Accelerated Tumor Growth Mediated by Sublytic Levels of Antibody-Induced Complement Activation Is Associated with Activation of the PI3K/AKT Survival Pathway

Xiaohong Wu¹, Govind Ragupathi¹, Katherine Panageas², Feng Hong¹, and Philip O. Livingston¹

Abstract

Purpose: We addressed the possibility that low levels of tumor cell-bound antibodies targeting gangliosides might accelerate tumor growth.

Experimental Design: To test this hypothesis, we treated mice with a range of monoclonal antibody (mAb) doses against GM2, GD2, GD3, and CD20 after challenge with tumors expressing these antigens and tested the activity of the same mAbs in vitro. We also explored the mechanisms behind the complement-mediated tumor growth acceleration that we observed and an approach to overcome it.

Results: Serologically detectable levels of IgM-mAb against GM2 are able to delay or prevent tumor growth of high GM2 expressing cell lines both in vitro and in a SCID mouse model, whereas very low levels of this mAb resulted in slight but consistent acceleration of tumor growth in both settings. Surprisingly, this is not restricted to IgM mAb targeting GM2 but consistent against an IgG mAb targeting GD3 as well. These findings were mirrored by in vitro studies with antibodies against these antigens as well as GD2 and CD20 (with Rituxan), and shown to be complement-dependent in all cases. Complement-mediated accelerated growth of cultured tumor cell lines initiated by low mAb levels was associated with activation of the phosphoinositide 3-kinase (PI3K)/AKT survival pathway and significantly elevated levels of both p-AKT and p-PRAS40. This complement-mediated PI3K activation and accelerated tumor growth in vitro and in vivo are eliminated by PI3K inhibitors NVP-BEZ235 and Wortmannin. These PI3K inhibitors also significantly increased efficacy of high doses of these four mAbs.

Conclusion: Our findings suggest that manipulation of the PI3K/AKT pathway and its signaling network can significantly increase the potency of passively administered mAbs and vaccine-induced antibodies targeting a variety of tumor cell surface antigens. Clin Cancer Res; 19(17); 4728–39. ©2013 AACR.

Introduction

A variety of monoclonal antibodies (mAb) against cancer antigens have resulted in prolongation of disease-free and overall survival in preclinical studies and in clinical responses when tumors known to be strongly positive for the relevant antigens were targeted. Several of these mAb have been U.S. Food and Drug Administration (FDA) approved for these purposes. Although not FDA approved, ganglioside-specific mAbs against gangliosides GD2 and GD3 have showed both preclinical efficacy and clinical responses (1–8) in patients with neuroblastoma and melanoma, respectively, in the setting of strongly antigen-positive tumors. On the other hand, randomized trials with a GM2-keyhole limpet hemocyanin (KLH) vaccine that consistently induces IgM and IgG antibodies against GM2 in patients with melanoma have shown either no benefit (9, 10) or an initial decrease in overall survival compared with no treatment controls (11, 12). GM2 is present in essentially all melanomas, but unlike GD3 and GM3, which are the most highly expressed melanoma gangliosides, it is expressed at only low levels in the majority of cases (13, 14) and very few melanoma cell lines can be lysed with complement and mAbs or immune sera against GM2 (15). We hypothesized therefore that: (i) induction of high titers of antibodies against GM2 results in only a low level of cell surface antibodies against GM2 because of the low level GM2 expression on most melanomas and (ii) this might result in sublytic levels of complement activation resulting in inflammation, angiogenesis, and tumor cell activation, as has been described for sublytic levels of cell surface complement activation in other settings (16–18).
Complement-Activating Antibodies Are a Two-Edged Sword

Translational Relevance

Therapy with tumor vaccines or complement-fixing monoclonal antibodies (mAb) inevitably results in sublytic levels of complement activation due to low antigen expression or periods of low antibody titers. We show here that while detectable levels of serum mAbs result in complement-mediated tumor cell lysis in vitro and tumor elimination in vivo, lower levels of antibody result in sublytic complement activation and more rapid tumor cell growth in vitro and in vivo. This sublytic complement activation was associated with activation of the phosphoinositide 3-kinase (PI3K)/AKT survival pathway. Both accelerated tumor cell growth and PI3K activation were eliminated by PI3K inhibitors NVP-BEZ235 and Wortmannin. These PI3K inhibitors also significantly increased efficacy of even high mAb doses.

