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ORIGINAL ARTICLE

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Generation of anti-idiotypic reagents in the EGFRvIII tumor-associated antigen system

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Abstract The use of anti-idiotype (anti-id) vaccines for immunotherapy of human cancers is attractive, as immunization with true anti-id reagents (Ab2β) has been shown to induce both cellular and humoral immunity, frequently when the original antigen does not, or when a state of anergy to the self-expressed tumor-associated antigen exists. The aim of this study was to investigate the potential of an anti-id vaccine approach to the glioma-associated antigen epidermal growth factor receptor variant III (EGFRvIII) for human clinical trials. By using conventional methodology, seven rat mAbs specific for the binding site of the murine anti-EGFRvIII-specific mAb Y10, as defined by the ability to inhibit the binding of mAb Y10 to EGFRvIII expressed on cells or as purified protein, were generated, and a subset (3/7) was found to be true Ab2β, as defined by the ability to induce the formation of antibody directed against EGFRvIII in two species (mouse and rabbit) when used as immunogen. The ability of these three Ab2β to elicit a protective anti-tumor response when used as a vaccine in the syngeneic, subcutaneous C57Bl/6-B16mseEGFRvIII tumor model was investigated. Following vaccination with one Ab2β mAb (2C7), 6/20 mice failed to develop tumor upon challenge, and 3/20 mice with outgrowing tumors exhibited dramatic regression of incipient tumors. Vaccination with a second mAb (5G8) resulted in one tumor-free survivor and one tumor regressor; vaccination with the third Ab2β mAb (7D3) did not confer protection, but did significantly increase the latency period until tumor outgrowth in all vaccinated recipients. The ability of Ab2β mAb 2C7 to induce an anti-EGFRvIII response in non-human primates was investigated by using the saponin adjuvant approved for human clinical trial, QS-21. Three of three macaques produced anti-EGFRvIII titers, as detected on EGFRvIII-expressing cells by both ELISA and fluorescence-activated cytometric analysis, following six immunizations with Ab2β mAb 2C7 and QS-21. The results obtained confirm that an anti-id response in the EGFRvIII antigen system can be induced in rodents, rabbits, and non-human primates, and it may prove a useful adjunct to immunotherapeutic approaches to EGFRvIII-positive gliomas, breast carcinomas, and non-small-cell lung tumors.

Keywords Immunotherapy · Gliomas · Anti-idiotypic response · EGFRvIII

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Introduction

Anti-id vaccine approaches to the therapy of human cancer have been under study for the past several years [3, 14, 21]. According to Jerne's network hypothesis [17], a mAb specific for antigen (Ab1) can be used as an immunogen itself to elicit the production of mAbs that bind to Ab1 (Ab2). Ab2 mAbs that inhibit the binding of Ab1 to antigen are designated as Ab2β, and Ab2β mAbs have an antigen binding site that conformationally mimics the antigen epitope recognized by Ab1. Upon administration as an antigen, Ab2βs can induce a tertiary response, both humoral (Ab3) and T-cell mediated (T3), to Ab2β. The subset of Ab3 mAbs directed to the binding site of Ab2β, which therefore recognize the original antigenic epitope, are designated Ab1′. Of most importance for vaccine approaches is the observation that Ab2β immunization induces both cellular and humoral immunity, frequently when the original antigen does not [5], or when a state of tolerance to an oncofetal antigen, such as forms of carcinoembryonic antigen (CEA) [10] or cancer antigen 125 (CA125) [27] exists. In several rodent experimental tumor systems, suppression or prevention of tumor growth by administration of

Ab2β has been demonstrated, even with target epitopes conventionally considered weakly immunogenic, such as gangliosides [7, 12, 18, 31]. Most encouraging is the induction of cell-mediated responses following anti-id immunization in situations where immunization with antigen has been ineffective [32].

As summarized by Herlyn et al [14], clinical studies with both polyclonal and monoclonal Ab2 mimicking the CO17-1A and GA733 epitopes associated with colon carcinoma have demonstrated the production of both humoral (Ab1′) and cellular (T1′) response in human colon cancer patients. Similarly, as summarized by Bhattacharya-Chatterjee et al [3], Foon′s group [10] reported that 13/23 colorectal cancer patients exhibited true Ab1′ to CEA following administration of the murine anti-id 3H1; sera from 11 of these patients mediated antibody-dependent cellular cytotoxicity (ADCC). Ten patients had idiotypic T cell responses, and five had T-cell responses to CEA, but none had objective clinical responses to the 3H1 vaccine. The overall median survival for the 23 treated patients was not significantly different from that in a similar Phase II trial of irinotecan, although toxicity in the latter trial was higher [10]. The tumor-associated antigen CA125, expressed by most malignant ovarian tumors, has been the subject of anti-id approaches because of the relative anergy of ovarian cancer patients to this highly expressed antigen. Schultes et al [30] reported the induction of the anti-id cascade in patients treated with the anti-CA125 (ACA125) Ab1 mAb B43.13; following intravenous injection of B43.13, 18/75 ovarian cancer patients developed ACA125 antibody activity that was capable of mediating ADCC. Reinartz et al [27] reported the production of Ab3 to the ACA125 Ab2 ACA125, and a strong increase of intracellular IFN-γ and IL-2 characteristic of a T helper 1 (Th1) cell-type immune response in seven patients receiving ACA125. The early appearance of IFN-γ and IL-2, followed by delayed IL-4 expression, characteristic of Th2 response, suggested an initial cellular response followed by delayed humoral reactivity.

The widely expressed ganglioside, GD3, which is present on the cell surface of most small cell lung carcinomas, has been the target of anti-id vaccine approaches using the murine Ab2 IgG_{2b} mAb BEC2, which structurally mimics GD3 [12]. Although only 5/15 patients demonstrated anti-GD3 activity, this group included those with the longest relapse-free survival.

We have extensively characterized the expression of a tumor-associated variant of the epidermal growth factor receptor (EGFRvIII) by human glioma cells using a library of polyclonal and monoclonal reagents [37, 38, 39]. Specific mAbs (Ab1) to EGFRvIII have been shown to localize to EGFRvIII-expressing tumors [28], and recently, the anti-tumor activity of unarmed Ab1 murine mAb Y10 following passive administration to mice challenged with an aggressive EGFRvIII-expressing syngeneic tumor was demonstrated [29]. An anti-id approach is, therefore, directly applicable for vaccination to EGFRvIII. We present here studies describing the isolation of Ab2β mimicking EGFRvIII, their capacity to induce humoral anti-EGFRvIII activity in a variety of species, and cellular reactivity and tumor protective effects in mice.

Materials and methods

Antigen targets, cell lines, and antibodies

Pep-3, a 13-amino-acid peptide corresponding to the predicted amino acid sequence of the EGFRvIII fusion junction, plus a carboxy terminal cysteine (LEEKKGNYVVTDH-C) and Pep-14 (RLSWTANEGVFDNF), an irrelevant peptide, were synthesized and purified by AnaSpec Inc. (San Jose, Calif.).

Cell lines used in this study included the myeloma fusion partner P3X63/Ag8.653 [35], target cell lines NR6 (untransfected NIH 3T3 NS1), NR6M (EGFRvIII transfected NR6), and NR6W (EGFRwt transfected NR6 [1, 38]). In addition, the B16F10 (C57Bl/6 melanoma) cell line and its subline, transfected to express the murine homologue of EGFRvIII, B16mseEGFRvIII, were used for in vitro assays; the murine xenograft derived from the B16mseEGFRvIII cell line was used for in vivo therapy protocols [29]. Cell culture and maintenance have been previously described [35]. The Ab1 mAbs used for immunization and inhibition analyses were fully characterized, as previously published [37]. Anti-EGFRvIII mAb L8A4 is a murine IgG_1 with a K_A of 3.8×10^7 and 2×10^9 for Pep-3 and EGFRvIII-positive cells, respectively. The mAb Y10 is a murine IgG_{2a} with a K_A of 8.5×10^6 and 2×10^8 for Pep-3 and EGFRvIII-positive cells, respectively. The polyvalent rabbit anti-EGFRvIII serum, rabbit anti-Pep-3, has been thoroughly described [36, 37]; this reagent is purified on a Pep-3 affinity column, then subsequently absorbed with A431 microsomal membrane preparations to yield a reagent reactive only with the 145 kDa EGFRvIII protein and unreactive with the 170 kDa EGFR wild-type protein, as determined by Western blot analysis [37]. Additional murine anti-EGFRvIII mAbs included P14 and X32, and irrelevant isotype control mAbs (IgG_1, P3X63Ag8.4 and P588, IgG_{2a}, M22.1, and RPC5.4 [American Type Culture Collection, Rockville, Md.; Wikstrand, unpublished data]). $F(ab')_2$ fragments of L8, Y10, P588, and M22.1 were prepared as previously described [8]. Rat immunoglobulins were purified by passage over a Protein G column, as specified by the manufacturer (Pierce Chemical Co., Rockford, Ill.), and quantified by specific anti-rat immunoglobulin capture ELISA using subclass-specific reagents (Caltag Laboratories, Burlingame, Calif.).

Rat immunization

Immunization protocols were designed as described by Herlyn et al [13]. Briefly, LOU/C female rats (Harlan), 12 weeks of age, were immunized with 200 µg of murine anti-EGFRvIII mAb (Y10 or L8) and Complete Freund's Adjuvant (CFA; Sigma, St. Louis, Mo.) intraperitoneally (i.p.) on day 0 (500 µl total volume), followed by the same dose plus Incomplete Freund's Adjuvant (IFA; Sigma) i.p. on day 22, subcutaneously (s.c.) on day 36, and intradermally (i.d.) on day 50; test bleeds were taken on days 33 and 60. For the production of idiotypic mAbs, an additional boost of 100 µg Ab1 in phosphate-buffered saline (PBS) was administered i.p. on day 130; a prefusion boost of 200 µg Ab1 in PBS was administered intravenously on day 159, followed by spleen harvest and fusion on day 162.

