chromosomal content compared with the parental and the CSP immunoselected cells. Most of the cells examined from IR clone 13-06-IR29 were hypertriploid (70–76 chromosomes), although occasional cells contained hypotriploid and hypopentaploid chromosome numbers (66–109 chromosomes) (Table 4; Fig. 5). Two cytogenetic subclones were identified within IR 13-06-IR30 cloned cells. Cytogenetic subclone A represented 61% of the cells, which were generally hypertriploid (69–74 chromosomes), with a few exceptions that reached hyperpentaploid chromosome numbers (126 chromosomes). Cytogenetic subclone B represented the remaining 39%, which comprised hyperdiploid to hypotetraploid cells (55–59 chromosomes).

The genomic imbalances of the glioma cell populations and clones are summarized in Table 3. Frequent clonal abnormalities (i.e., those existing in >60% majority of the cells) are marked

as such. Major features shared by all of the glioma cell populations and clones analyzed were gains of chromosome 7 and arm 17q and loss of arm 17p. Loss of chromosome 10, which was clonal but not as frequently displayed in the parental cells, became a common major feature to all the other glioma cell populations studied. Major imbalances common only to the IR clones were losses of chromosomes 4 and 6 and arm 2q. Gain of chromosome arm 20p was restricted to clonal variant 2 of parental and immunoselected cells and the IR clones. The most frequent genomic imbalance in parental and 13-06-CSP cells was a gain of chromosome 15; other, less frequent gains included chromosomes 11, 12, 18, and 21 and arm 1p. Major features that distinguished clone 1 from clone 2 in the parental and 13-06-CSP cells was the loss of chromosome 14 in clone 1 and gain of arm 3q in clone 2. Based on chromosomal imbalances alone, the IR clones exhibited cytogenetic patterns that distinguished them from the parental cells from which they were derived.

A list of structural abnormalities that were found in the glioma cell populations and clones is presented in Table 4, with those appearing in a majority of cells marked as such. Three frequently observed clonal structural anomalies shared by the parental 13-06-CSP cells and the IR clones were $\text{del}(17)(\text{q}12\text{q}21)$, $\text{i}(17)(\text{q}10)$, and $\text{del}(19)(\text{p}13)$. Six additional major structural abnormalities distinguished clone 2 from clone 1 in the 13-06-MG cells. Even upon immunoselection with aCTL, clones 1 and 2 were essentially retained with the same relative frequency in 13-06-CSP cells, even though both clones showed an overall reduction of rare abnormalities compared to the parental population. The 13-06-IR29 clone was monoclonal; 13-06-IR30 cultures contained two subclones, A and B. Interestingly, four anomalies, $\text{der}(4)\text{t}(4;7)(\text{q}21;\text{q}21)$, $\text{der}(4)\text{t}(4;9)(\text{p}11;\text{q}13)$, $\text{i}(14)(\text{p}10)$, and $\text{der}(16)\text{t}(3;16)(\text{q}13;\text{p}11.1)$ were found in very high frequencies in the 13-06-MG clone 2, 13-06-CSP clone 2, 13-06-IR29, and 13-06-IR30 subclones A and B. Three other abnormalities, $\text{der}(2)\text{t}(2;20)(\text{q}11.2;\text{q}11.2)$, $\text{der}(4)\text{t}(3;4)(?;\text{q}35)$, and $\text{rea}(7)$, were present in almost every cell in 13-06-IR29 and in 13-06-IR30 subclone A, but were never observed in 13-06-IR30 subclone B (Table 4; Fig. 5). Representative G-banded and SKY karyotypes of cells from the 13-06-IR29 clone are shown in Figs. 6a and 6b, respectively. The cells from the 13-06-IR29 clone contained the highest frequency of structural abnormalities; a representative G-banded karyotype even displays a translocation of three chromosomes (designated as $\text{t}1/4/13$; Fig. 6a). A SKY karyotype of a different 13-06-IR29 cell readily confirms multiple translocations and anomalies within one cell: $\text{der}(1)\text{t}(1;16)$, $\text{der}(2)\text{t}(2;20)$, $\text{der}(4)\text{t}(3;4)$, $\text{der}(4)\text{t}(2;4)$, $\text{der}(4)\text{t}(4;7)$, $\text{der}(4)\text{t}(4;9)$, $\text{der}(10)\text{t}(1;10)$, $\text{der}(16)\text{t}(3;16)$, $\text{del}(17)$, $\text{i}(17\text{q})$, $\text{del}(19\text{p})$, $\text{der}(20)\text{t}(7;20)$ (Fig. 6b).