Our findings suggest that manipulation of the PI3K/AKT pathway and its signaling network can significantly increase the potency of passively administered mAbs and vaccine-induced polyclonal antibodies targeting a variety of tumor cell surface antigens.

To test this hypothesis, we treated mice with a range of mAb doses against GM2, 2 other glycolipid antigens (GD2 and GD3), and a glycoprotein antigen (CD20) after challenging with tumors expressing these antigens and we explored the activity of the same mAbs in vivo. Our findings supported this original hypothesis and extend it beyond GM2 to a variety of other antigens. Here, we also explore a mechanism behind this complement-mediated tumor growth acceleration and an approach to overcome it.

Materials and Methods

Monoclonal antibodies and reagents

mAb PGNX (anti-GM2, murine IgM) was obtained from Progenics Pharmaceuticals Inc. mAb 3F8 (anti-GD2, murine IgG3) was provided by Dr. Nai-Kong V. Cheung (ref. 19; Department of Pediatrics, Memorial Sloan-Kettering Cancer Center (MSKCC), New York). mAb R24 (anti-GD3, murine IgG3) was provided by Dr. Paul Chapman (ref. 20; MSKCC). Rituxan (anti-CD20, chimeric IgG) was obtained from Genentech, Inc. mAbs against phosphorylated AKT (p-AKT), AKT, phosphorylated PRAS40 (p-PRAS40), and PRAS40 were obtained from Cell Signaling Technology. NVP-BEZ235 and Wortmannin were from Chemdesa. mAb anti-C9 was purchased from Abcam. Human sera (as a complement source) were purchased from Quidel.

Cell culture

CHLA136Luc, (luciferase-transduced CHLA136 human neuroblastoma cell line) was kindly provided by Dr. Robert C. Seeger (refs. 21, 22; Division of Hematology-Oncology, Children’s Hospital, Los Angeles, CA) and was maintained in Iscove’s modified Dulbecco’s medium supplemented with 15% FBS and ITS premix (BD Bioscience) at 37°C, in a 5% CO2 humidified chamber.

Hs445 lymphoma cell lines were kindly provided by Dr. Wim Bleeker, (Genmab B.V.). These last 3 cell lines were maintained in RPMI-1640 media supplemented with 10% FBS at 37°C in a 5% CO2 humidified chamber.

In vivo experiments

Animals. CB17 severe combined immunodeficient mice (SCID) mice (Taconic) 5 to 8 weeks old were housed 5 per cage. All protocols and procedures were approved by the MSKCC’s Institutional Animal Care and Use Committee.

Tumor challenge. Mice were placed under a heat lamp for 2 to 3 minutes and immobilized in a mouse restrainer; 0.5 x 10^6 CHLA136Luc cells in 100 µL were injected into the tail vein using a BD insulin syringe with 28G needle.

mAb and NVP-BEZ-235 administration. Mice were treated with murine mAbs 3F8 (5, 8), PGNX (25, 26), R24 (2) against GD2, GM2, GD3, and human mAb Rituxan against CD20 with or without NVP-BEZ235. Control mice, typically 2 cages of 5 mice were treated identically, received the same volume of PBS at the same intervals.

Imaging. Mice were anaesthetized using isoflurane and injected with 300 µg of d-Luciferin Firefly (Caliper Life Sciences). They were imaged 10 minutes later using the IVIS-200 in vivo imaging system (Caliper Life Sciences) over periods of time ranging up to 3 minutes using the software program “Living Image 3.0” (Caliper Life Sciences). Values are reported as photons per second.

