Cyclophosphamide Facilitates Antitumor Efficacy against Subcutaneous Tumors following Intravenous Delivery of Reovirus

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Abstract

Purpose: The purpose of the present study was to investigate whether it is possible to achieve truly systemic delivery of oncolytic reovirus, in immunocompetent hosts, using cyclophosphamide to overcome some of the barriers to effective intratumoral delivery and replication of i.v. injected virus.

Experimental Design: I.v. delivery of reovirus was combined with different regimens of i.p. administered cyclophosphamide in C57Bl/6 mice bearing established s.c. B16 tumors. Intratumoral viral replication, tumor size, and survival were measured along with levels of neutralizing antibody (NAb) in the blood. Finally, differential toxicities of the virus/cyclophosphamide regimens were monitored through viral replication in systemic organs, survival, and cardiac damage.

Results: Repeated i.v. injection of reovirus was poorly effective at seeding intratumoral viral replication/oncolysis. However, by combining i.v. virus with cyclophosphamide, viral titers of between 10^7 and 10^8 plaque-forming units per milligram were recovered from regressing tumors. Doses of cyclophosphamide that ablated NAb were associated with severe toxicities, characterized by viral replication in systemic organs—toxicities that are mirrored by repeated reovirus injections into B-cell knockout mice. Next, we restructured the dosing of cyclophosphamide and i.v. virus such that a dose of 3 mg cyclophosphamide was administered 24 h before reovirus injection, and this schedule was repeated every 6 days. Using this protocol, high levels of intratumoral viral access and replication ($\sim 10^7$ plaque-forming units per milligram tumor) were maintained along with systemically protective levels of NAb and only very mild, non–life-threatening toxicity.

Conclusion: NAb to oncolytic viruses play a dual role in the context of systemic viral delivery; on one hand, they hinder repeated administration of virus but on the other, they provide an important safety mechanism by which virus released from vigorous intratumoral replication is neutralized before it can disseminate and cause toxicity. These data support the use of cyclophosphamide to modulate, but not ablate, patient NAb, in development of carefully controlled clinical trials of the systemic administration of oncolytic viruses.

Ａ major goal of gene therapy/virotherapy is to generate vectors that can be delivered systemically to metastatic disease deposits that are inaccessible to direct intratumoral injection (1–4). In a virus-naive host, antiviral activities in the circulation (4, 5) include complement (6), preimmune IgM (7) and other components of the innate immune system that inhibit replication within tumors (8–10). Circulating vector must also avoid both nonspecific adhesion to multiple cells (11) as well as specific sequestration in organs such as the liver (12). Vectors must also extravasate specifically at the tumor (13). In a virus-immune host, NAb is a major additional inhibitor of oncolytic viruses (4), dependent, at least partly, upon the route of administration (14). In addition, in contrast to preclinical models, even vectors directly injected into human tumors do not migrate far from the end of the needle (15). These findings inspired the development of replication-competent vectors, which would, in theory, require only low levels of seeding in a tumor to initiate spreading infections to cover the tumor comprehensively (16, 17). However, significant problems still persist even in the context of direct intratumoral injections, including stromal and immune barriers to effective intratumoral spread (8, 18, 19), which may be partially overcome by combination with preexisting modalities (20, 21). In light of these considerations, an intact immune system should also be viewed as a critical component that contributes to the "tightness" of the oncolytic specificity of a virus.

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Reoviruses (respiratory enteric orphan) are double-stranded RNA viruses isolated from the respiratory and gastrointestinal tracts of humans but not linked to disease (22–24). They do, however, cause fatal infections in neonatal and severe combined immunodeficient nonobese diabetic (SCID/NOD) mice (24, 25), reiterating the importance of an intact immune system as a component determining oncolytic specificity. About 90% of patients are preimmune to reovirus. The use of reovirus as an oncolytic agent was proposed on the basis of findings that an activated Ras pathway in tumor cells prevents RNA-activated protein kinase from aborting infection, leading to lytic viral replication in tumors but not in normal cells (22, 26–28).

Based on these considerations, we have completed a phase I clinical trial of systemically delivered reovirus given to 32 patients as a 1 h i.v. infusion every 4 weeks (one cycle).[7] Dose escalation reached 3×10^{10} TCID$_{50}$ for 5 days every 4 weeks without dose-limiting toxicity and a maximum tolerated dose was not defined. No viral shedding at any dose was seen by reverse transcription-PCR in blood, urine, stool, and sputum. Patients fell into two distinct groups in terms of NAb. Those with preexisiting antireovirus antibody had titer increases of up to 250-fold (median 80-fold) by day 5. Titers in patients without preexisting antireovirus antibody increased by 700- to 6,500-fold. However, in all cases, final titers ended up close to the same level during the subsequent cycles.[8] In addition to showing that i.v. reovirus is safe and well tolerated, replicating reovirus could also be detected in some cases in metastases after systemic viral delivery.[7]