Fusion

Fusion was by our standard protocol [35], with the following modifications. Nucleated spleen cells were fused at a 5:1 spleen cell/murine myeloma cell ratio; following fusion, the hybrid cell population was incubated for 4 h in 10% FCS-ZO medium. Cells were

collected, pelleted, resuspended in standard HAT selection medium, and plated at a density of 4×10^4 myeloma cell equivalents per well in 96-well plates.

Analysis of anti-murine Ab1 activity

Screening for anti-Ab1 (Ab2) reactivity was performed in two stages. The first stage identified positive clones reactive with target Ab1 F(ab')$_2$ fragments by ELISA assay; positive supernatants were then tested for lack of reactivity for irrelevant isotype control F(ab′)$_2$ fragments; this assay was also used to determine Ab3 activity by substituting the correct species secondary reagent. Supernatants reactive with immunogen F(ab′)$_2$ fragments and unreactive with irrelevant control F(ab′)$_2$ fragments were then tested in ELISA assay for their capacity to inhibit the binding of immunogen or other anti-EGFRvIII mAbs to Pep-3, the immunizing synthetic peptide derived from the EGFRvIII sequence. The F(ab′)$_2$ target ELISA used was modified from those described by Perosa et al [23] and Cheung et al [7]. Briefly, F(ab′)$_2$ fragments were plated in 96-well flat-bottomed polyvinyl chloride microtiter plates at a concentration of 300 ng/well/50 µl in 0.1 M bicarbonate buffer (pH 9.6) and left to adsorb for 1 h at 37 °C or overnight at 4 °C; plates were washed with 0.05% Tween-PBS and left to block in 0.5% BSA-PBS for 1 h at room temperature. Supernatant samples and appropriate positive (hyperimmune serum from the spleen donor) and negative (culture medium; normal rat immunoglobulin) controls were incubated with the plates for 1 h at 37 °C. Following five washes with 0.05% Tween-PBS, biotinylated goat anti-rat IgG (mouse serum adsorbed, Caltag Laboratories) was added, followed by incubation for 1 h at 37 °C. Standard development with HRP-streptavidin and *o*-phenylenediamine was then performed [37]. The inhibition ELISA was performed by incubating test supernatants from Ab1 F(ab′)$_2$-reactive rat hybridomas (400 µl) with 5 µl of target anti-EGFRvIII mAb (Y10, L8, P14, X32) at a concentration representing the 50% endpoint of binding to Pep-3 corrected for the incubation volume; the 50% endpoint concentration was determined for each target murine mAb in ELISA assay vs Pep-3 [36]. Following a 1-h incubation of anti-EGFRvIII mAb and potential inhibitors, medium, or irrelevant rat immunoglobulin, the mixture was applied to Pep-3 antigen-coated ELISA plates, and the residual binding of murine anti-Pep-3 determined by standard ELISA, as described above, by using a biotinylated goat anti-mouse IgG Fc (rat serum absorbed; Sigma) secondary reagent. Percent inhibition of input binding was determined vs input controls incubated with either medium or irrelevant rat IgG. All hybridomas exhibiting $>20\%$ inhibition of Ab1 binding to Pep-3 were retained for cloning [35].

For cell line target assays to determine Ab1′ activity, as described specifically below, target cells were plated at a concentration of 2×10^4 cells per well in 96-well flat-bottomed cell culture microtiter plates, and incubated at 37 °C until confluent, at which time they were rinsed in PBS, fixed briefly (6 min) in 10% formaldehyde-PBS, then washed twice in 0.5% Tween-PBS before blocking in 1% BSA-PBS for 30 min at room temperature. All subsequent analyses were performed as described for anti-Pep-3 assays; secondary reagents were used as appropriate for the species from which the primary reagent was derived, as described above for anti-murine or rat immunoglobulin, or biotinylated goat anti-rabbit IgG (Zymed, North San Francisco, Calif.), or goat anti-monkey IgG or IgM (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) according to the manufacturer's instructions.

Immunization of mice, rabbits, and macaques for analysis of Ab3 and Ab1′ activity

Determination of Ab3 and Ab1′ activity in mice following Ab2 immunization was performed with serum obtained during the course of tumor protection vaccination protocols described below. The protocol for induction of Ab3 and Ab1′ in rabbits immunized with Ab2 followed the published procedures of Herlyn et al [13] and Bhattacharya-Chatterjee et al [2]. Briefly, New Zealand White rabbits (Duke University Farm; 2 per immunogen) were immunized with 500 µg of Ab2 in CFA on day 0, followed by the same dose plus IFA s.c. on day 14, with intramuscular boosts on days 45 and 59. Bleeds were obtained on day 0 (pre-immune), day 22 (post 2× immunization), and day 67 (post 4× immunization). Macaques (*Macaca fasicularis*, Osage Research Primates, L.L.C, Osage Beach, Mo.) were determined to be free of simian T-cell leukemia virus 1, simian immunodeficiency virus (SIV), and simian retroviruses (SRV-1, SRV-2, and SRV-3) by the vendor, and to be negative for tuberculosis and herpesvirus simiae (Herpes B) by the Duke Vivarium. Serum samples from these macaques were determined to be unreactive with potential test immunogens (rat immunoglobulin and EGFRvIII) by testing of pre-vaccination serum samples in our laboratory. The saponin adjuvant approved for human clinical trial, QS-21 (a generous gift of Aquila Biopharmaceuticals, Inc., Framingham, Mass.), was used for all intramuscular immunizations of macaques to determine efficacy and feasibility in primate recipients. Macaques were immunized with 500 µg of Ab2 2C7 and 50 µg QS-21 on days 0 and 14, and 250 µg 2C7 and QS-21 on days 28, 42, 79, and 93; sample bleeds were obtained pre-vaccination, and 10 days after the 2×, 4×, and 6× bleeds.

Tumor protection vaccination protocol

Two immunization protocols were used for determination of splenic cell proliferative activity. The first protocol, based on that by Jinnohara et al [18], was used to examine induced cell reactivity in mice immunized with prospective Ab2β *without* subsequent tumor cell challenge. For the Ab2β tested (2C7, 2F4, 4D12, 4F9, and 4F12), groups of 3 C57Bl/6 mice were immunized intraperitoneally with Ab2β [5 µg of keyhole limpet hemocyanin (KLH)] in CFA on day 1, and with the same dose in IFA on days 8, 15, and 22; spleens were harvested for assay on day 29, as described below. The protocol used for in vivo challenge experiments was based on successful vaccination regimens described by Pervin et al [24] and Raychaudhuri et al [26], and utilized the syngeneic C57Bl/6-B16mseEGFRvIII xenograft system established in this laboratory [19]. Ab2 at a final concentration of 0.5 mg/ml was conjugated to KLH by mixing in the presence of 0.05% glutaraldehyde, as described by Bona et al [4]. The vaccine was administered in 50-µg amounts per 100 µl PBS + CFA i.p. on day 0, and with IFA s.c. in the scapular area on days 7, 14, 21, 28, 35, and 42, for a total of seven immunizations. Test bleeds were obtained after the second, fourth, and sixth immunizations. On day 49, mice were challenged with 1.5×10^4 B16mseEGFRvIII cells s.c. in the flank, and they were monitored daily for tumor growth, general health, and absence of distress. Tumors were measured every third day with calipers, the first measurable tumors appearing on day 20. Calculation of tumor volume utilized the formula:

$$(a^2 \times b)/2$$

where a = the longer measured tumor diameter, and b the shorter. Once tumors exceeded 17,000 mm^3, animals were killed. Animals surviving tumor challenge were retained for subsequent determination of cellular reactivity to Ab2, Pep-3, and irrelevant control antigens. Results were analyzed in several formats: (a) percent survival and/or percent with regressing tumors, (b) latency (time to appearance of measurable tumor per animal per treatment group), and (c) comparison of Kaplan-Meier plots. All plots and statistical comparisons (unpaired *t*-test with Welsh correction for unequal variance, log rank analysis, and doubling-time estimations) were performed by using GraphPad Prism version 3.0 software (GraphPad Software, Inc., San Diego, Calif.).

Lymphocyte proliferation assay

According to the protocol for non-tumor-challenged mice described above, or six weeks following the euthanasia of the last recipient succumbing to tumor cell challenge, survivors (tumor

resistant) or vaccinated mice without tumors were euthanized, and responder leukocytes were isolated from the spleens by disaggregation, erythrocyte lysis, and Ficoll-Hypaque density gradient separation [9]. Next, 5×10^5 cells/well/200 μl were cultured in 5% FCS-ZO medium containing 4 mM L-glutamine (Life Technologies, Grand Island, N.Y.) and 5×10^{-5} M 2-mercaptoethanol (Sigma) with 0.125–8 μg/ml of protein or peptide in 96-well round-bottom plates (Corning, Corning, N.Y.). After 5 days of incubation at 37 °C in a humidified CO_2 incubator, cells were pulsed with [^{3}H]thymidine (1 μCi/well) for 18–24 h. Lymphocytes were then harvested, and the [^{3}H]thymidine incorporated into the cells was determined by counting in an LKB 1214 Rack Beta liquid scintillation counter (Pharmacia LKB, Uppsala, Sweden); all determinations were performed in triplicate. Data evaluation was performed by calculation of a stimulation index (SI) [24], calculated as the mean cpm from experimental cell-stimulator combinations divided by the mean cpm from T-cells in medium alone without stimulator. An SI >2.0 was considered positive reactivity, but further evaluation for significance was provided by paired *t*-test (comparison of splenic cells + specific stimulator vs splenic cells + irrelevant stimulator; GraphPad Prism).