In vitro experiments

ELISA assay. ELISA assays were conducted to determine IgM and IgG serum antibody titers against GM2, GD2, and GD3 after administration of mAbs targeting these gangliosides (8). Briefly, 0.1 µg ganglioside per well in ethanol was coated on ELISA plates overnight at room temperature. Nonspecific sites were blocked with 3% human serum albumin in PBS for 2 hours. Serially diluted sera drawn at 4 and 24 hours and then at 3- to 4-day intervals for 3 weeks after mAb administration were added to each well. After one-hour incubation, the plates were washed and alkaline phosphatase-labeled goat anti-mouse IgM or IgG added at 1:200 dilution. The antibody titer was defined as the highest dilution with absorbance of ≥0.1 over that of control mouse sera. Pretreatment sera were consistently negative (absorbance <0.1 at 1.5 dilution).

FACS. Flow cytometry with the indicated cultured cancer cell lines was conducted as previously described (26). Briefly, single-cell suspensions of 1 x 10^6 culture tumor cells per tube were washed in PBS with 3% FBS. Murine mAbs PGNX (IgM against GM2), 3F8 (IgG3, GD2), R24 (IgG3, GD3), and human mAb Rituxan (IgG1, CD20), were used to identify the respective antigens. mAb anti-C9 was used for detecting the terminal complement complex (TCC) formed.
on cell surface. After washing in 3% FBS, 20 μL of 1:25 diluted goat anti-mouse IgM or anti-human or IgG labeled with fluorescein isothiocyanate (FITC, Southern Biotechnology) was added and the mixture incubated for another 30 minutes on ice. After a final wash, the positive population and median fluorescence intensity of stained cells were differentiated using fluorescence-activated cell sort or (FACScan) (Becton & Dickinson). Cells stained only with goat anti-mouse IgM or IgG labeled with FITC were used to set the FACScan result at 1% as background for comparison with percentage of positive cells stained with primary mAbs.

**WST-1 assay.** WST-1 cell proliferation assay kit was used for detection of the extent of cellular proliferation as instructed in the company's manual. Briefly, 2 × 10⁴ cells in 100 μL of culture media as defined above were plated in a 96-well flat-bottom plate and incubated at 37°C in 5% CO₂ overnight. Antibody doses between 0.02 pg to 5 μg/mL were added to each well and incubated for one hour at 37°C, 5% CO₂, and 4 to 10 μL of human serum complement was then added to each well and incubated overnight. For assays testing the impact of phosphoinositide 3-kinase (PI3K) inhibitor, NVP-BEZ235 at 0.005, 0.5, or 5 μg/mL in dimethyl sulfoxide were added accordingly at same time when mAb was added. WST-1 agent (Roche Applied Science) was added at 1:10 ratio at the end of incubation and optical density was acquired by reading the plates at 415 nm 4 hours later. The Student t test was used for statistical analysis.

**Western blot analysis.** A total of 1 × 10⁶ cells were plated into 6-well plates and incubated overnight. Cells were then treated with NVP-BEZ235 (0.5 μg/mL), mAbs (0.001 μg/mL) and human sera complement (5%) for 4 hours. At the end of incubation, cells were collected and lysed with lysis buffer from Cell Signaling Technology, which contains protease inhibitor cocktail and phosphatase inhibitor cocktail (Calbiochem), each at 1:100 dilution (cocktails: lysis buffer). The cell lysates were then quantified using Bradford assay (Bio-Rad) according to the method. The cell lysates were then treated with NVP-BEZ235 (0.5 μg/mL) and incubated for one hour at 37°C, 5% CO₂, and 4 to 10 μL of human serum complement was then added to each well and incubated overnight. For assays testing the impact of phosphoinositide 3-kinase (PI3K) inhibitor, NVP-BEZ235 at 0.005, 0.5, or 5 μg/mL in dimethyl sulfoxide were added accordingly at same time when mAb was added. NVP-BEZ235 agent (Roche Applied Science) was added at 1:10 ratio at the end of incubation and optical density was acquired by reading the plates at 415 nm 4 hours later. The Student t test was used for statistical analysis.