The preliminary data from this, and other, clinical trials have suggested that transient immunosuppression may facilitate viral delivery to metastatic tumors by removing, or reducing, the protective blanket of NAb and other immune effectors. In this respect, cyclophosphamide has been used to facilitate delivery/efficacy of oncolytic viruses (6–10, 29, 30) and is already used as a chemotherapy at doses that are heavily immunosuppressive (>120 or >400 mg/kg in mice; refs. 31–33). Short-term exposure to cyclophosphamide suppressed innate immune responses (natural killer cells, macrophages, and IFN-γ), which inhibit intravascular delivery of herpes simplex virus to rat gliomas (7), producing significant improvements in therapy (8). At lower doses (<100 mg/kg), cyclophosphamide enhances immune responses against tumors (34–36) through selective, transient depletion of regulatory T cells that suppress antitumor CD8$^+$ T-cell responses (34–41).

In this report, we have used our clinical experience using systemic delivery of oncolytic reovirus to return to our preclinical model to test new protocols by which replicating vectors can be delivered to metastatic disease, in immune-competent tumor-bearing hosts, in the absence of direct access by a needle. Reovirus is an attractive experimental oncolytic virus because it replicates in murine tumor cells that are tumorigenic in immunocompetent mice (42–45). We have used the B16/C57Bl/6 immunocompetent model to test different regimens of administration of reovirus with cyclophosphamide, as either a single low dose (150 mg/kg = 3 mg per mouse), as a high dose (3 mg for 3 days), or as iterative

injections (metronomic dosing) of 3 mg once every 6 days (31–33, 35). Our data show that cyclophosphamide can facilitate high levels of i.v. administered virus to reach, and replicate in, s.c. tumors, associated with dramatic reductions in levels of circulating neutralizing antibodies (NAb). However, cyclophosphamide-mediated loss of protective NAb can also be associated with severe toxicity probably as a result of subsequent dissemination of the virus from the tumor to systemic organs, showing that NAb can also provide a safety barrier to the widespread dissemination/toxicity of oncolytic viruses. Finally, we show that iterative injections (metronomic dosing) of 3 mg cyclophosphamide and reovirus once every 6 days allows both high levels of viral access to tumors and minimal toxicity. These data will drive the development of further clinical trials for systemic delivery of reovirus to patients with metastatic disease.

Materials and Methods

Cells and virus. Murine B16 melanoma cells (H2-K^b; ref. 46) were grown in DMEM (Life Technologies) supplemented with 10% (v/v) FCS (Life Technologies) and L-glutamine (Life Technologies). All cell lines were monitored routinely and found to be free of *Mycoplasma* infection.

Reovirus used in these studies is a wild-type reovirus type 3 (Dearing strain). Virus stock titers were measured by standard plaque assays of serially diluted samples on L929 cells.

Antibody titration from mouse serum. Preheated mouse antiserum was mixed with an equal volume of reovirus (predetermined as killing 80% of target L929 cells) and incubated at 37°C for 2 h to allow antibody to bind to virus. The virus/antibody mix was transferred to L929 monolayers and cell survival was assayed at 48 h by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. An anti-reovirus polyclonal antiserum was used as a positive control. The neutralizing titer is the highest dilution of serum that blocks the killing of L929 cells.

Virus titration from tumor and organs. Tumor and organs recovered from mice were harvested and weighed and, within 2 h of removal from the mouse, were lysed by three cycles of freeze/thawing. Virus was recovered from the lysates and titers were determined on L929 cells and expressed as pfu of reovirus per milligram of tissue.

In vivo *studies.* All procedures were approved by the Mayo Foundation Institutional Animal Care and Use Committee. C57BL/6 or B6.129S2-*Igh-6tm1*^{Cgn}/j mice (Jackson 002288), which lack mature B cells and cannot, therefore, make antiviral antibodies, were age and sex matched for individual experiments and were purchased from The Jackson Laboratory at 6 to 8 weeks of age. To establish s.c. tumors, 2×10^5 B16 cells were injected s.c. (100 μL) into the flank region. Animals were examined daily until the tumor became palpable, after which the diameter, in two dimensions, was measured thrice weekly using calipers. Animals were killed when tumor size was ∼ 1.0×1.0 cm in two perpendicular directions. Mice were euthanized based on the double criteria of both active progression in measurable size over several days and of reaching a diameter of 1.0 cm. This is based on our histologic experience that tumors that have progressively increased in size to a final size of 1.0 cm diameter represent actively growing tumor rather than predominantly necrotic tumor destruction. To establish systemic metastatic disease, C57BL/6 mice were injected i.v. with 2×10^5 B16ova cells to form lung metastases. Mice were killed at the first sign of any distress.