Results

Generation of Ab2

LOU/C female rats immunized according to the protocols outlined in Materials and methods were selected for splenic fusion when the 50% serum endpoint titer vs immunogen (murine mAb L8A4 [IgG_1] or Y10 [IgG_{2a}]) was in excess of 1/5000. Specific reactivity to L8A4 was detectable following the second immunization, with 50% endpoint titers between 1/3000 and 1/6000 in 4/6 immunized rats; as repeated immunization did not increase the titers, fusion was performed following the seventh immunization. Spleen cell yield and fusion efficiency were low for this group of donors; in a representative fusion, approximately 300 hybridomas were generated for testing, of which 35 (12%) were positive for L8A4 $F(ab')_2$ and unreactive with irrelevant murine IgG_1 $F(ab')_2$ P588. Of these 35, undiluted supernatants of only 8 (23%) demonstrated weak and variable inhibition of mAb L8A4 binding to Pep-3 by 20–70%. Purification of analytic amounts (from ≤ 1000 ml culture supernatant) on Protein G columns (Pierce Chemical Co.) yielded known rat Ig concentration samples, which were then tested for reactivity vs L8A4 $F(ab')_2$ and irrelevant $F(ab')_2$ fragments; although titratable specificity for L8A4 $F(ab')_2$ was determined, inhibition of mAb L8A4 binding to EGFRvIII-positive cells did not consistently exceed 30%, and the Ab2 were considered unlikely to be true Ab2β mAbs (data not shown).

Conversely, titers obtained following immunization of four LOU/C female rats with murine mAb Y10 rose rapidly, with 50% endpoint titers in excess of 1/8000 in 3/4 recipients. The fusion of two rat spleens harvested following anti-murine mAb Y10 IgG_{2a} immunization yielded 427/1442 (30%) hybridoma supernatants reactive with Y10 $F(ab')_2$, 100 (23%) of which were subsequently found to be unreactive with irrelevant murine IgG_{2a} $F(ab')_2$ M22.1. Undiluted supernatants of 7/100 inhibited mAb Y10 binding to Pep-3 by $>20\%$; 6/7 consistently inhibited Y10 binding by $>80\%$.

Characterization of Ab2

The seven inhibitory rat anti-Y10 mAbs (Ab2) identified above were isotyped; mAbs 2F4, 4D12, 4F9, 4F12, and 5G8 are of the rat IgG_1 subclass, and 2C7 and 7D3 are of the IgG_{2a} subclass. Following purification of analytic amounts, all potential rat Ab2 mAbs were titrated vs $F(ab')_2$ fragments of the antigenic target Y10 $F(ab')_2$, as well as $F(ab')_2$ fragments of anti-EGFRvIII murine IgG_1 mAb L8A4, anti-GP 240 IgG_{2a} mAb Mel-14, and irrelevant murine IgG_{2a} mAbs M22.1 and RPC5.4, and irrelevant murine IgG_1 mAb P588. As shown in Fig. 1, all seven anti-Y10 $F(ab')_2$ rat Ab2 mAbs reacted with immunogen Y10 $F(ab')_2$, with 50% endpoint titers of $\leq$ 0.5–0.07 μg/ml; no reactivity to anti-EGFRvIII mAb L8A4 (IgG_1) $F(ab')_2$ or irrelevant IgG_{2a} RPC5.4 $F(ab')_2$ was observed. In addition, the Ab2 panel was unreactive with Mel-14, M22.1, and P588 $F(ab')_2$ (data not shown). Irrelevant rat IgG_{2a} was unreactive with all targets (Fig. 1). The purified rat Ab2 was again analyzed in inhibition assays, as described above, verifying inhibition of Y10 binding to Pep-3 at greater than 70% for 6/7 mAbs at 10–12.5 times molar excess of mAb Y10 (Fig. 2); one Ab2 mAb, 2C7, inhibited Y10 binding to Pep-3 by 65% at 10 times molar excess. Specificity of the inhibition assay was determined both by the inability of irrelevant rat IgG_1 and IgG_{2a} to inhibit mAb Y10 binding to Pep-3, and by the lack of inhibition of binding of anti-EGFRvIII mAb L8A4 (murine IgG_1) by the anti-Y10 rat Ab2 (Fig. 2). In addition, none of the rat Ab2 mAbs significantly inhibited the binding of anti-EGFRvIII IgG_1 murine mAbs H10 or P14 to Pep-3 (data not shown).

Inhibition of mAb Y10 binding to EGFRvIII, as expressed on the cell surface of purposefully transfected NR6M cells ($\sim 8\times10^5$ EGFRvIII receptors/cell [38]), was then measured in a fixed-cell ELISA, as described in Materials and methods (Fig. 3). Six of the seven Ab2 rat mAbs inhibited Y10 binding to EGFRvIII-expressing cells by 70–100% at molar ratios vs mAb Y10 of 10–100:1. Rat Ab2 mAb 4D12 (IgG_1) did not compete effectively with mAb Y10 for EGFRvIII (20–45% inhibition), which suggests that this Ab2 was probably not a true Ab2β, but was reactive with an epitope that permitted incomplete steric hindrance of mAb Y10 binding. As was demonstrated for inhibition of binding to Pep-3, the anti-Y10 rat Ab2 had no significant inhibitory effect upon the binding of anti-EGFRvIII mAb L8A4 to NR6M cells (Fig. 3).

Determination of Ab3 and Ab1′ activity

To distinguish between steric and true anti-id inhibition, candidate Ab2 rat mAbs were used to induce anti-Ab2

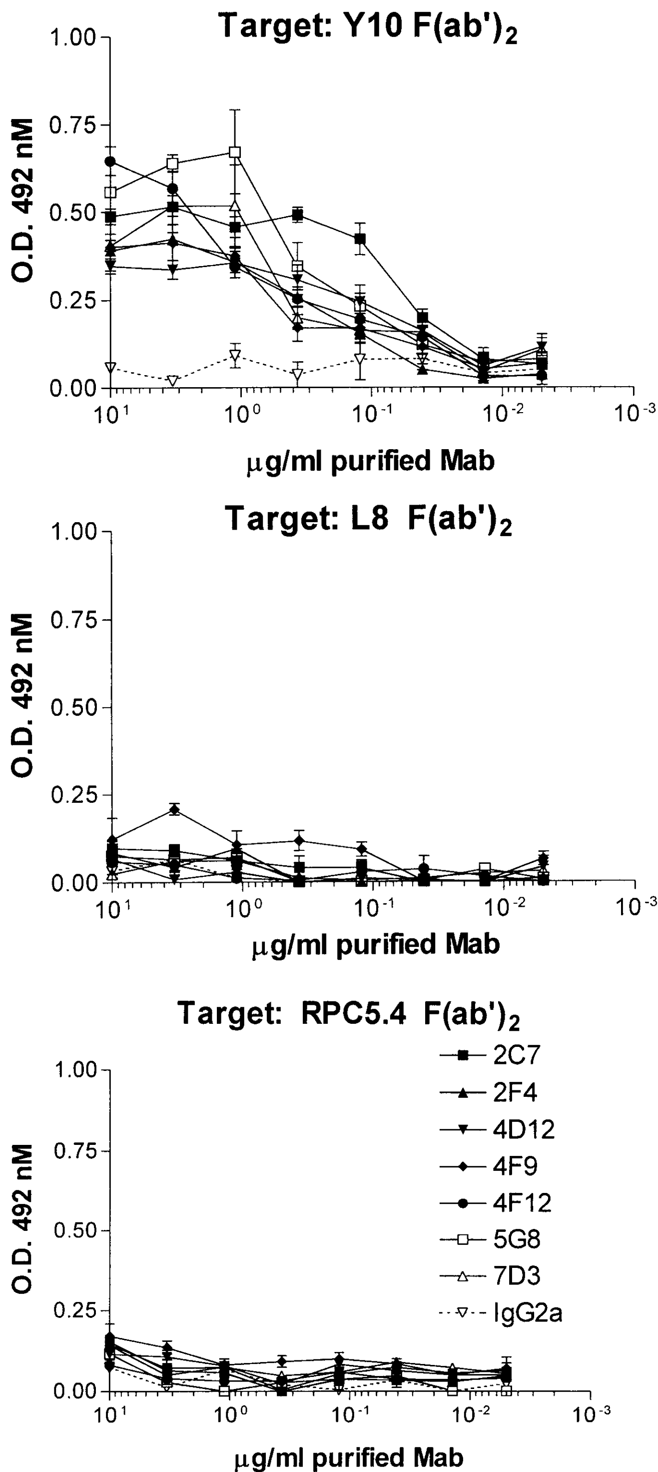


Fig. 1 Direct titration of rat Ab2 directed against Ab1 Y10 vs $F(ab')_2$ preparations of Y10, anti-EGFRvIII Ab1 L8 (L8A4), and irrelevant murine IgG_{2a} mAb RPC5.4 by ELISA