**Statistical analysis**

Overall survival was defined as the time from intravenous tumor cell challenge to date of death or day 160. Survival distributions were generated using Kaplan–Meier methodology (27) and comparisons between treatment group and control (PBS) were made via the log-rank test (using GraphPad Prism 5). For the combined analysis with each antibody, low-dose versus control was compared across all experiments carried out using a generalized linear model accounting for experiment.

**Results**

**In vivo experiments targeting GM2, GD2, GD3, and CD20**

Cell surface expression of GM2, GD2, and GD3 on neuroblastoma cell lines CHLA136Luc and Lan-1 and SCLC cell line H524 and CD20 expression on lymphoma cell lines Hs445 and Daudi were confirmed by flow cytometry (Supplementary Fig S1A)

Initial experiments focused on impact of low (1 or 5 μg) and high (50 μg) doses of mAbs administered weekly for 4 weeks beginning 2 days after intravenous challenge with 0.5 × 10⁶ CHLA136 cells. Survival was significantly prolonged by the 50 μg dose of PGNX (against GM2), 3F8 (GD2), or R24 (GD3), compared with untreated mice or mice receiving low-dose PGNX (Fig. 1A) and survival was more prolonged when the 3 mAbs were administered together. Although survival of mice receiving the 5 μg dose of PGNX was not significantly changed compared with the untreated control group, tumor growth measured by luciferase expression at 6 to 8 weeks was significantly increased (Fig. 1B). In subsequent experiments, the 1 μg doses of PGNX and R24 were found to be optimal for this growth enhancement at weeks 4 to 8 (Fig. 1C and D). Significant enhancement of early growth was seen at low mAb doses in 5 of 6 experiments for PGNX and 2 of 2 experiments for R24. Significantly decreased survival was seen at the 1 μg dose in 3 of 6 experiments for PGNX and 2 of 2 experiments for R24. The 1 μg and 2 μg doses of 3F8 and doses as low as 0.001 μg of Rituxan resulted in slight delay of tumor growth; accelerated in vivo tumor growth was not seen at doses down to 0.02 μg of 3F8 and 0.001 μg of Rituxan (data not shown).

Sera was drawn beginning 4 hours after administration of a high dose (50 μg) of mAbs PGNX, 3F8, and R24 and showed antibody titers between 1/160 and 1/1,280 at 4 hours which was still detectable at 24 hours (PGNX) and 2 weeks (R24). The 1 μg dose of R24 and PGNX that resulted in early accelerated tumor growth in vivo resulted in minimal or no detectable antibody titers, even at 4 hours.

**In vitro impact of high and low doses of mAbs and complement on tumor cell growth**

We confirmed the presence of bound antibody and complement activation at the CHLA136Luc cell surface after treatments with doses of PGNX mAb as low as 0.0002 μg/mL. Low-concentration of PGNX (0.0002 μg/mL) binds weakly but detectably to CHLA136-Luc (Fig. 2A) and TCC formation in the presence of complement (human serum) was PGNX dose-dependent and detectable down to the 0.0002 μg/mL dose level (Fig. 2B), but was not formed when C7-depleted human serum was used as complement source. Impact on cell growth was determined in parallel using the WST-1 assay. High antibody concentrations caused growth inhibition and the 0.0002 dose level caused growth acceleration, but only in
the presence of HuC'. Although 30% cell growth inhibition was seen with 20 \( \mu \)g/mL PGNX and C7-depleted human serum (C7dHuC'0) treatment, this was most likely due to direct antibody-mediated tumor growth inhibition as shown with high concentration of each of the mAbs tested below.