Histopathology of tumor sections. Tumors and major organs were harvested and fixed in 10% formalin in PBS, then paraffin-embedded and sectioned. H&E-stained sections were prepared for analysis of tissue destruction and gross infiltrate. A pathologist examining H&E sections, blinded to the experimental design, scored the degree of necrosis.

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[7] Vidal et al., submitted for publication.
[8] White et al., in preparation.

Statistics. Survival data from the animal studies were analyzed using the log-rank test (47), and the two-sample unequal variance Student's *t* test analysis was applied for *in vitro* assays. Statistical significance was determined at the level of $P < 0.05$.

Results

Reovirus is oncolytic in the B16 murine model. Direct intratumoral injection of reovirus induced significant regressions of established B16 tumors in fully immunocompetent C57Bl/6 mice, demonstrating that it is an effective oncolytic agent if sufficient levels of virus can be delivered to tumors ($P = 0.0027$ compared with heat inactivated virus; Fig. 1A). However, our ultimate goal is the treatment of metastatic disease not accessible to direct intratumoral injection. Therefore, reovirus was administered i.v. according to the protocol in Fig. 1B to treat either established lung metastases (Fig. 1C) or s.c. tumor (Fig. 1D). Over multiple, repeated experiments, i.v. delivery of reovirus to either lung metastatic, or s.c., B16 tumors never generated long-term cures of tumor-bearing animals. However, in the lung metastatic model, we were typically able

to obtain statistically significant, but very small, improvements in survival times with reovirus, compared with control treatments, in some experiments ($n = 5$), of which Fig. 1C is representative ($P = 0.0014$). In contrast, although some experiments generated significantly improved survival after i.v. delivery of reovirus to s.c. tumors, the more representative case ($n = 6$) was that there was no significant difference between virus and control treatments, of which Fig. 1D is representative ($P = 0.054$).

Combination of cyclophosphamide and virotherapy. Based on published studies in other models (6–8, 10, 30), we hypothesized that combination oncolytic virotherapy with cyclophosphamide would increase access of systemic virus to tumor sites. Hence, we investigated different regimens of cyclophosphamide + reovirus, as either a single low dose (150 mg/kg = 3 mg per mouse), or as a high dose (3 mg for 3 days; refs. 31–33, 35; Fig. 2A). In the model of i.v. injection of reovirus to treat B16 lung metastases, neither low-dose cyclophosphamide alone nor low-dose cyclophosphamide + reovirus showed any significant therapeutic benefits compared with PBS or heat-inactivated virus (data not shown). In
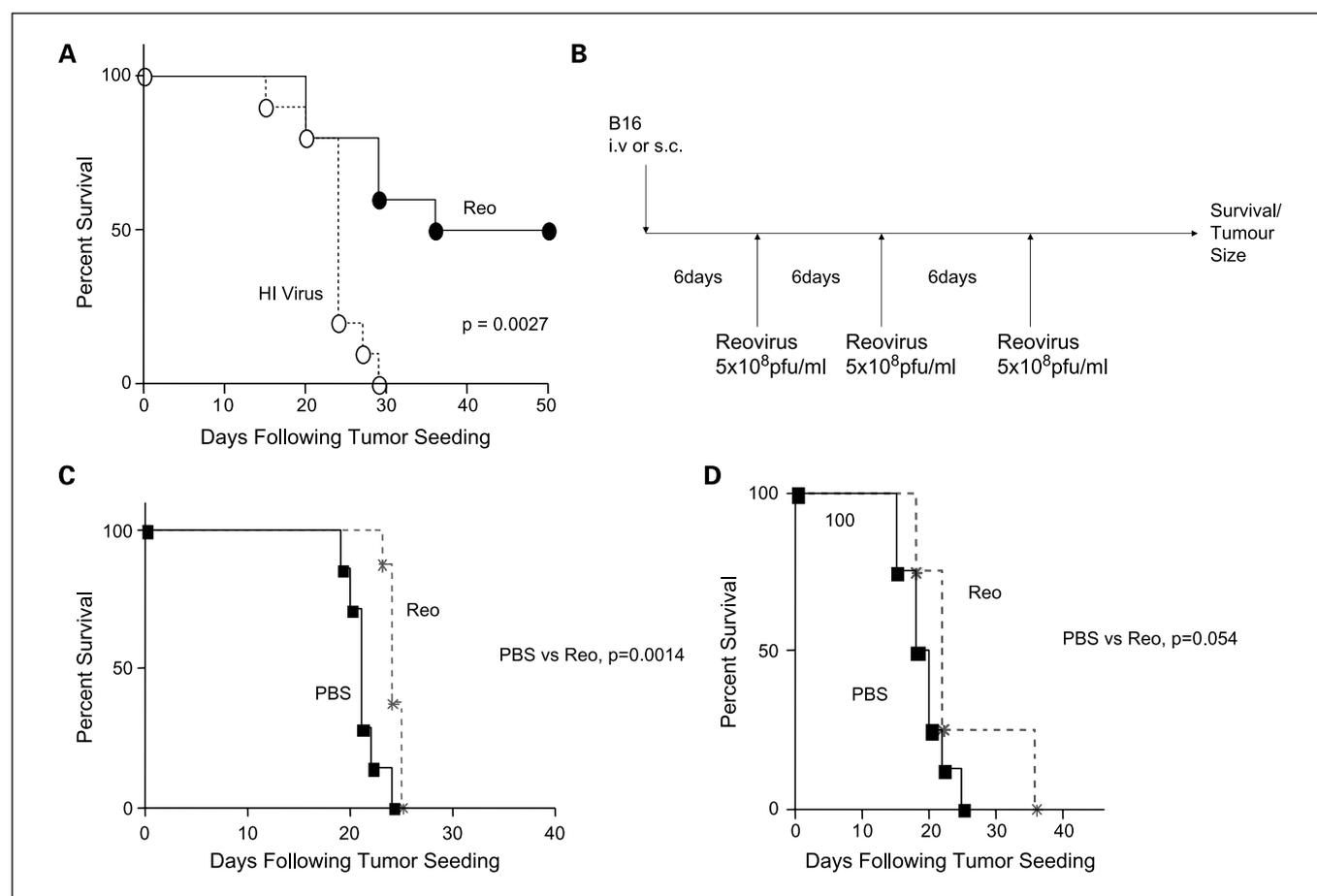

