

Featured Article

Impact of Human Neutralizing Antibodies on Antitumor Efficacy of an Oncolytic Adenovirus in a Murine Model

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ABSTRACT

Purpose: The purpose of this study was to assess the impact of anti-adenovirus neutralizing antibodies (AdNAbs) on the distribution, tolerability, and efficacy of intravenously administered oncolytic adenovirus. A translational model was developed to evaluate the impact of humoral immunity on intravenous administration of oncolytic adenovirus in humans.

Experimental Design: Initially, severe combined immunodeficient (SCID)/beige mice were passively immunized with various amounts of human sera to establish a condition of preexisting humoral immunity similar to humans. A replication-deficient adenovirus encoding β -galactosidase (rAd- β gal) was injected intravenously into these mice. An AdNAb titer that mitigated galactosidase transgene expression was determined. A xenograft tumor-bearing nude mouse model was developed to assess how a similar *in vivo* titer would impact the activity of 01/PEME, an oncolytic adenovirus, after intravenous administration.

Results: In SCID/beige mice, there was a dose dependence between AdNAbs and galactosidase transgene expression; 90% of transgene expression was inhibited when the titer was 80. A similar titer reconstituted in the nude mice with human serum, as was done in the SCID/beige mice, did not abrogate the antitumor efficacy of the replicating adenovirus after intravenous administration. Viral DNA increased in tumors over time.

Conclusions: In intravenous administration, preexisting AdNAb titer of 80 significantly attenuated the activity of a 2.5×10^{12} particles per kilogram dose of nonreplicating adenovirus; the same titer had no effect on the activity of an

equivalent dose of replicating adenovirus. Our results suggest that a majority of patients with preexisting adenovirus immunity would be candidates for intravenous administration of oncolytic adenovirus.

INTRODUCTION

Oncolytic replicating adenoviruses are emerging as a promising form of anticancer therapy. Oncolytic adenoviruses act by replicating within and lysing tumor cells. Intratumoral replication increases the local concentration of the virus at the tumor site (1, 2). To treat disseminated disease, it is preferable to deliver the adenovirus intravenously. However, previous reports on the effect of preexisting humoral immunity on intravenously administered adenovirus suggest that the neutralizing effect may prevent or significantly diminish the therapeutic value (3–5). In this study, we developed a model to test whether or not preexisting humoral immunity representative of that found in the general population would be sufficient to neutralize the antitumor efficacy of a replicating oncolytic adenovirus.

Antibodies to adenoviruses are common in the human population due to natural infection. Studies have shown that >90% of the human population is seropositive to adenoviruses of one or more serotype by enzyme-linked immunosorbent assay or by neutralization assay (3, 6–8). However, the anti-adenovirus antibody responses in the general population are quite variable, and anti-adenovirus neutralizing antibody (AdNAb) titers range from undetectable to very high (3, 7, 8). Therefore, modeling the effect of preexisting adenovirus immunity on systemic administration of therapeutic adenoviruses is challenging.

We wished to model the effect of preexisting humoral immunity to adenovirus on antitumor efficacy of an oncolytic adenovirus. To this end, a human tumor xenograft nude mouse model was developed to model preexisting humoral immunity to adenovirus observed in the human population. As a first step in model development, severe combined immunodeficient (SCID)/beige mice were passively immunized with different pools of human sera to establish a condition of preexisting immunity. Because SCID/beige mice lack a humoral immune response, the only source of AdNAb titer in this model was the human sera used for passive immunization. A nonreplicating adenovirus vector encoding β -galactosidase (rAd- β gal) was administered intravenously to determine the relationship between circulating AdNAb titer and vector gene expression in the liver.

A human tumor xenograft nude mouse model was developed to evaluate the inhibitory effect of human AdNAbs on the antitumor efficacy of an oncolytic adenovirus administered by the intravenous route. Preexisting immunity in this model was generated by passive immunization using the same procedure as performed in SCID/beige mice. Because nude mice are capable of mounting a humoral response in the absence of T-helper cells (9–13), mouse AdNAbs are induced after virus treatment and

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persist at increased neutralizing titers, analogous to the response seen in patients undergoing treatment with oncolytic adenoviruses (14–16). The data generated in these studies provide a method for estimating the feasibility of treating cancer patients with humoral immunity to adenovirus and suggest that preexisting neutralizing antibodies (NAbs) would not preclude intravenous administration of oncolytic adenovirus for >70% of the population.

MATERIALS AND METHODS

Adenovirus Vectors. Both rAd-GFP, which encodes green fluorescent protein, and rAd- β gal, which encodes β -galactosidase, are E1-deleted replication-deficient rAd vectors (17). The oncolytic adenovirus 01/PEME has been described previously (18). All vectors were purified by column chromatography (19) and quantified by Resource-Q high-performance liquid chromatography (20).

Neutralizing Antibody Assay. A previously described assay (6) was used to determine the titer of AdNAb in serum. The NAb titer, defined as ID_{50} , for each serum sample was reported as the reciprocal dilution that inhibited rAd-GFP-mediated transduction by 50%. Titer of <20 was extrapolated from the dilution curve.

Studies with Human Sera. Blood samples from healthy normal blood donors were collected for the study from the San Diego Blood Bank (San Diego, CA) in accordance with the San Diego Blood Bank informed consent guidelines. A total of 122 serum samples were collected over different times of the year. Sera were assayed individually for AdNAb titer. For passive immunization experiments, sera with titers of >320 were pooled into what is referred to as the high-titer group, and sera with titers of 20 to 320 were pooled into what is referred to as the low-titer group. Sera with AdNAb titers that were below the limit of quantification were not included in the study.