The impact of complement and these high and low concentrations of mAbs against all 3 glycolipids on tumor cell growth in vitro was determined using WST-1 assays. Rituxan was included to target a glycoprotein. mAbs PGNX, R24, 3F8, and Rituxan each inhibited tumor growth in vitro at high mAb concentrations and accelerated tumor cell growth at low mAb concentrations exclusively in the presence of complement (Fig. 3). Figure 3 represents multiple experiments with each of the cell lines. Of 7 experiments conducted on CHLA136 target cells, PGNX, 3F8, and R24 showed low-dose acceleration of growth in every case, which was statistically significant for PGNX and 3F8 five times each out of 7 experiments (combined analysis \( P < 0.001 \) in both cases) and four times out of 7 experiments (combined analysis \( P < 0.01 \)) for R24. Of 3 experiments conducted with LAN1, low-dose acceleration of growth was statistically significant twice with 3F8 and R24 (combined analyses 0.001 and 0.01, respectively). Five experiments were carried out on H524 with PGNX, 3F8, and R24. Low-dose accelerated growth was
statistically significant in 4 of these 5 experiments with PGNX, 3F8, and R24 (combined analyses $P < 0.001$ in each case). Six experiments were carried out with Hs445 and Rituxan. Low-dose Rituxan significantly accelerated growth 4 times (combined analysis $P < 0.001$) and also in the single experiment conducted on Daudi cells. In each case, high doses resulted in significantly diminished cell counts in every experiment, which was primarily complement-dependent. In each case, the low-dose acceleration of growth was seen with each of the 4 mAbs tested and was also complement-dependent (Fig. 3). No acceleration of tumor growth was detected in the absence of complement, though at the highest mAb doses, complement-independent tumor inhibition was detected with 3F8 on CHLA136Luc, Lan-1, and H524 cells (Fig. 3A–C), PGNX on CHLA136Luc (Fig. 3A), R24 on Lan-1 cells (Fig. 3B), and Rituxan (Fig. 3D and E). Comparable results were obtained when heat-inactivated serum was added to the mAb-alone wells (Supplementary Fig. S1B). Overall, this complement-dependent in vitro growth inhibition at high mAb doses and acceleration of growth at low mAb doses was true with 5 different human cell lines and included 1 IgM and 3 IgG mAbs targeting 3 glycolipid antigens (GM2, GD2, and GD3) and 1 protein antigen (CD20).

The PI3K/AKT pathway is activated by sublytic complement activation

We tested the association of the PI3K/AKT pathway with accelerated CHLA136Luc cell growth promoted by low-dose PGNX-mediated sublytic complement activation. A PGNX level of approximately 0.001 μg/mL resulted in more than 50% increase in p-AKT expression at 4 to 6 hours, whereas the highest doses of PGNX greatly decreased p-AKT expression (Fig. 4A and B). The impact of this increased p-AKT (AKT activation) on downstream events was also tested. PRAS40 is an AKT substrate and mTORC1 inhibitory binding protein. Phosphorylation of PRAS40 by p-AKT in the PI3K/AKT pathway dissociates this binding and recovers mTORC1 activity in regulating the cell growth. Treatment of CHLA136Luc cells with 0.001 μg/mL PGNX for 4 hours resulted in more than 50% increase in PRAS40 phosphorylation (Fig. 4B).
Figure 3. Percent change of in vitro cell growth was studied with a range of doses of mAbs and/or human serum as complement source (HuC') on the following 5 human cell lines: CHLA136Luc cells (neuroblastoma; A); Lan-1-Luc cells (neuroblastoma; B); H524 (SCLC; C); Hs455 (lymphoma; D); and Daudi (lymphoma; E); approximately 20,000 cells were plated in triplicate and treated with HuC' and different amounts of mAbs, mAbs alone, HuC' alone, or neither (cells only), as indicated for 24 hours. Cellular proliferation was quantitated using the WST-1 assay. Each bar represents the mean of triplicates. Student's t test results for statistical significance of comparison of antibody or antibody plus HuC'–treated cells to cells treated with HuC' or media alone are as indicated: (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
Impact of blocking PI3K/AKT on mAb induced in vitro growth inhibition and acceleration