Fig. 1. Reovirus is an effective oncolytic agent against B16 melanoma. *A*, C57Bl/6 mice (*n* = 8-10 per group) were injected s.c. with 2×10^5 B16 tumor cells on day 1. At days 10, 12, and 14, tumors were injected directly with 5×10^8 pfu of reovirus or heat-inactivated virus (*HI Virus*) or PBS. Survival of mice is shown with time after tumor seeding (mice were euthanized when tumors reached a size of 1.0 cm in the longest diameter). Heat-inactivated virus was indistinguishable from PBS treatment. Data are representative of multiple experiments. *B*, systemic viral therapy: s.c. B16 tumors were established by injecting 2×10^5 B16 cells s.c., or lung metastatic tumors were established by injecting 10^5 B16 cells i.v. in C57Bl/6 mice (*n* = 7-10 per group). On days 7, 13, and 19, mice were injected i.v. with 5×10^8 pfu of reovirus, heat-inactivated reovirus, or PBS. *C*, survival of mice in the lung metastatic model is shown after treatment of tumor-bearing mice with i.v. reovirus as shown in *B* (*P* = 0.0014 reovirus compared with PBS). *D*, survival of mice bearing a s.c. B16 tumor and treated with i.v. reovirus or PBS is shown with time after tumor seeding (*P* = 0.054 reovirus compared with PBS). In both *C* and *D*, heat-inactivated virus was indistinguishable from PBS treatment.
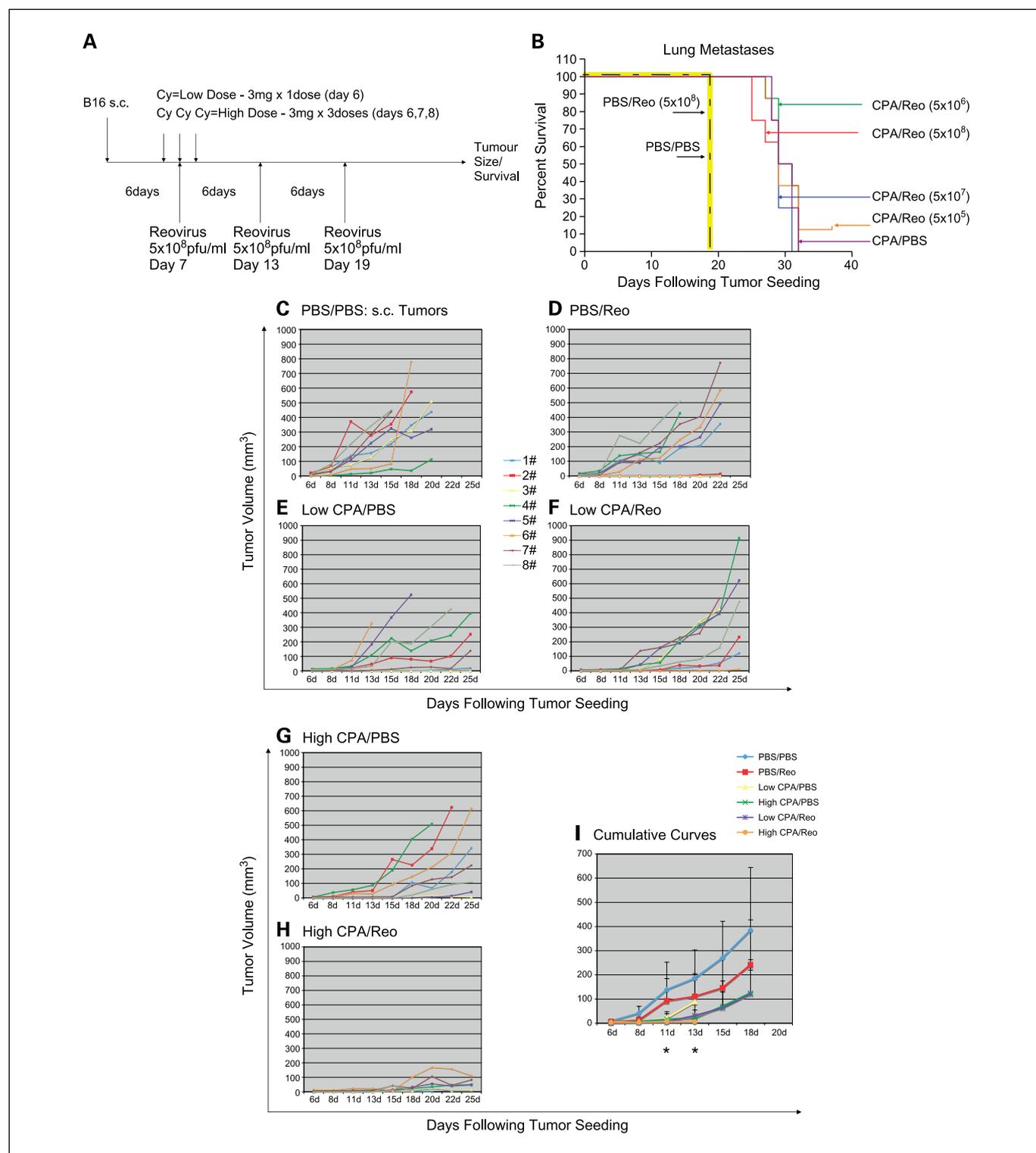