Passive Immunization of Human Sera in SCID/Beige C.B-17 Mice. Various dilutions of pooled high or low AdNAb titer human sera were used for passive immunization experiments. Sera (1.9–150 μ L) were diluted to a total volume of 500 μ L with vPBS (PBS supplemented with 3% sucrose and 2 mmol/L $MgCl_2$) and injected by intraperitoneal route into SCID/beige mice (Taconic, Germantown, NY; $n = 4$ mice for each condition). Mice were bled 3 hours after passive immunization to ensure the presence of AdNAbs in the circulation. The next day, all mice received 5×10^{10} particles of rAd- β gal by intravenous route. Mice were sacrificed at 72 hours. Livers were harvested and immediately frozen for polymerase chain reaction (PCR) analysis; portions of the livers were also fixed in OCT. Cryosections of the liver from OCT blocks were incubated with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) substrate to assess β -galactosidase activity as a measure of *in vivo* transduction efficiency. Naïve mice, sham passively immunized with vPBS, were used as controls.

Human Xenograft PC3 Tumor Model. Athymic nude mice were obtained from Harlan Laboratories, Inc. (Indianapolis, IN) between the ages of 6 and 8 weeks. Xenograft tumors were established by subcutaneous injection of 5×10^6 PC3 cells (human prostate adenocarcinoma, ATCC CRL-1435) in the flanks of athymic mice. Between days 7 and 10, tumors reached

100 mm³ in size, and the mice were randomized and divided into six groups of eight mice each. Fifty microliters of high-titer sera diluted in 500 μ L vPBS (1:10 dilution) were injected by intraperitoneal route into two groups of eight animals each (high-titer groups). The same procedure was performed with low-titer sera (low-titer groups). The control groups of sham passively immunized (naïve) mice received 500 μ L of vPBS. Three hours after passive immunization, all animals were bled to determine the *in vivo* AdNAb titers. The mice were then treated with two different regimens of 01/PEME: Each of the groups was treated with a single bolus dose of 5×10^{10} particles of 01/PEME per animal or 1×10^{10} particles of 01/PEME per animal per day for 5 consecutive days. Tumors from all groups of mice were measured every week after 01/PEME treatment. Tumor volume was calculated assuming spherical geometry. Mean tumor size for each treatment group \pm SE was plotted *versus* time after cell injection.

Quantitative Polymerase Chain Reaction. Real-time quantitative PCR (QPCR) and reverse transcription-PCR for rAd- β gal were described in detail previously (6), as well as the PCR for measuring adenovirus DNA with hexon primers (21). The result of QPCR is expressed as DNA copies per milligram of tissue.

Toxicity Evaluation. PC3 tumor-bearing athymic mice were monitored for general health appearance. Mice were observed daily for clinical symptoms such as anorexia and lethargy and weighed once a week. At the appropriate sacrifice time, blood samples were collected and tested for alanine aminotransferase (ALT) levels to evaluate liver function.

Statistics. Results are expressed as means \pm SE. Analysis of variance *post hoc* Fisher's protected least significant difference unpaired *t* test (Statview; Abacus, Berkeley, CA) was used to compare difference between groups. Statistical significance was defined as $P < 0.05$.

RESULTS

Characterization of Neutralizing Antibody Titers against Adenovirus in a Population of Healthy Blood Donors. A majority of the human population has preexisting humoral immunity to adenovirus due to natural infection. Analysis of AdNAb titers in the general population may provide an initial assessment of the percentage of the population that could benefit from gene therapy using adenovirus. Recently, we and others developed titration assays that measure neutralization of the Ad5-based vector rAd-GFP as reduction of green fluorescent protein (GFP) fluorescence in infected cells (6, 22). Results of these studies are in general agreement with studies using the plaque reduction assay (5, 14, 23) or the Ad- β gal neutralization assay (7) and show that approximately two thirds of study subjects had detectable AdNAbs against Ad5. In addition, we and others showed that in human sera, the reduction of GFP fluorescence in the *in vitro* assay was dependent on antibodies that recognized adenovirus capsid proteins (6, 16, 24). In the present study, we determined the midpoint titers (ID_{50}) of AdNAbs from 122 healthy blood donors using serial dilutions of sera in the rAd-GFP assay. Consistent with previous studies, we found that the AdNAb titers (ID_{50}) from our study population could be divided into three groups. Based on our criteria, 48