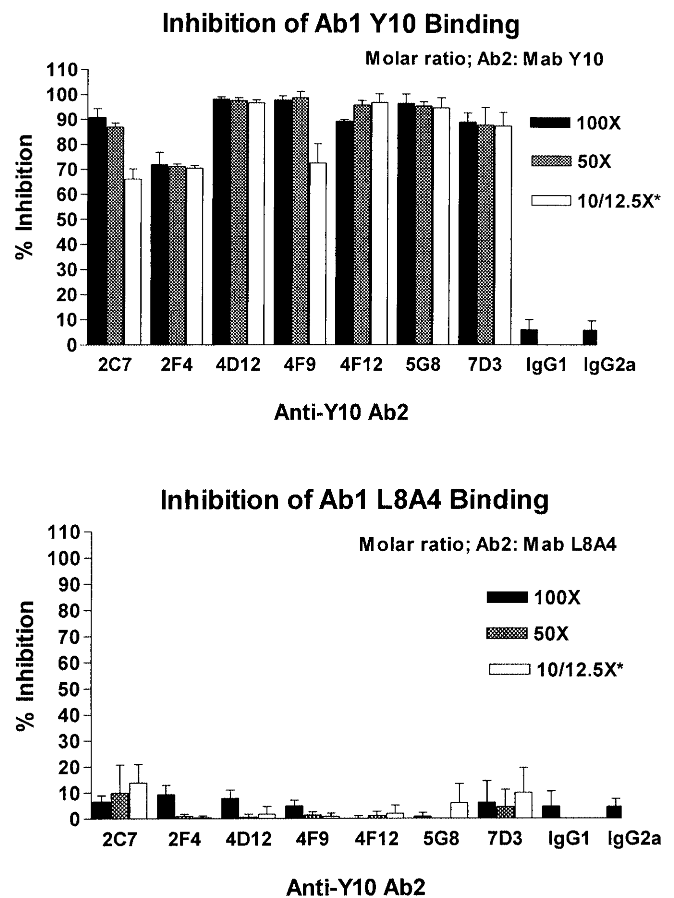


Fig. 2 Inhibition of Ab1 Y10 and anti-EGFRvIII mAb L8 binding to Pep-3 by pre-incubation of Ab1 mAb Y10 with rat Ab2 mAbs in 10 or 12.5, 50, and 100× molar excess; detection of residual murine mAb Y10 binding by rat Ig-adsorbed goat anti-mouse Ig secondary reagent

(Ab3 and, potentially, Ab1′ antibodies) in mice, rabbits, and macaques. Groups of 3 (preliminary screen of Ab2 mAbs 2F4, 4D12, 4F9, 4F12, and irrelevant control rat IgG_1 or IgG_{2a}) or 10 C57Bl/6 mice (Ab2 mAbs 2C7, 5G8, and 7D3) were immunized as described in the tumor protection protocol. Sample bleeds were obtained after the second, fourth, and sixth immunizations. Following the sixth immunization, greater than 1/10,000 50% endpoint titers for Ab2 2C7 were detectable (vs 1/500 for rat IgG_{2a}), which established the induction of Ab3 in immunized mice following Ab2 2C7 immunization. Results obtained with serum samples analyzed from mice immunized with the other Ab2 mAbs were variable in terms of percentage (30–70% response; 50% endpoint titers of 1/500–1/8000); mice immunized with irrelevant rat IgG_1, IgG_{2a}, or PBS had no Ab3 response to any of the Ab2β targets as opposed to irrelevant Ig, response being identified as twofold greater activity for Ab2β targets as opposed to irrelevant isotype at the highest negative control value.

These antiserum samples were then titrated vs NR6M cells (EGFRvIII-positive) and untransfected, non-EGFRvIII-expressing parental NR6 cells; representative data are presented in Fig. 4. A single example of antibody binding activity from each immunogen group with 50% Ab3 titers in excess of 1/1000 is presented; anti-EGFRvIII activity of the two reactive 2F4-, one reactive 4F9-, and two reactive 4F12-induced Ab3 for EGFRvIII-positive cell targets was negligible. Interestingly,

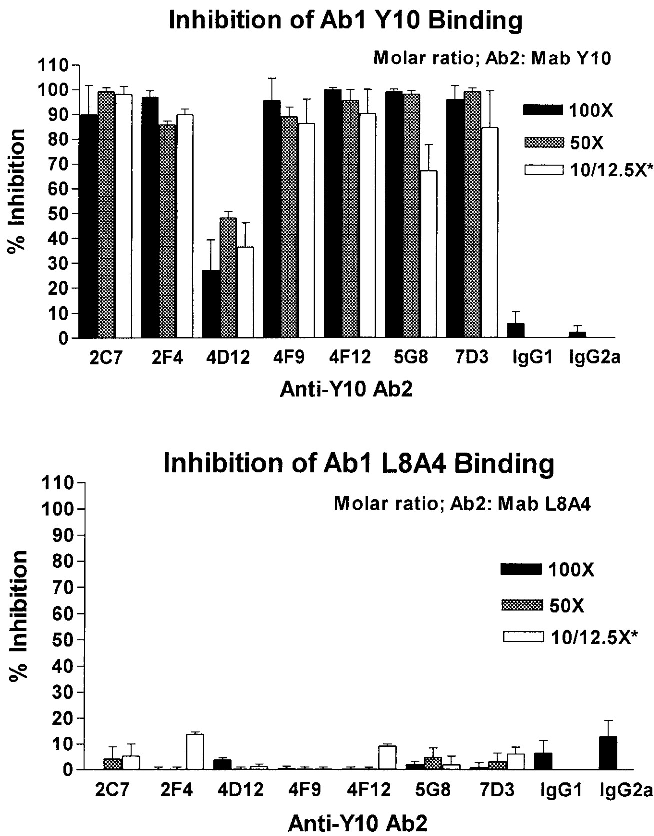


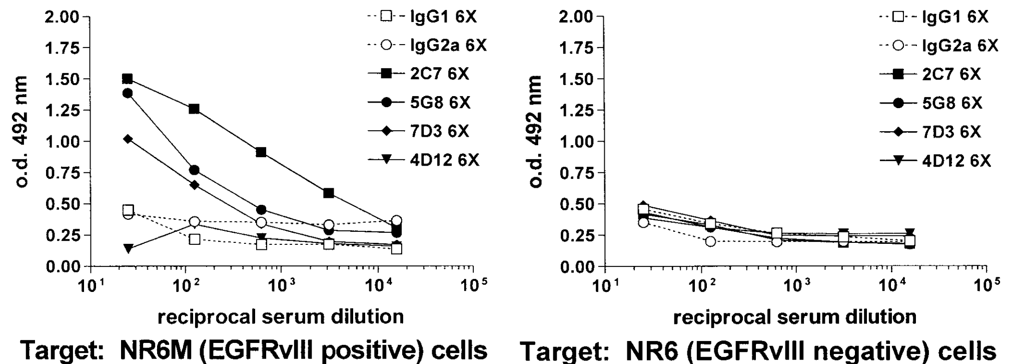
Fig. 3 Inhibition of Ab1 Y10 and anti-EGFRvIII mAb L8 binding to EGFRvIII-expressing NR6M cells by pre-incubation of Ab1 mAb Y10 with rat Ab2 mAbs in 10 or 12.5, 50, and 100× molar excess; detection of residual murine Mab Y10 binding by rat Ig-adsorbed goat anti-mouse Ig secondary reagent

none of the Ab3 serum samples could distinguish between the Pep-3 EGFRvIII-distinctive peptide (linear epitope) and irrelevant Pep-14 (data not shown). Thus, it appears that of the seven rat Ab2 investigated for the ability to induce Ab1′ in mice, only three are potentially true Ab2β: 2C7 and 7D3 (both rat IgG_{2a}) and 5G8 (rat IgG_1).

The ability of these selected Ab2 to induce Ab3 and Ab1′ in additional species was examined. As shown in Fig. 5, rabbits immunized with Ab2 7D3 exhibited specific 50% endpoint titers in excess of 1/10,000 following only the second immunization. Patterns obtained following immunization with both 2C7 and 5G8 were similar (data not shown). As with the murine response, rabbits immunized with these potential Ab2β failed to react with Pep-3 as opposed to irrelevant Pep-14 (data not shown), which supports the hypothesis that a true anti-anti-id response is primarily directed against conformational, not linear, epitopes. As shown in Fig. 6, however, rabbits do respond to the rat Ab2 immunization with a detectable Ab1′ response: 50% endpoint titers ≥1/10,000 were obtained in all rabbits vs cell line NR6M, with significantly lower levels of activity detected vs the non-EGFRvIII-expressing cell line NR6. The capacity of these rabbit Ab1′ antibodies to bind to the cell surface of EGFRvIII-expressing NR6M cells or human glioma U87MG.ÄEGFR, but not with non-expressing NR6 cells, was also demonstrated by indirect immunofluorescence. After only two immunizations with rat Ab2 2C7, rabbit Ab1′ (dilution 1/100) readily detected EGFRvIII as expressed on purposefully transfected murine and human cells (data not shown).