Fig. 2. High-dose cyclophosphamide enhances the antitumor efficacy of systemically delivered reovirus. *A,* the systemic delivery of reovirus was combined with either an i.p. single dose of cyclophosphamide (*Cy*) on day 6 (*Low Dose*; 150 mg/kg = 3 mg per mouse), or with three i.p. doses of 3 mg per mouse on days 6, 7, and 8 (*High Dose*) to treat either s.c. or lung metastatic tumors established as described in Fig. 1B. *B,* mice bearing established lung metastases were treated with either PBS or the high-dose cyclophosphamide (*CPA*) regimen along with three injections i.v. of either PBS or reovirus (days 7, 13, and 19) at doses of 5×10^5, 5×10^6, 5×10^7, or 5×10^8 pfu as shown (*n* = 8 per group). Survival of mice with time is shown. *C* to *H,* mice (*n* = 8) bearing established s.c. B16 tumors were treated with PBS, low-dose cyclophosphamide, or high-dose cyclophosphamide along with three i.v. injections of either PBS or 5×10^8 pfu of reovirus as indicated. Tumor size was measured with time after tumor seeding as shown. *I,* the cumulative data of *C* to *H,* also displayed with a mean measurement of tumor size and SD. Tumor growth curves of each group are terminated on the day of the first death in that group. *, significant differences exist between control groups (PBS or reovirus alone) and both low- and high-dose cyclophosphamide at day 11 (*P* = 0.02) and between PBS and high-dose cyclophosphamide from days 13 to 18; significant differences exist between the PBS group and reovirus + low-dose cyclophosphamide from day 13 (*P* = 0.035) onward until day 18 (when the first animals needed to be euthanized); tumor size in the high-dose cyclophosphamide + reovirus is significantly different from all other groups at days 11 and 13.