During the cytogenetic analyses, we also observed a high number of single-cell abnormalities within the related glioma cell populations (data not shown). Out of ~30 cells analyzed of each population, we counted 11, 12, 18, and 18 single-cell abnormalities from the 13-06-MG, 13-06-CSP, 13-06-IR29, and 13-06-IR30 cells, respectively. These data also demonstrate glioma cell heterogeneity and genomic instability.

In summary, based on these findings, a hypothetical model can be presented for the clonal progression that likely occurred during immunoselection with aCTL (Fig. 5). Cytogenetic clones 1 and 2 present in the parental population were essentially retained in the immunoselected cultures upon the application of CSP from six aCTL populations. The 13-06-CSP cells were then exposed to six additional aCTL populations prior to the isolation of two aCTL-resistant clones, 13-06-IR29 and 13-06-IR30. The IR clones shared more clonal rearrangements with clone 2 than clone 1 that were contained within the 13-06-MG parental and 13-06-CSP cell populations. Four additional clonal anomalies $\text{der}(2)\text{t}(2;20)$, $\text{der}(4)\text{t}(2;4)$, $\text{der}(4)\text{t}(3;4)$, and $\text{rea}(7)$ distinguished the IR clones from clone 2 cells within 13-06-CSP. The 13-06-IR29 had the highest frequency of structural anomalies; 14 of the 30 were nonshared structural aberrations. IR clone 13-06-IR30 cells were composed of two cytogenetic subclones, A and B. Subclone A was distinguished by six nonshared abnormalities, two of which were

frequently observed. Based on the abnormalities seen, we cannot tell whether subclone B originated independently from clone 2 of the 13-06-CSP cells or whether it was derived from subclone A of 13-06-IR30 cells. Overall, these data suggest that the selective pressure applied caused cytogenetic changes that contribute to, or are associated with, immunoresistance. Finally, the data support the concept of glioma cell heterogeneity and genomic instability.

4. Discussion

Cellular immunotherapy of recurrent malignant glioma patients employing aCTL has been explored as an adjuvant treatment strategy [7,8]. We wanted to explore the possibility that repeated administrations of aCTL, derived from different donors, may result in the expansion of stably IR tumor cell variants that could eventually render the immunotherapy ineffective. For instance, Topalian and colleagues [11] reported that four cycles of immunoselection produced a melanoma variant completely resistant to autologous tumor infiltrating lymphocytes and the resistant phenotype persisted for 13 months. In vitro ISP and CSP with aCTL were applied to a human glioma cell explant. We found that the immunoselected glioma cells initially displayed a decreased sensitivity to aCTL lysis (Fig. 1), but upon continued selection the decreased sensitivity was not maintained (Fig. 2), similar to immunoselected melanoma and ovarian carcinoma cells that displayed transient IR to allogeneic and autologous effectors, respectively [12,13]. Much like the approach that we used clinically for aCTL administration [7,8], repeated intermittent immunoselection applied from multiple aCTL preparations in vitro did not result in a stable IR glioma cell population. With CSP, IR clones were eventually isolated that displayed a better stability to resistance, but even those clones eventually reverted to a more susceptible phenotype (Figs. 3 and 4). It is advantageous therapeutically if the frequency of IR glioma cells is low and that the stability to the resistance can revert (i.e., IR cells do not develop a tolerance to the treatment). It is possible that the selection with multiple aCTL populations derived from different donors may increase the number of antigenic epitopes recognized by the aCTL, making it difficult for the small number of IR glioma cells to adapt to the aCTL. Our data suggest that glioblastoma patients may benefit from adjuvant adoptive aCTL immunotherapy because the resistance is relatively transient. On the other hand, the frequency of IR cells was 1–6 per 10,000 cells, and thus could be as high as 600 cells in a million. If one assumed that even after surgical debulking of tumor as much as 1 cm³ of tumor tissue remained, adjuvant therapies would have about 10⁹ cells to contend with (assuming 1 cm³ = 10⁹ cells). In that residual tumor there could be up to 6 × 10⁵ resistant cells, which could grow to the original 10⁹ cells in about 3–4 weeks, assuming a 2–3 day doubling time. It could explain adjuvant therapy failure. Also of concern is the development of cells cross-resistant to other therapies upon in vivo immunoselection. Therefore, an apparently small number of IR cells could play a fundamental role in the poor outcome of brain tumor treatment. Of course, our analysis comes from examination of one glioma explant obtained from a patient at initial diagnosis. Other gliomas, especially those coming from patients who have had other treatments, may respond differently to immunoselection in terms of resistance and reversibility. Study of a larger panel of immunoselected glioma cells is needed to confirm these observations.