The impact of mAb-mediated sublytic complement activation on PI3K/AKT/mTOR pathway activation was further shown by its inhibition using the PI3K and mTOR dual inhibitor NVP-BEZ235. NVP-BEZ235 inhibited both p-AKT and p-PRAS40 expression (Fig. 4C). NVP-BEZ235 decreased not only PI3K/AKT/mTOR pathway activation but also CHLA136Luc and DaudiLuc cell growth in vitro, especially in the presence of mAbs (Fig. 5). NVP-BEZ235 at 0.5 µg/mL, combined with 3F8 and Rituxan at various doses, significantly enhanced mAb cytotoxicity of CHLA136Luc and DaudiLuc cells compared with each treatment alone. When combined with low-dose 3F8 (0.001 µg/mL) and Rituxan (0.0001 µg/mL), NVP-BEZ235 eliminated accelerated CHLA136Luc and DaudiLuc cell growth induced by these low doses of mAbs. These findings were unchanged when heat-inactivated complement was used as negative control in place of no complement (data not shown).

These results with 3F8, R24, PGNX, and Rituxan were consistent over several experiments with $P$ values compared with antibodies and human complement alone ranging between 0.015 and 0.0001. Wortmannin (another PI3K inhibitor) also abrogated CHLA136Luc-accelerated cell growth induced by low-dose 3F8, when compared with low-dose 3F8 alone and human complement alone but the impact was less striking (data not shown; $P$ values, 0.04–0.008).

Impact of PI3K inhibitor on mAb-induced accelerated tumor growth in vivo

The impact of treatment with PGNX and/or 3F8 alone or in combination with NVP-BEZ235 on the growth of CHLA136Luc was tested in a SCID xenograft model (Fig. 6). Addition of NVP-BEZ235 alone resulted in some reduction of CHLA136Luc growth and prolonged survival. The combination of NVP-BEZ235 and PGNX and/or 3F8 resulted in more marked prolongation of survival compared
with antibody alone. NVP-BEZ235 also eliminated the early tumor growth acceleration induced by low-dose PGNX.

Discussion

Concerns that the immune system is a two-edged sword that sometimes inhibits and sometimes enhances tumor growth have focused on both the B-cell and T-cell arms of the immune system for over 40 years. While the clinical value of adoptively transfused T cells in a variety of clinical settings is well established (28), recent concerns have focused on regulatory mechanisms able to diminish the antitumor T-cell response and even enhance tumor growth (29). Antibodies and the B-cell response have long been associated with this dichotomy as well (30). Since FDA approval of mAbs such as Rituxan and Herceptin, and their widespread use, there is no doubt about the clinical value of immune effector mechanisms such as complement activation and antibody-dependant cellular cytotoxicity (ADCC), which these antibodies are known to mediate (8, 31). Despite the obvious value of adoptively administered antibodies and T cells, the role of vaccine-induced antibodies and T cells targeting cancer antigens remains less clear.

While one vaccine (Sipuleucel-T) was FDA approved for use in patients with prostate cancer (8, 31), its mechanism of action remains unclear. On the other hand, several recent randomized trials with whole-cell vaccines or carbohydrate-conjugate vaccines have shown no clinical benefit or an initial shortened time-to-recurrence compared with control groups (9–12). The shortened time-to-recurrence seen in patients receiving the whole irradiated melanoma cell vaccine Canvaxin is difficult to dissect, as its mechanism of action remains unclear.
action (B-cell or T-cell mediated) and relevant target antigens is unclear and any single immune response was detectable in only a minority of patients. Two of these trials targeted GM2 ganglioside using a GM2-KLH–conjugate vaccine compared with IFN-α (10) or no treatment (11, 12). This vaccine is known to induce only an antibody response and only against GM2 and to induce this response in essentially every vaccinated patient. The significantly decreased progression-free and overall survival identified during the initial 1 to 2 years of follow-up (9, 11), although not after longer term follow-up (10, 12), in these trials is assumed to be a consequence of the vaccine-induced antibodies targeting GM2.