We further investigated the specificity of the rabbit Ab1′ antiserum to inhibit the binding of the Ab1 immunogen, murine mAb Y10, to NR6M cells. Serial dilutions of either pre-immune rabbit antiserum, rabbit anti-Pep-3 antiserum, or post second immunization rabbit anti-Ab2 antiserum were incubated with a fixed concentration (10 µg/ml) of Y10 prior to incubation with NR6M or NR6 cells and subsequent development with biotinylated, rabbit serum-absorbed goat anti-mouse IgG. Results of a typical assay with rabbit antiserum #762 vs Ab2 7D3 are shown in Fig. 7. Results with Ab1′ antibodies elicited following Ab2 2C7 or 5G8 immunization were similar, 50% inhibition of Y10 binding being achieved with 1/50 and 1/20 dilutions of Ab2 2C7 and 5G8, respectively. As no inhibition of L8A4 binding by Ab3 to NR6M cells was observed (data not shown), the relative binding of Ab3 to Ab2β vs irrelevant isotype control rat IgG_{2a} was more than 300-fold higher for Ab2β (Fig. 5), and rabbit Ab1′ was demonstrated in all immunization protocols with 50% endpoint titers > 1/10,000, the results strongly suggest the presence of Ab3 antibodies that share idiotypes with

Fig. 4 Titration of serum samples obtained from C57Bl/6 mice following 6× immunization with various irrelevant isotype control or Ab2 mAbs plus CFA, vs EGFRvIII-expressing (NR6M) and non-expressing (NR6) cells; detection of Ab1′



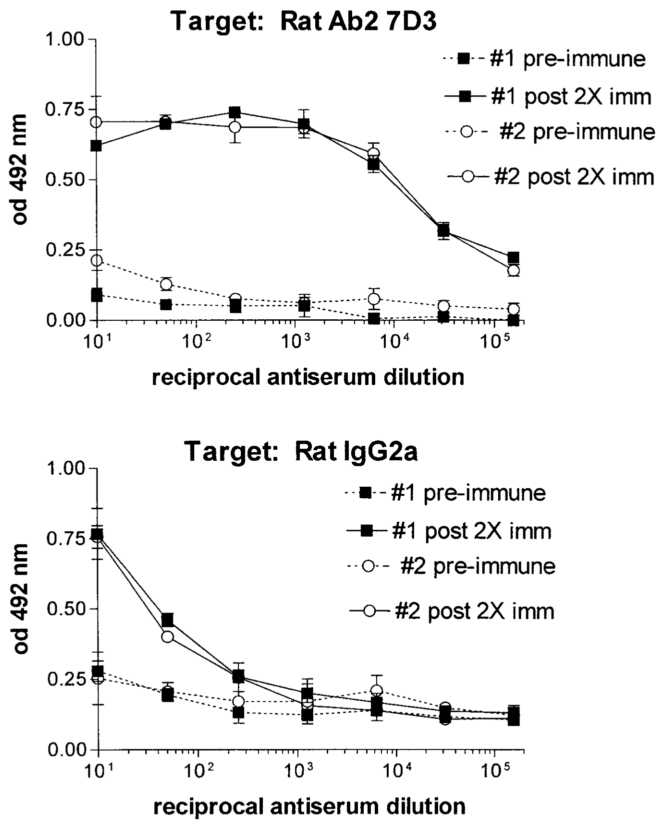


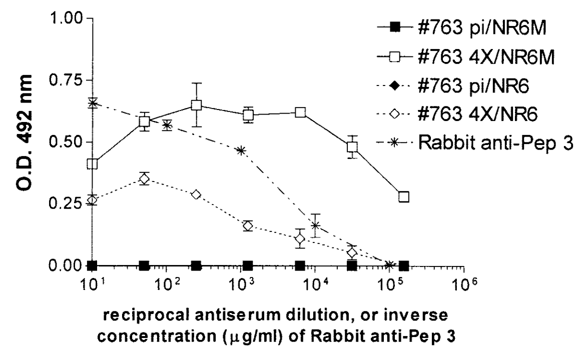
Fig. 5 Titration of serum samples obtained from rabbits before (pre-immune) and following 2× immunization with Ab2 mAb 7D3 plus CFA vs Ab2 mAb 7D3 and isotype control rat IgG_{2a}; detection of Ab3

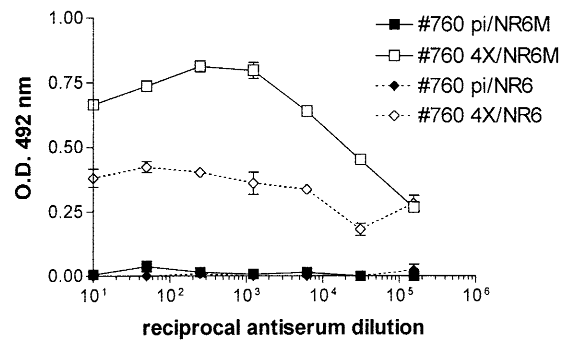
Ab1, although a contribution of steric hindrance by non-anti-Ab2β antibodies (Fig. 5) cannot be totally excluded.

In vivo tumor protection experiments

Two separate tumor protection experiments were performed. In Series 1, 7 groups of 10 mice were evaluated: groups immunized with rat Ab2 2C7, 2F4, 4D12, 4F9, or 4F12; one group receiving PBS; and one group not immunized. Because Ab2 2F4, 4D12, 4F9, and 4F12 did not elicit Ab1′, these immunoglobulins served as irrelevant IgG_1 controls.

Results are presented in Fig. 8 as the mean volume of tumors ± SD in each group over time. In *panel A*, results of IgG_1, IgG_{2a}, and 4F12 immunized recipients were superimposable over the PBS and sham-immunized groups; for clarity, these latter groups are not shown. Interestingly, the single Ab2 that failed to inhibit Ab1 Y10 binding to B16mseEGFRvIII cells (Ab2 4D12; Fig. 3) exerted no protective effect and was even marginally promotive of more rapid growth, although this difference was not significant (≤ .05) at day 38 from the sham-immunized controls. In *panel B*, tumor outgrowth in Ab2 2C7-immunized mice is plotted; 5/10 immunized





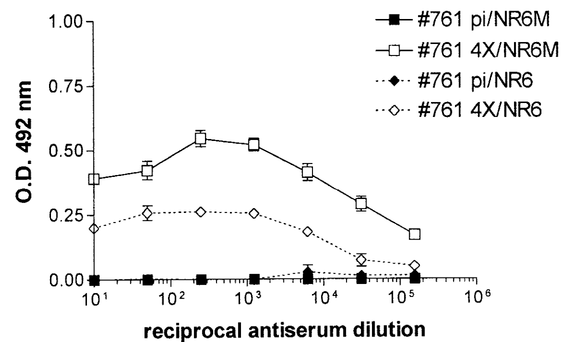


Fig. 6 Titration of serum samples obtained from rabbits before (pre-immune) and following 2× immunization with Ab2 mAb 2C7 plus CFA vs EGFRvIII-expressing (NR6M) and nonexpressing (NR6) cells; detection of Ab1′

mice remained tumor-free through day 100; the remaining 5/10 developed tumors with the same latency and outgrowth patterns as irrelevant Ig or PBS-sham-immunized control animals.

In Series 2, 4 groups of 10 mice were evaluated; rat Ab2 2C7, 4D12 (irrelevant rat Ig), 5G8 or 7D3, or PBS sham immunizations were performed; the mean and standard deviation of the latencies for 4D12 and PBS sham-immunized recipients were 19.3 ± 3.5 and 20.3 ± 2.4, respectively, while Ab2-immunized recipients exhibited greater mean and median latencies, with a larger range (2C7, 26.7 ± 6.4; 5G8, 27.9 ± 6.1; and 7D3, 26.1 ± 9.1). The differences in mean latency between the

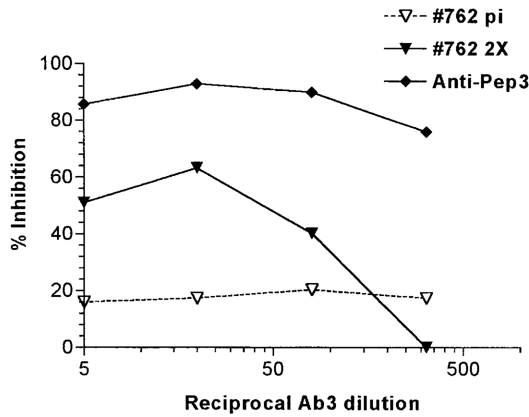


Fig. 7 Competition for Ab1 mAb Y10 binding to NR6M cells by Rabbit 762 anti-Ab2 7D3 pre-immune and post 2× immunization serum; comparison with EGFRvIII reactive rabbit anti-Pep-3 serum. Competing rabbit antibodies at the serum dilutions given were combined with a fixed concentration of Ab1 mAb Y10, and residual Y10 binding detected with rabbit immunoglobulin absorbed goat anti-mouse Ig. Results are expressed as percent inhibition of Ab1 mAb Y10 binding

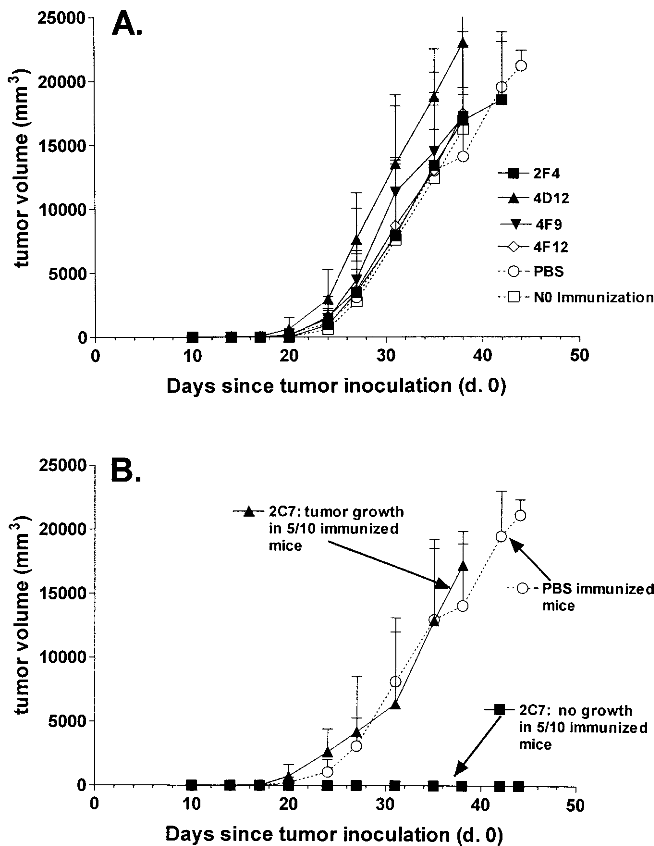


Fig. 8 Growth curves of B16mseEGFRvIII subcutaneous tumors in syngeneic C57Bl/6 mice vaccinated with various Ab2 mAbs; Series 1

2C7-, 5G8-, and 7D3-immunized groups were insignificant, as were those between irrelevant Ig (4D12) and PBS sham-immunized control groups; the differences between 2C7- and 5G8-immunized recipients and the irrelevant Ig control-immunized group were significant ($P<0.009$, $P<0.005$, respectively, by unpaired t-test, two-tailed, with Welch's correction).