The isolation of IR glioma cell models offers a potentially useful tool to examine factors that contribute to the resistance. As an example, the enhanced secretions of IL-6 and IL-8 cytokines obtained with immunoselected gliomas relative to the parental glioma cells upon coincubation with aCTL might indicate different recognition and response patterns between the effector and targets. It may be an antiapoptotic survival response by the tumor cells [37,38]. To clarify this further, experiments with secretion inhibitors are ongoing to define whether the effector or target is responsible for the IL-6 and IL-8 cytokine secretions during coincubation. Additionally, the cytogenetic analyses, besides confirming the genetic instability in glial tumors, can be used to correlate structural anomalies and genomic imbalances with microarray

data that can point to gene products responsible for playing a role in the IR. Therapy-related chromosomal changes were reported in gliomas [39,40]. When we analyzed our data for relative gross chromosomal changes, we found that IR cells displayed preferential losses of chromosomes 4 and 6 and arms 2q (Table 3). Although the contribution of these changes to the resistance is presently unknown, knowledge of the location of genes involved in glioma-T cell interactions and induction of cell death may point to potential mechanisms of aCTL resistance. As an example, HLA class I alleles reside on chromosome 6, thus determining whether downregulation or loss of HLA class I expression contributed to the resistance was warranted based on the cytogenetic findings [41]. Another example is the relative gain of chromosome 12 observed in the parental and 13-06 CSP cells, compared to no gain or loss in the IR clones (Table 3). Altered expression of the proapoptotic factor Apaf-1, whose gene resides on chromosome 12 [42,43], correlated with our cDNA microarray analysis of apoptosis-related pathway gene expression. A 20-fold or greater downregulation of the *APAF1* gene within the IR clones was revealed, compared with the parental cells [41]. Further examination of the cytogenetic findings along with functional assays and microarray analyses may lead to the identification of factors responsible for providing mechanisms of escape to the immunotherapy. This knowledge would then allow for the development of drugs or gene therapy strategies to overcome the resistance.

Acknowledgements

This work was supported by National Institutes of Health grants F31 CA94834 to GGG, NS-046463 to CAK, The R. Herbert & Alma S. Manweiler Memorial Research Fund, and The La Jolla Foundation for Molecular Medicine Research. GGG performed the experimental work in partial fulfillment for his doctoral dissertation work required in the Experimental Pathology Program at the University of Colorado. Chiron, Inc. supplied the IL-2 for these studies. CAK and MVG were members of the University of Colorado Cancer Center. The Immunology, Cytogenetics and Radiological Core Facilities of the University of Colorado Cancer Center were utilized. We thank Dr. Lin Chen of the Cytogenetics Core for her technical expertise with the karyotyping.

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Fig. 1.

Immunoselected glioma cells display a reduced sensitivity to lysis by aCTL compared to parental 13-06-MG glioma cells. Anti-13-06 aCTL lysis of parental (13-06-MG), intermittently immunoselected (13-06-ISP), continuously immunoselected (13-06-CSP), and unrelated target (DBTRG-05MG) glioma cells. Triplicate wells of glioma cells (10^4) were coincubated with 5×10^5 , 2.5×10^5 , and 1.25×10^5 aCTL cells for 4 hours. The percent lysis $\pm$ standard error of the mean (SEM) of glioma cell targets is shown at the multiple effector to target (E:T) ratios. An asterisk indicates statistical significance ($P \leq 0.05$) was reached relative to parental 13-06-MG cells by paired *t*-test.

Fig. 2.