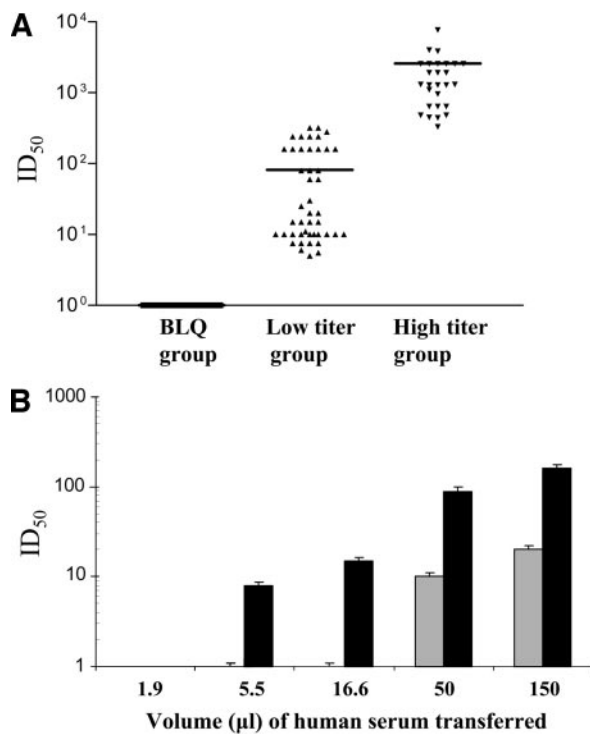


Fig. 1 Characterization of neutralizing antibody titers against adenovirus in human sera and in SCID/beige mice sera passively immunized with human sera. **A**, The neutralizing activities in sera of normal individuals were defined by ID₅₀ titers using an *in vitro* rAd-GFP base assay. Sera from 122 donors were assayed, and three patterns of neutralizing titers emerged: 48 donors had titers below the limit of quantification, 45 had low titers (ID₅₀ < 320), and 29 had high titers (ID₅₀ > 320). **B**, passive immunization using pooled high-titer and pooled low-titer human sera in SCID/beige mice. ID₅₀ titer values of sera taken from low-titer (■) and high-titer (■) groups of SCID/beige mice that were passively immunized with different volumes of pooled low-titer or high-titer human sera.

donors had titers that were below the limit of quantification, 45 donors had a low AdNAb titer with an ID₅₀ range of 20 to 320, and 29 had a high AdNAb titer with ID₅₀ > 320 (Fig. 1A).

AdNAb Titer Measured in Sera of Severe Combined Immunodeficient/Beige Mice after Passive Immunization with Human Sera. To avoid any bias associated with possible idiosyncratic antibody responses, we pooled sera from four representative donors from either the high- or low-titer groups and ran full dilution curves using the *in vitro* rAd-GFP assay (data not shown). The resulting titers of the pooled low- and high-titer sera were determined to be 80 and 1,560, respectively. Increasing volumes of the pooled low- and high-titer sera were administered intraperitoneally to SCID/beige mice to generate humoral immunity to adenovirus by passive immunization. Three hours after administration of the pooled human sera, serum samples were taken from passively immunized mice with the equivalent of 1.9, 5.5, 16.6, 50, and 150 µL of human sera. The respective titers determined in the high-titer groups were as follows: below limit of quantification, <20, <20, ~89, and ~160 (Fig. 1B). In mice that were passively immunized with low-titer serum, only the groups

that received 50 and 150 µL had quantifiable titers, and both were <20. The ID₅₀ titers determined in sera from the passively immunized mice correlated with the amount of input pooled high- and low-titer human sera. As expected, the circulating titers in the mice were lower due to dilution in the mouse body fluids as well as absorption of antibodies into the tissues during homeostasis.

Effect of Human Neutralizing Antibodies on Adenovirus Vector Distribution after Intravenous Administration. In the mouse, >90% of the input dose of adenovirus vector is distributed to the liver after intravenous administration (25). To determine whether this distribution was affected by circulating anti-adenovirus antibodies, we challenged the passively immunized SCID/beige mice with intravenous rAd-βgal and measured the levels of rAd-βgal DNA in livers by PCR 3 days later. For the low-titer groups, there was no statistically significant reduction in the amount of rAd-βgal DNA distributed to the livers with increasing doses of serum used for passive immunization (data not shown). In contrast, in groups passively immunized with high-titer serum, there was a modest dose-dependent drop in viral DNA in the livers of groups passively immunized with 1.9, 5.5, and 16.6 µL of serum (Fig. 2A). In mice that received 50 µL of serum, viral DNA in the liver was reduced by half compared with the vehicle-treated group. In mice that received 150 µL of serum, there was a significant inhibition of viral DNA uptake in the livers of animals that received the 150-µL dose of serum ($P < 0.05$).

Effect of Human Neutralizing Antibodies on Adenovirus Vector Function after Intravenous Administration. We assessed the impact of AdNabs on vector function by measurement of βgalactosidase activity in liver homogenates and by measurement of βgalactosidase activity in liver sections. Although rAd-βgal DNA was detected in the livers of animals in all of the groups, βgalactosidase activity was only detected in groups that received <50 µL of high-titer serum. Consistent with the βgalactosidase activity, βgal RNA was significantly reduced in the group that received 50 µL of high-titer serum; this corresponded to a 90% inhibition of βgal activity at an *in vivo* titer of ≥80 (Fig. 2B). No reduction in βgal RNA was observed using pooled low-titer sera up to the maximum dose tested of 150 µL, which had a titer of ≤20 (data not shown). Overall, there was a good correlation between circulating antibody titers measured in the *in vitro* rAd-GFP assay (Fig. 1B) and inhibition of βgal RNA expression in mouse liver after intravenous challenge with 5×10^{10} particles of rAd-βgal. In addition, the levels of βgal RNA in liver homogenates were in good agreement with the level of βgalactosidase expression as measured by X-Gal staining of liver sections (Fig. 2C). Together, these results showed that passive immunization with 50 µL of pooled human high-titer sera provided levels of circulating AdNabs sufficient to inhibit rAd-mediated gene expression in the livers by 90% and further demonstrated that titers measured using the *in vitro* rAd-GFP neutralization assay could be inversely correlated to levels of *in vivo* transgene expression after intravenous administration of a nonreplicating adenovirus vector.