contrast, in the same model of lung metastases, high-dose cyclophosphamide alone showed significant chemotherapeutic efficacy over PBS treatment (Fig. 2B). However, combination of high-dose cyclophosphamide + reovirus added no additional significant benefit to treatment with cyclophosphamide alone (Fig. 2B). This held true irrespective of the dose of reovirus (from 5×10^5 to 5×10^8 pfu per injection; Fig. 2B).

In the s.c. model, i.v. reovirus did not produce statistically significant benefits compared with PBS or heat-inactivated virus (Fig. 2C and D). Treatment with either low- or high-dose cyclophosphamide alone significantly slowed tumor progression (with respect to PBS), consistent with its use as a chemotherapeutic agent (refs. 32, 35; mean tumor volumes at day 13, $P = 0.01$; Fig. 2E and G). Combination of low-dose cyclophosphamide + reovirus was statistically superior to PBS, or reovirus alone (mean tumor volumes at day 13, cyclophosphamide + reovirus compared with reovirus alone, $P = 0.035$), in slowing tumor growth, although not different from cyclophosphamide alone. However, high-dose cyclophosphamide + reovirus led to complete tumor regressions in five of eight mice and a highly significant reduction in tumor size at all time points after 8 days compared with other groups (Fig. 2H).

High-dose cyclophosphamide + reovirus leads to severe toxicities. However, by day 25 after treatment, mice in this group had to be euthanized due to severe toxicities. In several of the treated mice, tails became blackened and, in one case, detached. To further investigate the basis of these systemic toxicities, we recovered tumor and major organs from mice treated with the different regimens. Although no virus could be recovered from any body organs after i.v. delivery of three doses of 5×10^8 pfu of reovirus in normal C57Bl/6 mice, very low levels of virus were detected from s.c. tumors of two of four mice (Fig. 3A). Cotreatment with low-dose cyclophosphamide did not increase the levels of virus detected in s.c. tumor but did facilitate the recovery of virus from the heart in detectable amounts ($P < 0.001$ compared with i.v. reovirus alone; Fig. 3B), suggesting that cyclophosphamide may enhance access of virus into normal heart and/or its replication therein. In contrast, very high levels of virus were recovered from s.c. tumors from mice treated with both i.v. reovirus and high-dose cyclophosphamide (Fig. 3C). In addition, however, viral replication was also detected in several other organs and, predominantly, in the heart of these mice (Fig. 3C). These viral distribution studies were consistent with pathologic analysis of mice euthanized after treatment with high-dose cyclophosphamide + reovirus. Histopathologic analysis of all major organs indicated that the principal toxicity of the treatment was due to severe cardiotoxicity (Fig. 3D and E) associated with diffuse, severe non-suppurative myocarditis, and calcification, (Fig. 3E; Table 1). Multifocal inflammatory cellular infiltrates, with slight necrosis, were also reported in the liver (not shown). Hearts of mice treated with reovirus alone or cyclophosphamide alone (high or low dose) showed no abnormalities over control mice, showing that the toxicities observed were due to the combination of virus and cyclophosphamide.

NAb acts both to inhibit access of virus to the tumor and to protect against systemic dissemination. In our clinical trial of systemic reovirus administration to cancer patients, we observed that antireovirus NAb titers in patients without preexisting antireovirus antibody increased by 700- to 6,500-fold.[7,8] Therefore, we hypothesized that NAb to reovirus will limit antitumor efficacy in regimens where viral administrations are separated by several days. To test this in this preclinical model, we titrated NAb to i.v. delivered reovirus in C57Bl/6 mice with time. Mice injected i.v. with reovirus typically show no detectable neutralizing activity in serum until days 3 to 7. Subsequently, NAb increase sharply to plateau by day 9 where they remain stable as far out as day 60 postinjection (not shown). These data suggest that viral doses administered after 6 to 10 days after the first virus dose will encounter very high antiviral antibody titers, and so may be of little therapeutic value. Therefore, we investigated the effects of cyclophosphamide in this model on levels of NAb to the virus. Consistent with the results of the clinical trial in nonimmune patients, serum from mice treated with three consecutive injections of 5×10^8 pfu reovirus contained high levels of NAb (Table 1). In contrast, undiluted serum from mice treated with high-dose cyclophosphamide and 5×10^8 pfu reovirus contained no detectable NAb in the L929 neutralization assay (equivalent to preincubation of reovirus with medium alone; Table 1). These data suggested that both the therapeutic effects (enhancing access to the tumor by circulating virus through inhibition of NAb) and the toxicity (widespread viral dissemination in the absence of NAb) associated with high-dose cyclophosphamide + reovirus might be explained in large part by the effects of cyclophosphamide on NAb.

To test this hypothesis, the experiments of Fig. 1B (with no added cyclophosphamide) were repeated in B6.129S2-Igh-6tm1^{Cgn}/j mice, which lack mature B cells and cannot, therefore, make antiviral antibodies. B6.129S2-Igh-6tm1^{Cgn}/j mice not bearing tumor but administered reovirus i.v. did not develop any detectable toxicity. However, within 2 days of just the second virus administration all tumor-bearing mice receiving reovirus had to be euthanized because of very similar toxicities as were observed in C57BL/6 mice treated with high-dose cyclophosphamide + i.v. reovirus (Table 1). Pathologic analysis confirmed that these mice had identical cardiac toxicity. Consistent with this, the pattern of virus dissemination through the B-cell knockout mice was very similar to that observed in C57BL/6 mice treated with high-dose cyclophosphamide + i.v. reovirus (Fig. 3F and C). Thus, very high levels of reovirus were recovered from tumors from B-cell knockout mice treated with i.v. reovirus, and very significant viral titers were also detected from a variety of organs, including the heart (Fig. 3F; no detectable titers could be recovered from these organs in normal immunocompetent mice; Fig. 3A).