Results are presented for each recipient as the mean volume of tumor over time in Fig. 9; these data illustrate the wider range in latency in the Ab2β-immunized groups, but the similar growth rate pattern once tumor outgrowth exceeded 5000 mm^3. In contrast to Series 1, in which 5/10 Ab2β 2C7-immunized mice remained tumor-free, in Series 2, 1/10 Ab2β 2C7-immunized mice was tumor-free, with an additional 2/10 recipients exhibiting total and rapid tumor regression following initial outgrowth. A similar, but more modest effect was seen in Ab2β 5G8-immunized mice; 1/10 recipients remained tumor-free, and 1/10 exhibited tumor regression. Immunization with Ab2β 7D3 did induce a longer and more variable latency period, but ultimately, all 10 animals succumbed to tumor challenge.

To determine if detectable immune responses to EGFRvIII had been induced by Ab2β immunization, tumor-challenged, tumor-free mice in both Series 1 and 2 were observed through 150 days following tumor challenge, then spleens harvested for determination of splenic cell proliferative response.

The splenic cell proliferative response to antigens associated with Ab2β immunization was assessed in nonimmunized, Ab2-vaccinated, and non-tumor-challenged mice, and in the 5/10 long-term survivors from tumor protection Series 1. As demonstrated in Fig. 10 for splenocytes obtained from Ab2β 2C7-immunized, tumor cell challenge survivor #1, but representative of all survivor cell donors tested, positive stimulation (SI $\geq$ 2.0) by specific Ab2β vs control IgG_{2a} was seen at all concentrations; all were significantly positive by unpaired t-test, with the exception of the stimulatory dose of 4 µg/ml, which was just below the level of significance. In no instance was a significant difference seen between the SI obtained vs Pep-3 and Pep-14. A more extensive presentation of splenic cell stimulation is provided in Table 1. Data obtained with B16mseEGFRvIII and B16 parent cells as stimulators are provided here; it must be noted, however, that in the case of tumor cell-challenged survivors, the exposure to the initial tumor cell inoculum would be expected to induce a response to non-EGFRvIII, but B16 melanoma-associated, antigens. In sum, the data presented in Fig. 10 and Table 1 suggest the following.

(1) The short-term (4×) immunization protocol without tumor cell challenge consistently induced a detectable anti-rat immunoglobulin response in the majority of mice immunized; however, the differences between specific Ab2β and rat isotype control immunoglobulin were not significant. Following this protocol, no detectable sensitization to the linear epitope represented by Pep-3, nor to the irrelevant peptide, Pep-14, was seen.

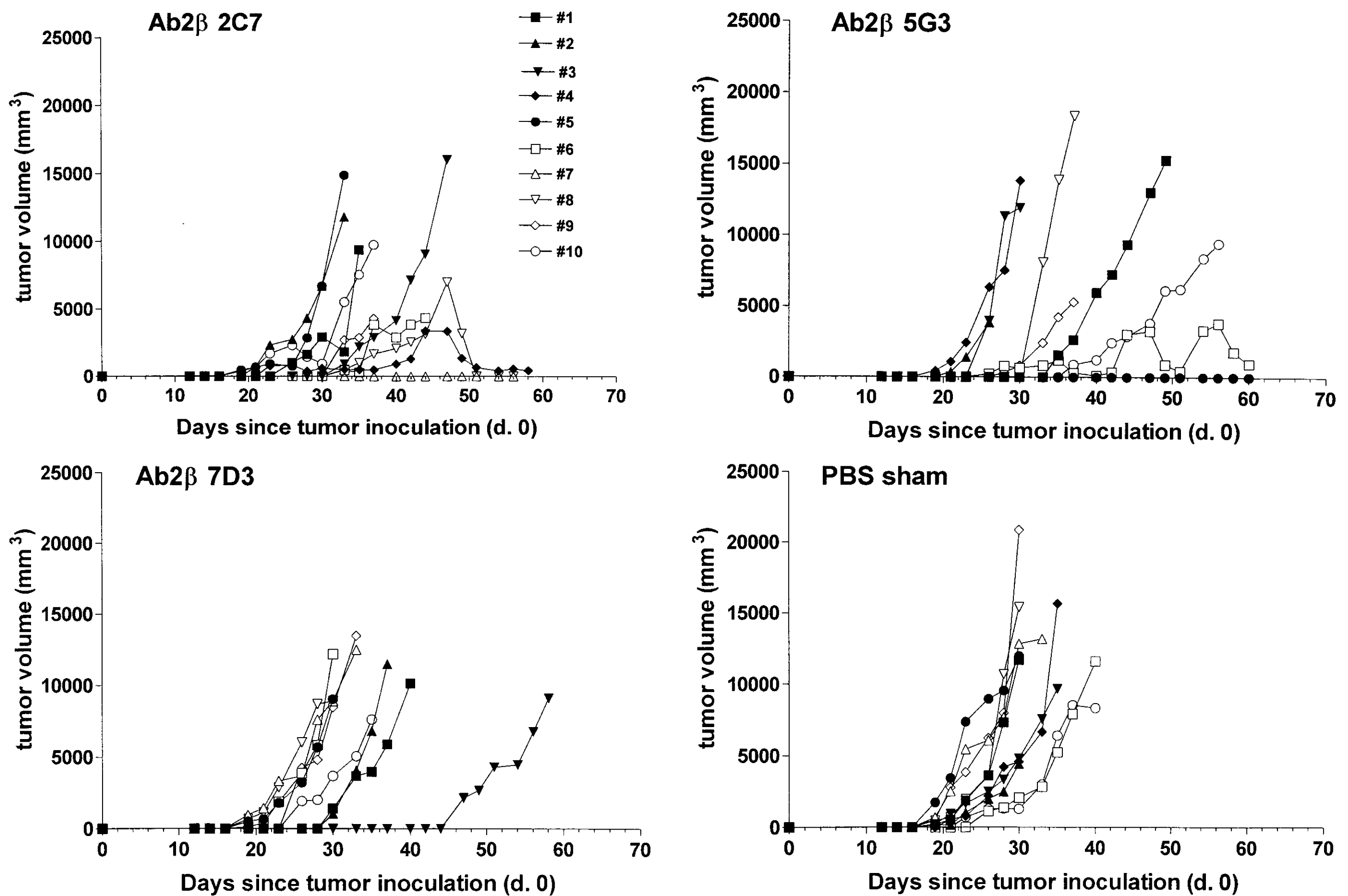


Fig. 9 Individual growth curves of B16mseEGFRvIII subcutaneous tumors in syngeneic C57Bl/6 mice vaccinated with various Ab2 mAbs; Series 2. Mice were killed when tumors exceeded 17,000 mm^3, or observed through day 150

(2) Immunization with Ab2β 2C7, however, induced significant, specific splenic cell recognition of B16mseEGFRvIII cells as opposed to the B16 parent melanoma cell line; as these animals were never exposed to tumor cell challenge, this is strong evidence for the EGFRvIII mimotope capacity of Ab2β 2C7. Data obtained with spleen cells from mice immunized with Ab2β 2F4 and 4F9 were similar to those for 4F12 (data not shown). In the case of Ab2β 2C7-immunized, tumor cell-challenged, surviving mice, 5/5 exhibited positive stimulation for Ab2β 2C7 as opposed to rat IgG_{2a}. No significant response to Pep-3 was detected. Four of five survivors exhibited positive splenic cell stimulation by B16mseEGFRvIII cells; however, in only 2 cases was this activity significant as compared to stimulation by B16 parent cells, indicating the probable primary sensitization of these mice to B16 tumor-associated epitopes unrelated to EGFRvIII. As the short-term Ab2β 2C7-immunized mice did exhibit specific B16mseEGFRvIII activity, it is probable that an EGFRvIII-specific response was induced.

Investigation of Ab3 and Ab1′ immunogenicity in nonhuman primates

To assess the potential efficacy of Ab2β 2C7-immunization for humans, we employed the saponin adjuvant QS-21, approved for human clinical trials, for the immunization of nonhuman primates (*Macaca fascicularis*). As described in Materials and methods, macaques received 6 immunizations with Ab2β 2C7-QS-21; serum samples were obtained prior to immunization and following immunizations 2, 4, and 6. Fifty percent endpoint titers were determined for all antiserum-target combinations; in no case did this endpoint vs irrelevant target (rat IgG_{2a} or cell line NR6) exceed 1/100 (Fig. 11). Following the sixth immunization, all three macaques exhibited a 50% endpoint titer $\geq$1/250 against NR6M cells ($>$5-fold greater than the respective titers vs NR6 cells). Ab3 titers were present in 2/3 macaques (50% endpoints of 1/1000) following the sixth immunization; the third macaque (#3) exhibited equivalent reactivity to both mAb 2C7 and rat IgG_{2a} (Fig. 11).