Passive Immunization against Adenovirus in a Nude Mouse Tumor Model. Oncolytic adenoviruses such as 01/PEME have shown significant antitumor activity in animal models using intravenous administration (21). Because hu-

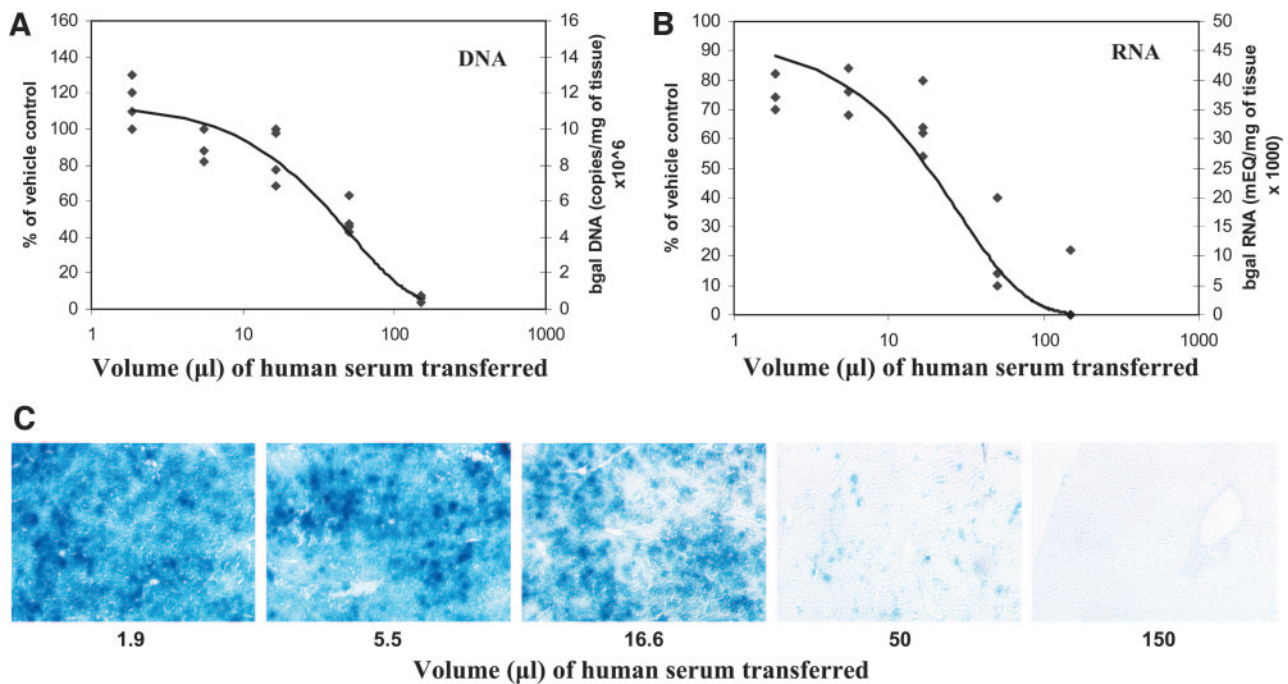


Fig. 2 Effect of AdNABs on adenovirus vector distribution and transgene expression after intravenous administration of 5×10^{10} particles of rAd- β gal. Three days after intravenous administration of rAd- β gal in SCID/beige mice passively immunized with different volumes of pooled high-titer human sera, livers were harvested, and rAd- β gal DNA was assessed by PCR (A), and rAd- β gal RNA was assessed by reverse transcription-PCR (B). The percentage of vehicle control is calculated based on result from the sham passively immunized group. Assessment of rAd- β gal activity was done by X-Gal staining of liver sections (C).

man adenovirus does not replicate in mouse cells or murine tumors, most preclinical studies with oncolytic adenovirus have been done using human tumor xenografts in nude mice. To explore the effect of preexisting humoral immunity on oncolytic adenovirus distribution and function in the human tumor xenograft/nude mouse model, we passively immunized tumor-bearing nude mice as described above for the SCID/beige mouse studies. Human PC3 prostate tumor-bearing mice were passively immunized by intraperitoneal injection of pooled human sera from either the high-titer or low-titer group. The 50- μ L dose of human serum was selected because in the previous study (Fig. 2C) this provided an approximately 90% reduction of virus gene expression for the high-titer group. Samples of serum were taken from the passively immunized mice 3 hours after administration for determination of circulating neutralizing titers. The circulating titers for mice passively immunized with the high- and low-titer human sera were very comparable with those observed in the SCID/beige mice using comparable doses of sera (Fig. 3A). Mice from the high-titer group had circulating AdNab titers of 100, mice from the low-titer group had circulating titers of <20 , and mice injected with vehicle had titers that were below the limit of quantification.

Effect of Human Neutralizing Antibodies on Distribution and Replication of Oncolytic Adenovirus after Intravenous Administration. We evaluated the impact of circulating AdNABs on distribution of an oncolytic adenovirus, 01/PEME (18), to the liver and tumor in the human PC3 xenograft/nude

mouse model using passive immunization with pooled human sera as described above. Oncolytic adenovirus 01/PEME was then administered by tail vein injection. Doses of either 1×10^{10} or 5×10^{10} particles of 01/PEME were administered as a single bolus intravenous injection. The 1×10^{10} -particle dose was selected because it was previously shown to be the efficacious dose in the PC3 tumor model in nonimmunized nude mice (21). A 5-fold higher dose also was selected because of the anticipated reduction in antitumor efficacy due to passive immunization with human AdNABs.