While GM2 is expressed on most melanomas, it is expressed in only small amounts in most cases; less than 20% of melanoma cell lines can be lysed with high doses of anti-GM2 antibodies and human complement (13). Consequently, previous clinical trials with the GM2-KLH vaccine induced sublytic levels of cell surface complement activation in most cases. We show here that in a setting where high-dose PGNX (an IgM monoclonal antibody targeting GM2) is able to delay or prevent growth of strongly GM2-positive tumor cells both in vivo and in vitro, low (sublytic) levels of the same mAb accelerates initial tumor growth in both settings. Surprisingly, these findings were not limited to IgM mAb PGNX against GM2. These in vitro and in vivo findings were also present with IgG mAb R24 against GD3. This low antibody dose acceleration of cell growth in vitro was also seen with two other IgG mAbs, 3F8 (against GD2) and Rituxan (against the protein antigen CD20). Both inhibition and acceleration of tumor cell growth in vitro with high or low doses of these 4 mAbs was shown on 5 different cell lines and was shown to be complement-dependent. With 3F8 and Rituxan, however, accelerated growth was not seen in vivo, possibly because of the over-riding role for ADCC in protection mediated by these 2 antibodies in this murine model.

The high-dose (50 mg) of these mAbs is comparable with doses of mAbs commonly used in patients on a per kilogram basis and results in antibody titers in mice at 4 and 24 hours.

Figure 6. Impact of NVP-BEZ235 alone, PGNX and/or 3F8 alone, or the combination in two separate in vivo experiments. Mice received NVP-BEZ235 25 mg/kg in experiment 1 (A and B) or 12.5 mg/kg in experiment 2 (C) by gavage beginning 4 days after intravenous challenge with 0.5 × 10⁶ CHLA136Luc cells and continuing daily for 2 weeks. PGNX and/or 3F8 at the indicated doses were injected intravenously (PGNX) or intraperitoneally (3F8) starting a day later (5 days after tumor challenge) and reinjected once a week for 4 weeks. A and C, comparison of experimental group survivals to control group by Kaplan–Meier methodology in 2 experiments. B, student t test used for statistical comparison of tumor growth in (A) measured by luciferase expression at 8 weeks in experimental groups compared with control mice. All groups contain 4 or 5 mice except the control group in experiment 1 (A and B) which contains 10 mice.
We show here that the accelerated cell growth induced by treatment with low-dose mAbs was associated with activation of the PI3K/AKT/mTOR pathway. Treatment with low-dose PGNX (0.001 μg/mL) and human complement induced increased AKT phosphorylation and also increased release of the p-AKT substrate PRA540, a raptor binding protein that inhibits mTORC1 kinase activity. These data suggest involvement of the PI3K/AKT/mTOR pathway in low-dose mAb sublytic complement activation-induced accelerated CHLA136Luc cell growth. Testing with the inhibitors of this pathway supported this finding. NVP-BEZ235 is a dual-PI3K and mTOR inhibitor, inhibiting both the catalytic subunit (P110) of PI3K and mTORC, whereas Wortmannin is a more specific PI3K inhibitor, binding only to the P110 catalytic subunit of PI3K. We showed that constitutive expression and low-dose mAb-induced increased expression of p-AKT and p-PRA540 in CHLA136-Luc cells was inhibited by NVP-BEZ235. NVP-BEZ235 and Wortmannin eliminated in vitro tumor growth acceleration induced by low doses of 3F8, R24, PGNX, and Rituxan and significantly enhanced in vitro tumor cytotoxicity with high doses of these same mAbs in a dose-dependent manner. NVP-BEZ235 also increased the efficacy of mAbs PGNX and 3F8 against CHLA136Luc cells in vivo, significantly increasing survival of challenged SCID mice compared with high-dose PGNX and 3F8 alone, and preventing the early tumor growth acceleration seen with low-dose PGNX.