Alloreactive CTL selected glioma cells display a reduced sensitivity to lysis that is not maintained upon continued application of selective pressure. The sensitivity of parental glioma cells (13-06-MG; solid bars) to aCTL-mediated cytotoxicity is compared to those given intermittent selective pressure (13-06-ISP; hatched bars) or continuous selective pressure (13-06-CSP; striped bars). The cytotoxicities were obtained after the 13-06-ISP and 13-06-CSP populations had been immunoselected with 10 (panels a and e), 12 (b and f), 13 (c and g), or 15 (d and h) aCTL preparations. The percent lysis $\pm$ SEM of tumor targets is shown at multiple E:T ratios after a 4-hour coincubation with aCTL.

Fig. 3.

Glioma clones were isolated that significantly resisted lysis by aCTL at all of the E:T ratios examined ($P \leq 0.01$). The percent lysis $\pm$ SEM of the parental glioma cells (13-06-MG) is compared to the immunoresistant 13-06-IR29 clone or to the 13-06-IR30 clone after coinubation with aCTL for 4 hours at several E:T ratios.

Fig. 4.

The maintenance of resistance to different aCTL preparations by clones a) 13-06-IR29 and b) 13-06-IR30 after removal of immunoselective pressure is shown over time relative to the number of cell doublings that occurred. aCTL were placed into 4-hour cytotoxicity assays at a 25:1 E:T ratio with immunoresistant clones 13-06-IR29 (open bars) or 13-06-IR30 (striped bars). For each experiment, lysis of 13-06-MG (solid bars) parental cells was adjusted to 100% and lysis of the clones was expressed as a percent of the parental cell lysis $\pm$ SEM.

Fig. 5.

Hypothetical model of the clonal progression occurring in the related 13-06-MG cell populations during immunoselection with aCTL based on the cytogenetic findings.

Fig. 6. Representative G-banding (a) and SKY (b) karyotypes of immunoresistant clone 13-06-IR29. The lower portion of panel b is the inverted 4',6-diamidino-2-phenylindole (DAPI) image of the cells stained with SKY reagent.

Table 1

Cytokines secreted by individual and aCTL/glioma cell cocultures

Effector	Target ^a	GM-CSF	IFN γ	TNF α	IL-1 β	IL-4	IL-5	IL-6	IL-8	IL-10
Anti-13-06 aCTL	none	40	164	37	14	45	886	81	1,245	483
None	13-06-MG	16	14	21	15	47	16	635	3,431	10
Anti-13-06 aCTL	13-06-MG	96	284	51	16	51	1,112	2,007	5,644	578
None	13-06-CSP	44	22	26	22	43	26	4,593	9,398	12
Anti-13-06 aCTL	13-06-CSP	196	275	71	39	49	710	14,051	15,093	400
None	13-06-ISP	20	16	21	13	45	22	1,272	2,410	10
Anti-13-06 aCTL	13-06-ISP	108	312	55	19	49	996	7,671	7,923	516
None	DBTRG-05MG	12	26	23	13	45	24	47	511	12
Anti-13-06 aCTL	DBTRG-05MG	76	309	45	17	43	1,102	217	2,003	518

Anti-13-06 aCTL (25×10^4) or glioma target cells (10^4) were incubated alone or cocultured for 18 hours (25:1 E:T ratio) before clarifying supernates for analysis. Simultaneous detection of multiple human cytokines present in the clarified supernates was achieved with a 10 plex cytokine kit and the Luminex 100 flow cytometer. Concentrations of cytokines in pg/mL are given after subtracting medium-alone values. IL-2 is not reported, because it was a component of the growth medium.

^aRelevant, related parental and immunoslected gliomas (13-06-MG, 13-06-ISP, and 13-06-CSP), and irrelevant, unrelated DBTRG-05MG glioma (with minimal 2 HLA, AB allele overlap) were tested with the anti-13-06 aCTL.