The amount of 01/PEME viral DNA in livers and tumors was measured by QPCR 3 hours and 5 days after administration. These time points were selected based on previous studies showing that distribution of 01/PEME to PC3 tumors in nude mice was detectable at 3 hours and that replication of 01/PEME DNA within the tumors peaked at day 5 (21). At the 3 hour time point, similar levels of viral DNA were detected in the livers of PC3 tumor-bearing nude mice, regardless of their circulating AdNab titers (Fig. 3B). As expected, the level of viral DNA in the liver was lower in the 1×10^{10} -particle dose groups compared with the 5×10^{10} -particle dose groups. By day 5, there was a significant decrease in viral DNA in the livers of all of the groups that received the 1×10^{10} -particle dose ($P < 0.05$; Fig. 3B, \blacksquare). For the 5×10^{10} -particle dose group, viral DNA levels on day 5 remained similar to those observed at the 3 hour time point and persisted until day 12 (data not shown).

Viral DNA distributed to the tumor was approximately $500\times$ less than that observed in the liver at the 3 hour time

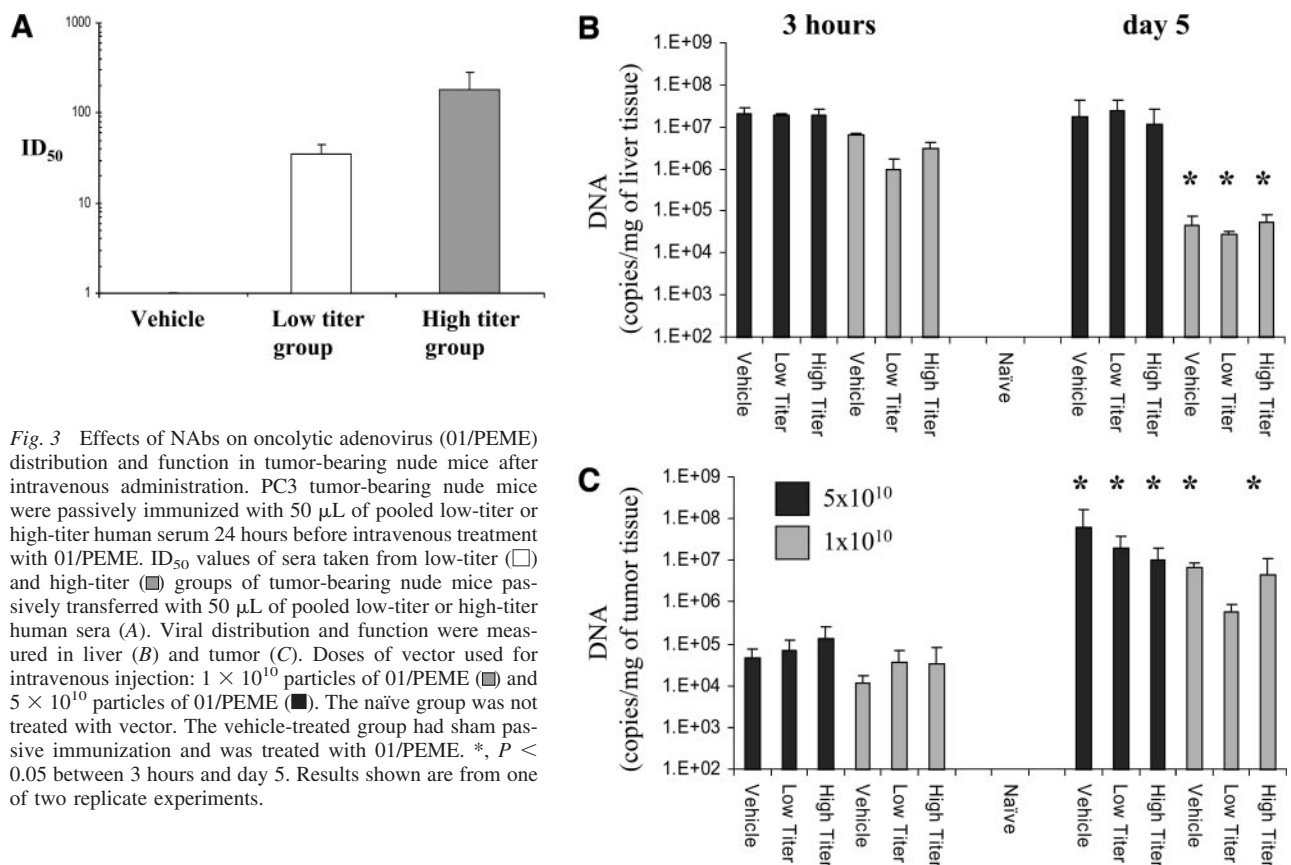


Fig. 3 Effects of NAbs on oncolytic adenovirus (01/PEME) distribution and function in tumor-bearing nude mice after intravenous administration. PC3 tumor-bearing nude mice were passively immunized with 50 μ L of pooled low-titer or high-titer human serum 24 hours before intravenous treatment with 01/PEME. ID₅₀ values of sera taken from low-titer (□) and high-titer (■) groups of tumor-bearing nude mice passively transferred with 50 μ L of pooled low-titer or high-titer human sera (A). Viral distribution and function were measured in liver (B) and tumor (C). Doses of vector used for intravenous injection: 1×10^{10} particles of 01/PEME (■) and 5×10^{10} particles of 01/PEME (■). The naïve group was not treated with vector. The vehicle-treated group had sham passive immunization and was treated with 01/PEME. *, $P < 0.05$ between 3 hours and day 5. Results shown are from one of two replicate experiments.