Discussion

We initially selected two specific anti-EGFRvIII Ab1 with different in vitro and in vivo characteristics for investigation of anti-id approaches to glioma immunotherapy. The first mAb, L8A4, a murine IgG_1, was

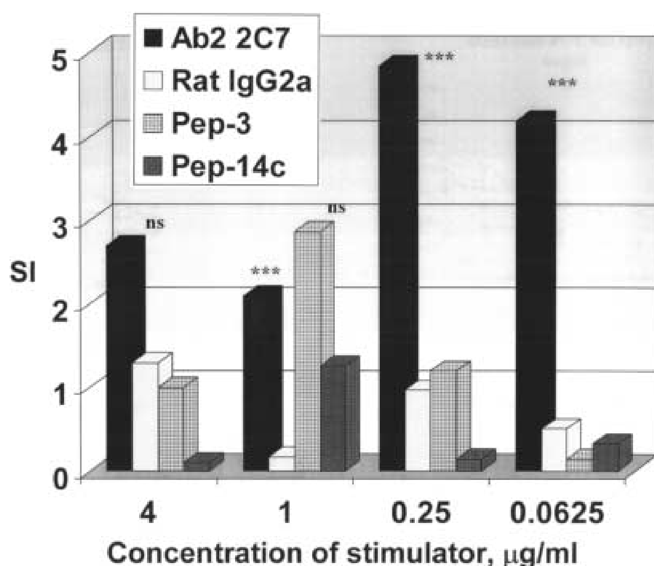


Fig. 10 T cell proliferation of splenocytes from Series 1 tumor challenge survivor 2C7-1 vs Ab2β 2C7, rat IgG_{2a}, Pep-3, and Pep-14. Results are presented as the SI (mean cpm experimental T cell-stimulator/mean cpm T cell-medium). A stimulation index >2.0 is considered positive reactivity; positive indices are presented in *bold*. *P* values are indicated by the code (*ns* >0.05, * 0.01–0.05, ** 0.001–0.01, *** <0.001) next to the positive stimulator value, compared with the irrelevant control stimulator. The range of ^3H uptake by splenocytes from nonimmunized mice with all stimulators ranged between 0.1 and 1.7

characterized by high affinity for cell-expressed EGFRvIII under a variety of conditions (live cells, fixed cells, denatured lysate preparations), and under conditions of continuous, passive administration, by the capacity to protect mice from EGFRvIII-expressing tumor cell challenge [29]. Ab1 mAb Y10, a murine IgG_{2a}, while exhibiting lower affinity and less robust binding to fixed antigen, exhibited not only the in vivo protective effects demonstrated by L8A4, but also induced long-lasting protection against tumor cell outgrowth. In addition, Y10 was capable of mediating ADCC in vitro [29]. Only the Ab2 elicited vs Ab1 Y10 consistently inhibited the binding of the immunogen Ab 1 Y10 to antigen (Figs. 2 and 3). As the humoral mimotopic activity of Ab2β is presumably conformational in nature [16], the difference between these two Ab1 in generating functional Ab2β is most likely a reflection of a less immunogenic topography of mAb L8A4.

The seven rat anti-Ab1 mAb Y10 Ab2 mAbs were all capable of inhibiting the binding of Y10 to Pep-3, the linear peptide representation of the EGFRvIII fusion junction [36, 37], and 6/7 significantly inhibited Y10 binding to EGFRvIII-expressing cells. We have previously demonstrated that Y10 has an approximately 25-fold lower affinity (K_A) for Pep-3 than for cell-expressed EGFRvIII (8.5×10^6 as opposed to 2.1×10^8 [37]). The relative inability of Ab2 4D12 to inhibit the binding of Y10 to cell-expressed EGFRvIII is attributable either to low affinity of Ab2 4D12 for the Y10 antigen binding site, or to weak steric hindrance of Y10 binding to conformationally correct antigen.

By classical definition of Ab3 response, each of the seven Ab2 mAbs induced the formation of Ab3 specific for immunogen Ab2 as opposed to irrelevant rat IgG in at least one murine recipient of those immunized. Titers (50% endpoint titers in excess of 1/10,000) induced following 6× immunization with Ab2 2C7 were higher than previously reported titers for induced Ab3 to murine Ab2 [2, 24]. Although the Ab2 mAbs used here were of

Table 1 Summary of T cell proliferation assays: reactivity of splenic T cells from immunized, non-tumor-challenged mice, and immunized, tumor-challenged survivors

Stimulation index[b]:							
Immunogen	Mouse #	Ab2β	IgG control	Pep-3	Pep-14	B16mseEGFRvIII	B16 parent
Experiment 1: Splenocytes from Ab2β immunized mice; no tumor challenge[a]							
2C7	#1	7.0^{ns}	7.7	1.0	1.5	**6.9*****	0.1
	#2	**5.3**^{ns}	**5.7**	**1.2**	1.4	**10.1*****	0.9
4D12	#1	**11.0**^{ns}	**4.1**	1.6	1.7	**5.7**^{ns}	4.8
	#2	**6.1**^{ns}	**4.5**	1.3	1.7	1.2	1.1
4F12	#1	1.8	1.9	0.8	0.9	0.2	0.3
	#2	**4.3**^{ns}	**2.1**	0.3	0.4	0.2	0.4
Experiment 2: Splenocytes from Ab2β 2C7 immunized mice that survived B16mseEGFRvIII tumor cell challenge[c]							
2C7	#1	**2.7**^{ns}	1.3	1.0	0.1	**10.0**^{ns}	**9.3**
	#2	**5.2****	0.4	**2.2**^{ns}	0.2	0.5	0.1
	#3	**2.3**^{ns}	1.1	0.4	0.8	**4.4**^{ns}	**3.1**
	#4	**6.1***	**3.1**	0.1	**2.3**	**4.1***	1.3
	#5	**4.0**^{ns}	**2.9**	0.2	1.0	**3.2****	0.3

[a]Immunization protocol based on that of Jinnohara et al [18], described in Materials and methods
[b]Combinations were tested in triplicate; data from stimulator concentrations of 0.25 μg/ml (proteins) or 1.6×10^6 cells per well (tumor cells) are presented as a stimulation index (mean cpm uptake from triplicate test combinations/mean cpm uptake from triplicate effectors in medium alone [24]. A stimulation index >2.0 is considered positive reactivity; positive indices are presented in *bold*. *P* values, calculated as in Fig. 10, are indicated by the code (*ns* >0.05, * 0.01–0.05, ** 0.001–0.01, *** <0.001) next to the positive stimulator value, compared with the irrelevant control stimulator. The range of ^3H uptake by splenocytes from non-immunized mice with all stimulators ranged between 0.1 and 1.7
[c]Tumor protection vaccination protocol based on that of Pervin et al [24] described in Materials and methods