Table 2

[illegible]

Clonal genomic imbalances observed in 13-06-M6, 13-06-CSP, 13-06-IR29, and 13-06-IR30 cells based on the SKY and G-banded karyotypic analyses

Numerical imbalance Chromosome number Ploidy	13-06-MG		13-06-CSP		13-06-IR29	13-06-IR30	
	Clone 1 72-86 hyper 3 <i>n</i> to hypo 4 <i>n</i>	Clone 2 76-83 hyper 3 <i>n</i> to hypo 4 <i>n</i>	Clone 1 72-87 hyper 3 <i>n</i> to hypo 4 <i>n</i>	Clone 2 74-84 hyper to hypo 4 <i>n</i>	66-76/109 hyper 3 <i>n</i>	Subclone A 69-74/126 hyper 3 <i>n</i>	Subclone B 55-59 hyper 2 <i>n</i> to hypo 3 <i>n</i>
Gain of X	X	—	X	—	—	—	—
Gain of 1	—	—	—	—	X	X	X
Gain of 1p	X	X	X	X	—	—	—
Gain of 2	X	—	—	—	—	X	—
Gain of 2p	—	—	—	—	XX	XX	XX
Loss of 2q	—	—	—	—	—	—	—
Gain of 3	X	—	X	—	—	—	X
Loss of 3	—	—	—	—	—	—	—
Gain of 3q	—	XX	—	XX	—	—	—
Loss of 4	—	—	—	—	XX	XX	XX
Gain of 5	X	X	X	X	X	X	—
Loss of 6	—	—	—	—	XX	XX	XX
Gain of 7	XX	XX	XX	XX	XX	XX	XX
Gain of 7p	XX	XX	XX	XX	XX	XX	XX
Gain of 8	X	—	X	—	—	—	—
Gain of 9	X	—	X	—	—	—	—
Loss of 10	X	X	XX	XX	XX	XX	XX
Gain of 11	X	X	X	X	—	—	—
Loss of 11	—	—	—	—	—	—	X
Gain of 12	X	X	X	X	—	—	—
Loss of 12	—	—	—	—	—	—	X
Gain of 13	X	—	X	—	X	—	—
Loss of 14	XX	—	XX	—	—	—	—
Gain of 15	XX	XX	XX	XX	—	—	—
Loss of 15	—	—	—	—	—	—	X
Gain of 16q	—	XX	—	XX	XX	XX	—
Loss of 16p	—	—	—	—	—	—	XX
Loss of 17p	XX	XX	XX	XX	XX	XX	XX
Gain of 17q	XX	XX	XX	XX	XX	XX	XX
Gain of 18	X	X	X	X	—	—	—
Loss of 18	—	—	—	—	—	X	XX
Gain of 19q	X	X	X	X	X	X	X
Gain of 20	X	—	X	—	—	—	—
Gain of 20p	—	X	—	XX	XX	XX	XX
Gain of 21	X	X	X	X	—	—	—

An "X" indicates that the numerical imbalance was observed in at least three of the analyzed cells and thus was a feature of the cytogenetic clone. A bolded "XX" indicates numerical imbalance existed in a majority (>60%) of the cells analyzed.

Table 4
Clonal chromosomal structural abnormalities found in 13-06-MG, 13-06-CSP, 13-06-IR29, and 13-06-IR30 cells