point. Differences in administered dose of 01/PEME (1×10^{10} versus 5×10^{10} particles) resulted in corresponding differences in the levels of viral DNA detected in tumors at the 3 hour time point (Fig. 3C). Circulating AdNab titers had no statistically significant impact on the amounts of viral DNA distributed to tumors at this time point; the amount of virus delivered was not correlated with AdNab titers. However, significant increases in viral DNA in tumors were observed at day 5 in all treatment groups ($P < 0.05$), regardless of the status of preexisting immunity or the administered dose. The group with the 1×10^{10} -particle dose in the low-titer group had a modest increase in viral DNA on day 5 ($P < 0.07$).

Endogenous Anti-Adenovirus Neutralizing Antibodies Were Induced in Tumor-Bearing Nude Mice after Intravenous Administration of 01/PEME. Induction of NAbs to oncolytic adenovirus has been described in several clinical studies (14, 15, 26). Although we were unable to model induction of human AdNabs in mouse models, it was possible to model some aspects of induction of AdNabs in response to therapy. Athymic nude mice are deficient in most T-cell-mediated immune responses but remain capable of mounting humoral responses to antigen. To evaluate the development of an endogenous humoral response to adenovirus in mice that had preexisting humoral immunity, we measured circulating AdNab titers in sera of tumor-bearing nude mice 5 days after intravenous administration of 01/PEME. As shown in Table 1, AdNab responses to adenovirus developed by day 5

in the sham passively immunized mice injected with saline vehicle instead of human serum. The circulating neutralizing titer (due to endogenous mouse antibodies) induced in the sham passively immunized groups exceeded the circulating neutralizing titers generated by passive immunization with human serum. The vehicle group injected with the 5×10^{10} -particle dose showed a higher AdNab response (ID₅₀ = 300) than the group injected with the 1×10^{10} -particle dose (ID₅₀ = 152). Interestingly, in both the 1×10^{10} - and 5×10^{10} -particle dose groups, mice that were passively immunized with the low-titer human sera showed higher AdNab response than either the high-titer or the vehicle groups. We had determined previously that the

Table 1 AdNab titers pre and post 01/PEME treatment

Treatment (particles/mouse)	Group	Pretreatment (ID ₅₀)*	Posttreatment (ID ₅₀)†
1×10^{10}	Vehicle	BLQ	152 \pm 25
1×10^{10}	Low titer	<20	520 \pm 6
1×10^{10}	High titer	100	166 \pm 17
None	Naïve	BLQ	BLQ
5×10^{10}	Vehicle	BLQ	300 \pm 13
5×10^{10}	Low titer	<20	640 \pm 27
5×10^{10}	High titer	100	320 \pm 10

Abbreviation: BLQ, below the limit of quantification.

* Titers measured 3 hours after passive immunization.

† Titers measured 5 days after 01/PEME IV administration.

half-life of *in vivo* anti-AdNAb in human passively immunized sera was 14 days (data not shown); thus, the circulating neutralizing activity present by day 5 in the passively immunized groups was due to mouse endogenous AdNAb plus residual human AdNAb.

PC3 Efficacy Tumor Model in the Presence of Established Humoral Immunity to Adenovirus. In a study of pharmacological indicators of antitumor efficacy of 01/PEME, efficient distribution to the tumor site was found to be a critical factor for efficacy of oncolytic adenoviruses (21). Similar to what was seen in the SCID/beige model, circulating AdNAb titers of ≤ 80 did not substantially impact 01/PEME distribution to the tumor or to the liver in the human xenograft nude model. However in the SCID/beige mice, circulating AdNAb titers of ≥ 80 were found to inhibit adenovirus transgene expression in the liver by 90%. To determine whether AdNAb would inhibit the antitumor efficacy, we followed tumor growth over time in passively immunized human xenograft-bearing nude mice (as described above) treated with 01/PEME.

To examine the effect of preexisting AdNAb titer on the outcome of 01/PEME antitumor efficacy, tumor-bearing nude mice were passively immunized with high-titer and low-titer human sera before receiving 01/PEME therapy. Two regimens of therapy were used for the antitumor efficacy study: a single bolus intravenous injection of 5×10^{10} particles of 01/PEME, or five consecutive daily injections of 1×10^{10} particles of 01/PEME. As seen in Fig. 4A, by day 37, all groups receiving 01/PEME had tumor growth inhibited by 70%, regardless of preexisting AdNAb titer status (derived from passive immunization of human sera) and regardless of the induction of endogenous AdNAb by day 5 (derived from intravenous administration of 01/PEME).

Impact of Preexisting Immunity on Vector-Induced Toxicity after Intravenous Administration of 01/PEME. Progression of PC3 tumors in this nude mouse model was associated with progressive loss of body weight and other signs of cachexia. Systemic administration of oncolytic adenoviruses was also associated with elevation of serum levels of liver enzymes (*e.g.*, ALT) and inflammatory cytokines, including tumor necrosis factor α (25, 27–30).