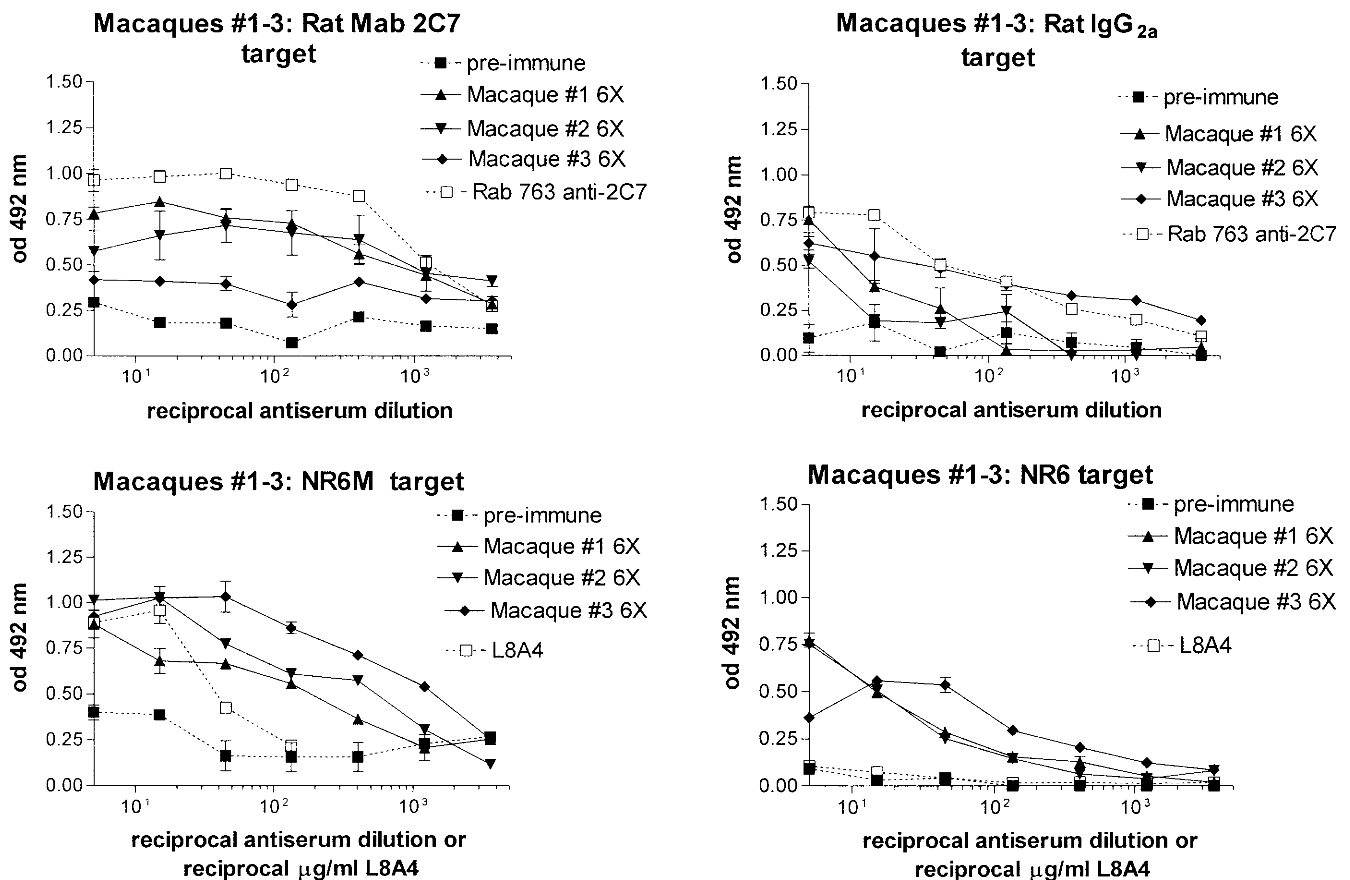


Fig. 11 Ab3 and Ab1′ responses of macaques vaccinated with Ab2β 2C7 plus QS-21 adjuvant; 50% endpoint titers were determined as that dilution yielding 50% o.d. values corrected for background binding. Minimum of a 3-fold difference between titers to specific vs irrelevant target was considered positive

rat origin, it is evident from the 300-fold lower 50% endpoint titer of the Ab3 for irrelevant rat IgG_{2a} that the predominant response is to the Ab2 2C7 variable region, and not to rat allotypic epitopes. Of the seven Ab2 mAbs used for successful Ab3 immunization, only three (Ab2 2C7, 5G8, and 7D3) were capable of eliciting detectable reactivity to EGFRvIII, as expressed on the transfected murine fibroblast cell line NR6M; negligible activity for the antigen-negative parental line NR6 was observed (Fig. 4). Ab1′ titers obtained were similar to those reported by previous investigators [24]. These results (3/7 Ab2 mAbs being identified as true Ab2β) are consistent with the previous report by Cheung et al [7] that 3/6 Ab2 mAbs induced anti-GD2 Ab1′ activity, and they are superior to the 1/5–1/7 experience in other primary antigen systems (CEA [15] and HIV gp 120 [33]).

The Ab2β mAbs identified by induction of Ab1′ in murine recipients were administered to rabbits, and similar results were obtained in terms of induction of specific, high-titer Ab3 (Fig. 5) and Ab1′ (Fig. 6). Interestingly, as with the murine Ab3 response, rabbit Ab3 serum did not bind to Pep-3, the linear amino acid 14-mer representing the EGFRvIII unique fusion junction sequence, but did recognize EGFRvIII on cell membranes, as determined by (a) ELISA vs EGFRvIII-positive and -negative cells (Fig. 6), (b) FACs analysis of purposefully transfected EGFRvIII-positive murine fibroblast and human glioma cells, and (c) competitive inhibition of Y10 binding to NR6M cells (Fig. 7). The results in both species support the proposal that the induction of Ab1′ activity may be primarily via conformational epitope parameters, rather than primary amino acid sequence, largely, but not solely based on the capacity of Ab2 vs anti-carbohydrate Ab1 to induce anti-carbohydrate reactive Ab1′ [7, 12, 16, 18, 34]. As the anti-EGFRvIII activity was restricted to cell-expressed EGFRvIII, and not to linear peptide, the assumption would be that secondary and tertiary conformational structures are important in the anti-id cascade described here. It should be noted, however, that primary structure homology has been described for at least two Ab2-antigen systems: Ab2 87.92.6/mammalian reovirus type 3 hemagglutinin-neutralizing antigen [6] and Ab2 BR3E4/CO17-1A [21], indicating that for some protein epitopes, antigen mimicry by antibodies may be achieved by sharing primary structure [6].

Production of Ab1′ reactivity is not, however, necessarily associated with biologically relevant tumor effect [21, 25, 26]. As demonstrated in Figs. 8 and 9, the ability of representative Ab2 and Ab2β to induce a tumor-

protective effect was variable. As expected, vaccination with those Ab2 mAbs without demonstrable Ab1′ induction capacity (2F4, 4D12, 4F9, 4F12) did not alter the pattern of outgrowth of EGFRvIII-expressing tumors, as established in null or sham-vaccinated mice (Fig. 9), whereas prechallenge vaccination with Ab2β 2C7, 5G8, or 7D3 resulted in protection from tumor outgrowth (Figs. 8 and 9), delayed tumor outgrowth, and/or regression of initially growing tumors (Fig. 9). However, the protective effect was not complete: 9/20 Ab2β 2C7-vaccinated mice were tumor-free and 2/10 Ab2β 5G8-vaccinated mice were regressors, whereas all Ab2β 7D3-vaccinated mice eventually succumbed to tumor, albeit with delayed latency (Fig. 9). The experience of Raychaudhuri et al [26] was similar to that reported here; of six Ab2β demonstrated serologically as internal image Ab2 to the mouse mammary tumor virus tumor-associated antigen gp52, only one induced protective immunity and was effective in immunotherapy. Herlyn's group has reported that two Ab2 mAbs generated vs Ab1 recognizing the CO17–1A GA 733 antigen, despite excellent immunogenicity in terms of Ab3 and Ab1′ humoral activity and delayed hypersensitivity, could not elicit proliferative T cell responses or tumor-protective effects [20, 21]. We also saw a lack of correlation between the presence of induced humoral reactivity and tumor protection; sera from survivors showed low levels of activity for EGFRvIII, as measured on NR6M vs NR6 cells (data not shown). As these animals were challenged with C57Bl/6 EGFRvIII-positive murine tumor cells, it is arguable that the failure to detect Ab1′ in the animals succumbing to tumor was due to an antigen sink effect of even a transiently growing xenograft.

As presumed mechanisms of idiotypic cascade-induced tumor protection are presumably cell mediated, however [3, 12, 21], the measurement of cell proliferative responses is most informative. As shown in Fig. 10 and Table 1, the responses of Series 1 tumor challenge succumbing or surviving mice to Ab2β and irrelevant control IgG are indicative of a strong induction of Ab3 and antigen-specific cellular activity, as previously established. In addition, the tumor-challenge protective Ab2β 2C7 induced a cellular response to EGFRvIII, whether or not the mice so vaccinated were challenged with B16mseEGFRvIII.

The feasibility of vaccinating human patients with the Ab2β 2C7 without the concomitant administration of a powerful adjuvant such as CFA was investigated by immunizing *Macaca fascicularis* with Ab2β 2C7 and the FDA-approved adjuvant QS-21. As shown in Fig. 11, positive Ab3 responses were detected in 2/3 macaques, and Ab1′ response was detected in 3/3, at 50% endpoint titers of approximately 1/300, verifying the previously reported ability of QS-21, a mild adjuvant, to support immunization of nonhuman primates [31] and humans [3, 11, 22] with the elicitation of Ab3 and Ab1′ activity. Although objective clinical responses in the latter studies have been minimal, long-term evaluation is required to determine if an effect on disease progression and survival will become apparent.

As stated by Bhattacharya-Chatterjee et al [3] in a comprehensive review, "the greatest challenge in immunotherapy by means of anti-Id antibodies is to identify the right network antigen for a tumor associated antigen." As numerous investigators cited above and these data have shown, the demonstration of tumor antigen-associated (Ab1′) immunogenicity is not necessarily sufficient for induction of a tumor protective response. Also, the immunomodulatory activity of Ab2 demonstrated in animal model systems is not necessarily predictive of the ultimate activity in patients [13]. This dichotomy is best illustrated with the Ab2β VF2 derived from rats immunized with anti-colorectal carcinoma Ab1 CO17-1A; whereas polyclonal goat anti-CO17-1A induced Ab3 and Ab1′ in rabbits and patients, Ab2β VF2 induced relevant biologic responses only in mice and rabbits, and was totally ineffective when administered with alum to nine patients with colorectal carcinoma. This group has made similar observations with the Ab2β BR3E4, also directed vs CO17-1A, in a series of studies comparing the single-epitope anti-id vaccine with multiple-epitope tumor antigen (GA733) vaccine, both administered with alum as adjuvant. In sum, the full-length antigen expressed by recombinant adenovirus was a more potent modulator of humoral and cellular immune responses than Ab2 in mice [21], which suggests that in the case of highly immunogenic tumor antigens, such as EGFRvIII, for which antigenic forms exist (peptide, protein extracellular domain), selection of the optimal vaccine component (antigen or Ab2β) may need to be made following examination in nonhuman primates.

In summary, numerous experimental model and human clinical trial studies have established the potential of anti-id approaches for immunotherapy of human neoplasia, even for antigens of compromised immunogenicity or lack of tumor specificity. The study reported here has established the ability to elicit antigen-specific activity with an anti-id network approach to the EGFRvIII glioma-associated antigen. As passive, unarmed Ab1 transfer has been demonstrated to protect against EGFRvIII-positive tumor cell outgrowth (100% protection for subcutaneous implants, 60% for intracerebral implants) in mice [29], determination of the optimal format for anti-EGFRvIII-directed therapy in human patients – vaccination with antigen, Ab2β, or administration of Ab1 capable of mediating tumor cell arrest – will need to be addressed by elucidation of the immune effector mechanisms operant in each of these formats in rodents and primates.

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