Structural abnormality Chromosome number Cells number analyzed (% of total)	13-06-MG		13-06-CSP		13-06-IR29		13-06-IR30	
	Clone 1	Clone 2	Clone 1	Clone 2			Subclone A	Subclone B
	72-86 20 (67%)	76-83 10 (33%)	72-87 18 (78%)	74-84 5 (22%)	66-76/109 29 (100%)	69-74/126 17 (61%)	55-59 12 (39%)	
del(1)(p12)	X	—	X	—	—	—	—	—
del(1)(q12)	X	X	X	X	X	X	—	—
del(1)(q31)	—	—	—	—	—	—	—	—
der(1;10)(q12;q11.2)	X	X	—	—	—	—	—	—
der(1)(t(1;11)(q12;p11)	X	—	—	—	X	—	—	—
der(1)(t(1;11)(q44;q14)	X	—	—	—	—	—	—	—
der(1)(t(1;15)(q12;q11)	X	—	—	—	X	—	—	—
der(1)(t(1;16)(q21;?)	—	—	—	—	X	—	—	—
dup(1)(q32q44)	X	—	—	—	—	—	—	—
i(1)(p10)	X	—	—	—	—	—	—	—
i(1)(q10)	X	—	—	—	—	—	—	—
der(2)t(2;20)(q11.2;q11.2)	—	—	—	—	XX	XX	—	—
der(2)t(2;21)(q11.2;p13)	—	—	—	—	—	—	—	—
del(3)(q11)	X	—	—	—	—	—	—	—
der(4)t(2;4)(p11.2;p12)	—	—	—	—	XX	XX	XX	XX
der(4)t(3;4)(?;q35)	—	—	—	—	XX	XX	—	—
der(4)t(4;7)(q21;q21)	—	XX	XX	XX	XX	XX	XX	XX
der(4)t(4;9)(p11;q13)	—	XX	XX	XX	XX	XX	XX	XX
del(7)(q21)	—	X	—	—	—	—	—	—
der(7)t(X;7)(q13;p11)t(X;14)(q28;q21)	—	—	—	—	X	—	—	—
der(7)t(X;7)(q13;p11)t(X;15)(q28;q12)	—	—	—	—	—	—	—	—
der(7)t(7;10)(q32;q11.2)	X	—	—	—	—	—	—	—
der(7)t(7;11)(q22;q25)	—	—	—	—	X	—	—	—
der(7;15)(q22;q26)dup(7)(q22q36)	—	—	—	—	—	—	X	—
i(7)(p10)	X	—	—	—	—	—	—	—
rea(7)	—	XX	—	—	XX	XX	—	—
der(9)t(9;10)(q34;q11.2)	X	—	—	—	—	—	—	—
der(10)t(1;10)(q21;q26)	—	—	—	—	X	—	—	—
der(12)t(10;12)(q24.3;q21)	X	—	—	—	—	—	—	—
der(12)t(9;12)(q12.3;q24.3)	—	—	—	—	X	—	—	—
der(12)t(12;13)(q1;q11)dup(12)(q4q23)	—	—	—	—	X	—	—	—
der(12)t(12;14)(q24.3;q21)	—	—	—	—	X	—	—	—
der(13)t(X;13)(?;p13)	—	—	—	—	X	—	—	—
der(13)t(4;13)(q12;p13)t(1;4)(q21;q21)	—	—	—	—	—	—	—	—
der(13)t(5;13)(q12;p11)	—	—	—	—	—	X	XX	XX
der(13)t(13;17)(p11;p11)	—	—	—	—	—	—	—	—
der(16)t(6;16)(?;q13.1)	—	—	X	XX	—	—	—	XX
i(14)(q10)	—	XX	—	XX	XX	XX	XX	XX
der(15)t(1;15)(q21;p11)	—	—	—	—	—	X	—	—
der(15)t(7;15)(p11;p11)	—	—	—	—	—	—	—	—
der(15)t(15;21)(p13;p11)(17;21)(q11;q22)	—	—	—	—	—	—	—	—
der(16)t(3;16)(q13;p11.1)	—	XX	XX	XX	XX	XX	XX	XX
del(17)(q12q21)	XX	XX	XX	XX	XX	XX	XX	XX
i(17)(q10)	XX	XX	XX	XX	—	—	—	—
del(19)(p13)	—	—	—	—	—	—	—	—
der(20)t(7;20)(p21.2;q11.2)	—	—	—	—	—	—	—	—
der(21)t(5;21)(p13;p13)	—	—	—	—	—	—	—	—
der(21)t(13;21)(q11;p13)	—	—	—	—	—	XX	XX	XX
der(21)t(19;21)(q1;p11)	—	—	—	—	—	XX	XX	XX
der(22)t(1;22)(q12;p13)	X	—	—	—	X	XX	—	—

Structural abnormality Chromosome number Cells number analyzed (% of total)	13-06-MG		13-06-CSP		13-06-IR29		13-06-IR30	
	Clone 1	Clone 2	Clone 1	Clone 2			Subclone A	Subclone B
	72-86 20 (67%)	76-83 10 (33%)	72-87 18 (78%)	74-84 5 (22%)	66-76/109 29 (100%)		69-74/126 17 (61%)	55-59 12 (39%)
der(22)t(4;22)(q12;p13)	—	—	—	—	—		X	—
der(22)t(19;22)(p11;p13)	—	—	—	—	X		—	—

An "X" indicates that the structural abnormality was observed in at least three of the analyzed cells and thus was a feature of the cytogenetic clone. A bolded "XX" indicates that the structural abnormality was observed in a majority (>60%) of the analyzed cells.