The antitumor effect of 01/PEME was correlated with weight gain in animals treated with 01/PEME, regardless of circulating AdNAb titer (Fig. 4B). No signs of anorexia or lethargy were observed in the 01/PEME-treated animals. In contrast, untreated animals lost body weight as their tumors progressed.

We assessed the effect of circulating AdNAb on serum liver enzyme ALT levels in mice treated with a single bolus intravenous administration of 1×10^{10} or 5×10^{10} particles of 01/PEME. Sera were collected from 01/PEME-treated mice 3 hours and 5 days after intravenous administration. In animals from all groups that received 1×10^{10} particles of 01/PEME by intravenous treatment, the serum ALT levels were within the normal range (ALT, 20–60 units/liter) measured at 3 hours and on day 5 (data not shown). At the 5×10^{10} -particle dose of 01/PEME, ALT levels were within normal range at 3 hours (ALT, 20–50 units/liter) but rose significantly by day 5 in all of the 01/PEME-treated groups (ALT, 199–530 units/liter; $P <$

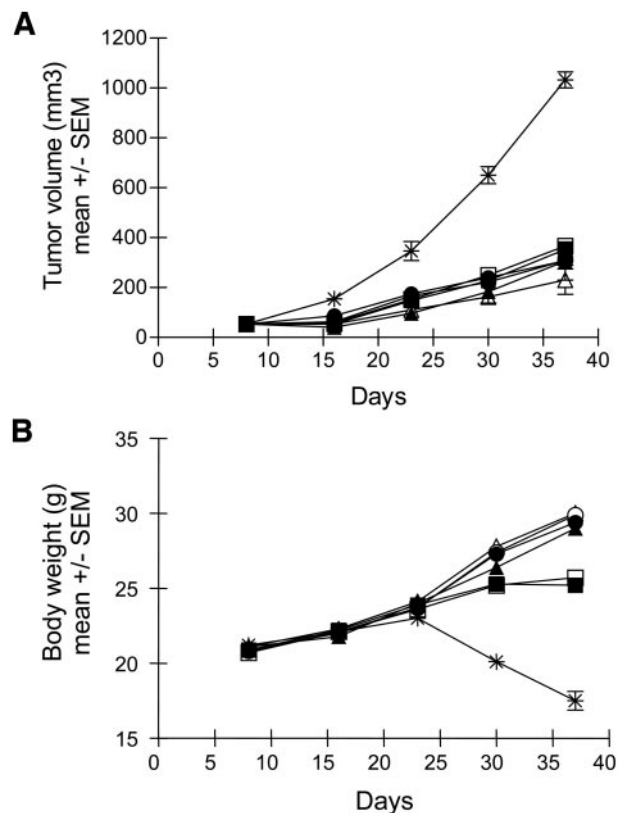


Fig. 4 Tumor sizes (A) and body weights (B) of animals treated with 01/PEME. PC3 tumor bearing mice were treated with either single bolus of 5×10^{10} particles of 01/PEME (open symbols) or 5 daily injections of 1×10^{10} particles of 01/PEME (filled symbols). Mice were passively immunized with low-titer (circles), high-titer (triangles), or vehicle (sham immunized; squares). Unimmunized, untreated group was included as controls (*).

0.05; Fig. 5). The circulating AdNAb in the passively immunized mice had no significant effect on ALT response when compared with sham immunized mice at either the 1×10^{10} or 5×10^{10} -particle dose of 01/PEME.

DISCUSSION

Preclinical models used to study oncolytic adenoviruses such as 01/PEME for cancer therapy test for the ability of the adenovirus to be delivered to the tumor site, replicate in the tumor, and inhibit tumor growth in a dose-dependent manner. Because replication of oncolytic adenoviruses is restricted to human cells, preclinical testing is generally performed using human tumor xenografts in immune-compromised mice. These approaches have yielded useful information on the relative safety and efficacy of systemic delivery of oncolytic adenovirus. However, the immune-compromised mouse models omit many aspects of the host immune response that may be relevant to the distribution, tolerability, and efficacy of oncolytic adenovirus.

In contrast to the very potent neutralizing humoral response to adenovirus seen in immune-competent mice, we found that a majority of humans exposed to adenovirus through natural infection did not have high levels of AdNAb present in the serum

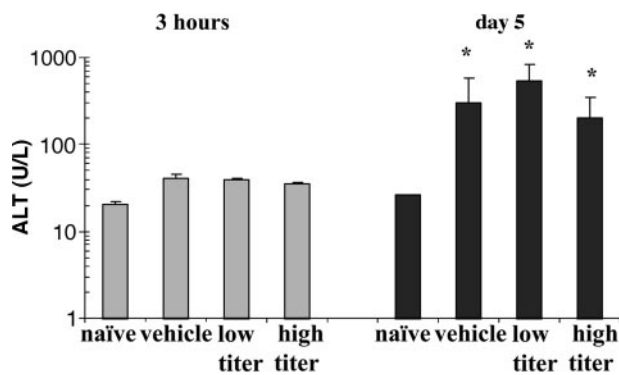


Fig. 5 ALT measured in sera of 01/PEME-treated PC3 tumor-bearing nude mice passively immunized with high-titer human sera at 3 hours (■) and on day 5 (■) after intravenous administration of 5×10^{10} particles of 01/PEME. *, $P < 0.05$ between 3 hours and day 5.