In summary, complement-activating antibodies are a two-edged sword, showing potent antitumor activity at high (clinically relevant) doses and weak tumor-enhancing or -accelerating activity at very low doses. Therapy with complement-activating antibodies should be restricted to treatment of antigen-rich tumors. Sublytic complement activation, which can result from a low level of antibody or low antigen expression, results in increased activation of the PI3K/AKT survival pathway and accelerated tumor growth. This can be eliminated by treatment with PI3K inhibitors such as NVP-BEZ235 and Wortmannin, which also increase the efficacy of even high doses of these mAbs. Our findings suggest that manipulation of the PI3K/AKT pathway and its signaling network can potentially increase the potency of passively administered mAbs and vaccine-induced antibodies targeting a variety of tumor cell surface antigens.

**Disclosure of Potential Conflicts of Interest**

G. Ragupathi has ownership interest (including patents) and is consultant/advisory board member in MabVax Therapeutics Inc. P.O. Livingston has ownership interest (including patents) and is the chief scientific officer in MabVax Therapeutics Inc. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: X. Wu, G. Ragupathi, F. Hong, P.O. Livingston

Development of methodology: X. Wu, G. Ragupathi

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Wu, G. Ragupathi, K. Panagras, F. Hong, P.O. Livingston

Writing, review, and/or revision of the manuscript: X. Wu, G. Ragupathi, K. Panagras, F. Hong, P.O. Livingston

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Wu, F. Hong, P.O. Livingston

Study supervision: G. Ragupathi

in the 1/160 to 1/1,280 range. The low dose (0.01–1 μg) resulted in little or no detectable serum antibody even at 4 hours. Long-lasting antibody titers in the 1/320 to 1/1,280 range against these same antigens are induced in most patients by KLH conjugate vaccines (32). This suggests that if used in the setting of high antigen–expressing tumors, the monovalent vaccines should be beneficial, not detrimental, and that polyvalent vaccines inducing antibody titers against several cell surface antigens should be even more beneficial.

Low doses of naturally present, affinity-purified human anti-Neu5Gc antibodies were shown to accelerate growth of Neu5Gc-containing tumors in Neu5Gc-deficient mice (33), whereas at higher doses, these same antibodies elicited tumor cytotoxicity (34). The high-titer anti-Neu5Gc antibodies present in human sera were shown to deposit complement on Neu5Gc-expressing human cells in vitro. Complement activation or ADCC were suggested as potential mechanisms behind this low-dose/high-dose dichotomy (35). Adding to uncertainty over the impact of complement activation are reports that complement activation can promote an immunosuppressive environment in the tumor and can compete with (or inhibit) natural killer cell-mediated ADCC (36, 37).

No previous studies exploring sublytic complement activation have involved tumor cells and mAbs or immune sera targeting cancer antigens. Early studies exploring the high-dose/low-dose dichotomy showed consistently that the difference between tumor facilitation and inhibition could be quantitative, the same anti-trinitrophenyl or anti-human immune sera that mediated tumor facilitation at low dose in the presence of complement could inhibit tumor growth at high dose (17, 30). The results of the studies presented here with mAbs targeting a variety of naturally expressed tumor antigens are consistent with these results. High doses of antibodies against each of the glycolipid antigens and one protein antigen that we tested all decreased tumor cell growth in vitro in the presence of human complement, whereas low doses of each antibody increased tumor growth. Clearly, these in vitro findings are independent of the tumor environment or impact on ADCC, which complicate analyses in vivo.

Sublytic complement activation at the cell surface has long been known to activate a variety of metabolic processes resulting in adherence, aggregation, mitogenesis, and proliferation of a variety of nonmalignant cell types. Enhanced HIV infection (38), glomerular mesangial cell proliferation associated with glomerulonephritis (39), and protection from subsequent lytic complement doses (17) have been shown as consequences. Several signal transduction pathways have been responsible for the cell-cycle activation, antiapoptotic, and differentiation properties associated with sublytic complement levels. These include primarily activation of the PI3K/AKT pathway (18, 40, 41). Involvement of the PI3K/AKT signaling pathway in accelerated tumor growth induced by sublytic levels of antibody-mediated complement activation has not previously been explored.
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4738

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Xiaohong Wu, Govind Ragupathi, Katherine Panageas, et al.


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