Metronomic dosing of virus with cyclophosphamide reduces toxicity and generates antitumor efficacy. Because of the different balances between levels of NAb, tumor clearance, intratumoral viral replication, and toxicity that we observed between the low-dose cyclophosphamide + reovirus and high-dose cyclophosphamide + reovirus treatments, we hypothesized that there may be a threshold level of NAb at which i.v. delivered virus can still access tumor upon repeat administration but at which antibody is still able to control systemic spread (and toxicity). Therefore, we altered the high-dose cyclophosphamide + reovirus protocol to a metronomic dosing regimen (32, 35) in which cyclophosphamide treatments were administered 1 day before each virus administration (Fig. 4A). This metronomic cyclophosphamide regimen alone was very
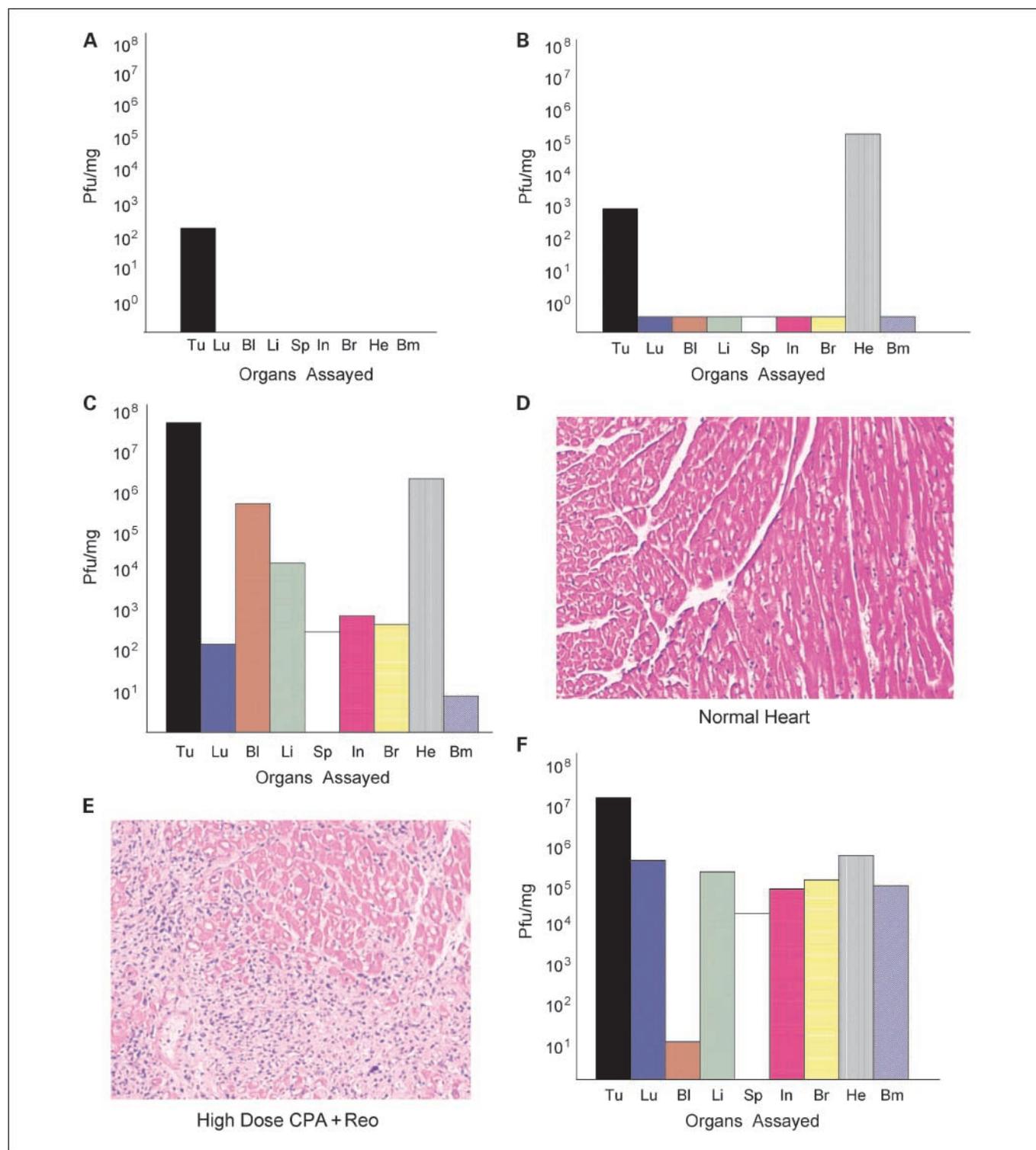
Fig. 3. Cyclophosphamide significantly alters the distribution of virus *in vivo* after i.v. reovirus delivery. C57Bl/6 mice bearing 6-d established s.c. B16 tumors were treated with (*A*) three i.v. injections of 5×10^8 pfu of reovirus (as described in Fig. 1B); (*B*) with low-dose cyclophosphamide + three i.v. injections of 5×10^8 pfu of reovirus (as described in Fig. 2A); or (*C*) with high-dose cyclophosphamide + three i.v. injections of 5×10^8 pfu of reovirus (Fig. 2A). Upon euthanasia due to either tumor size (*A* and *B*) or systemic toxicity (*C*), tumor (*Tu*) and organs [lungs (*Lu*), blood (*Bl*), liver (*Li*), spleen (*Sp*), intestines (*In*), brain (*Br*), heart (*He*), and bone marrow (*Bm*)] were harvested, weighed, and lysed by freeze thawing. Virus titers recovered were determined on L929 cells and are shown as pfu of reovirus per milligram of tissue. Results represent data from one mouse in each group and are representative of viral titers from two to four mice per group. *D,* hearts harvested from mice treated as in *A* (i.v. reovirus) showed normal architecture and no pathologic abnormalities. *E,* in contrast, hearts harvested from mice treated as in *C* (high-dose cyclophosphamide + i.v. reovirus) showed widespread and diffuse abnormalities throughout the heart (see text). *F,* B6.129S2-*Igh-6tm1^Cgn^*/j mice (Jackson 002288), which lack mature B cells and cannot, therefore, make antiviral antibodies, bearing 6-d established s.c. B16 tumors were treated with two i.v. injections of 5×10^8 pfu of reovirus at days 7 and 13 (see Fig. 1B). Two days after the second i.v. virus injection, mice had to be euthanized due to toxicity. Upon euthanasia, tumor and organs (lungs, blood, liver, spleen, intestines, brain, heart, and bone marrow) were harvested, weighed, and lysed by freeze thawing. Virus titers recovered were determined on L929 cells and are shown as pfu of reovirus per milligram of tissue. Results shown represent data from one mouse and are representative of viral titers from four different mice.