(3, 6, 7). The model used in this study was designed to address the impact of naturally acquired preexisting immunity in cancer patients undergoing adenovirus gene therapy. In the human tumor xenograft nude mice model, we could assess the delivery and replication of 01/PEME in the presence of preexisting AdNAb (from passive immunization of human serum) and in the presence of newly induced mouse AdNAb from the host (in response to intravenous administration of 01/PEME), conditions analogous to those experienced by cancer patients undergoing virus therapy. From this model, we were able to follow the distribution, tolerability, and the efficacy of 01/PEME after intravenous administration in the presence of humoral immune responses.

The use of human sera in these translational models addressed how the different AdNAb titers found in the human population would affect systemic oncolytic adenovirus therapy. Our data showed that the presence of anti-AdNAbs did not contribute or prevent hepatic toxicity as reported in studies using preimmunized animal models (5, 31). Rather, our data were very similar to several human clinical trials involving different replication-competent adenoviruses delivered by various intravascular routes (30). Increased toxicity such as transaminitis was associated with a higher dose of virus used (as in our 5×10^{10} -particle dose) and was not associated with the presence of AdNAbs found in the host.

Our data were consistent with previous reports from clinical trials with systemic administration of oncolytic adenoviruses. AdNAb titers increased after intravenous administration, regardless of the preexisting humoral immunity (14–16, 30, 32). However, the *in vivo* titrations of AdNAbs in our SCID/beige study allowed evaluation of the effect of preexisting immunity on distribution of the virus to the liver. Gene expression in the liver was inversely proportional to the *in vivo* AdNAb titers, and 90% of the viral function was inhibited when the *in vivo* titer was >80 . This indicated that AdNAbs have a substantial effect on virus function after intravenous administration and that measuring virus particles in the blood or target organs during viral therapy by QPCR alone would not give sufficient assessment of the bioactivity of the virus. For oncolytic adenovirus therapy, functional assays that can quantify infectious viral

particles in the presence of autologous patients' sera may help to elucidate some aspects of viral drug activities

In the nude mouse/xenograft model, antitumor efficacy was achieved in the presence of a blocking AdNAb titer. The replication of 01/PEME in tumors in naïve mice was similar to that observed in tumors in mice passively immunized with either low- or high-titer sera (Fig. 3B). These results suggest that AdNAbs may not play as critical a role in inhibiting the activities of the 01/PEME within the tumor microenvironment as they do in the periphery. It is likely that without the replication ability of the adenovirus, AdNAbs would attenuate the small number of viral particles distributed to the tumors after intravenous delivery. Alternatively, some aspect of the host innate immune responses may contribute to antitumor efficacy (33–35). Additional studies are needed to elucidate the host immune mechanisms involved in the antitumor efficacy of 01/PEME.

There are limitations in our models. Immune responses induced by oncolytic adenovirus in systemic administration would best be done in immune-competent models, as demonstrated recently by Hallden *et al.* (34). However, it would be difficult to replicate the condition of natural infection by human adenovirus in laboratory animals. Our models are also limited in assessing induced humoral immunity resulting from systemic administration of oncolytic adenovirus because the induced antibodies are of mouse origin. Although induction of mouse AdNAbs recapitulates the increase in neutralizing titer seen in patients treated with oncolytic adenoviruses, the antigen specificity, avidity, and isotype preference of the induced mouse antibodies may differ from those induced in humans.

Our models highlighted some of the hurdles of systemic delivery of 01/PEME in assessing distribution, tolerability, and antitumor efficacy. Antitumor response with 01/PEME is a balance between dose-dependent toxicity and dose-dependent efficacy. We have shown that 01/PEME is at least a 1,000-fold more potent than a nonreplicating rAd vector such as rAd-p53 in the same tumor model (21); it is possible to achieve efficacy with a lower dose of 01/PEME and therefore avoid hepatic toxicity. However, a lower dose of 01/PEME may not be efficacious in the presence of AdNAbs. Reduction of AdNAbs by either direct inhibition of AdNAb formation or, as proposed by our previous study, lowering the titer of AdNAbs by immunoplasmaferesis would preserve virus activities (6, 36, 37).

Our models were able to provide some insights into the relationship between AdNAb titer and drug activity that have been difficult to obtain from clinical data, possibly because of variability in preexisting immunity between patients. Our models demonstrated a correlation between circulating AdNAb titer and viral (drug) activity for replication-defective vectors but also showed that the AdNAb threshold for inhibition of oncolytic adenovirus activity is increased, most likely due to the ability of virus to replicate at the tumor site; delivery, replication, and antitumor efficacy of 01/PEME were maintained in the presence of AdNAb titers capable of blocking rAd- β gal gene expression in the SCID/beige model. Because the AdNAb titers in the passively immunized mice at the time of intravenous administration of 01/PEME were higher than those found in $>70\%$ of healthy human donors, these results suggest that preexisting humoral immunity will not preclude systemic administration of 01/PEME in the majority of cancer patients.

Importantly, our study suggests that the newly induced antibodies in patients during viral therapy should be lower than a titer of 80 before the next round of therapy. Given the safety profile accumulated thus far with oncolytic adenovirus in intravascular delivery, modulating some aspects of the host immune responses during therapy should be tested for this type of drug to be used for systemic anticancer therapy.

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