Table 1. NAb response and systemic toxicity of i.v. reovirus in combination with different cyclophosphamide regimens

Serum from mice treated with:	Range of neutralizing titer	Toxicities observed
PBS	None*	NDT
Reo	>1:1,280	NDT [†]
Reo/low CPA	1:160-1:320	NDT [‡]
Reo/high CPA	None*	Lethargic; thin; difficulty in breathing; fluid build up s.c. and in organs; found dead; swollen tails; tail detached; severe myocarditis/calcification
Reo/metro CPA	1:40-1:80	No gain of body weight

NOTE: C57Bl/6 mice bearing 6-d established s.c. B16 tumors were treated with PBS; three i.v. injections of 5×10^8 pfu of reovirus (Reo; as described in Fig. 1B); with low-dose cyclophosphamide + three i.v. injections of 5×10^8 pfu of reovirus (Reo/low CPA; as described in Fig. 2A); with high-dose cyclophosphamide + three i.v. injections of 5×10^8 pfu of reovirus (Reo/high CPA; Fig. 2A); or with metronomic dosing of reovirus + cyclophosphamide (Reo/metro CPA; Fig. 4A). NAb was measured from the serum of treated mice as described in Materials and Methods upon euthanasia of the mice due to either systemic toxicity or tumor size. The ranges of typical levels of NAb are shown to indicate any difference between mice. The major toxicities observed in different treated groups are described; not every mouse treated necessarily developed each of the symptoms.
Abbreviations: NDT, no detectable toxicity; CPA, cyclophosphamide; Reo, reovirus.
*Preincubation of serum with reovirus was not able to reduce the toxicity to L929 cells to any greater extent than DMEM.
[†] No detectable toxicity was observed upon three doses of 5×10^8 pfu of reovirus administered 6 d apart; however, when three doses of 1.1×10^{10} pfu were administered 6 d apart, one of nine mice developed severe toxicity including loss of weight and a swollen and red tail that eventually became detached.
[‡] No detectable toxicity was observed upon three doses of 5×10^8 pfu of reovirus administered 6 d apart; however, when three doses of 1.1×10^{10} pfu were administered 6 d apart, nine of nine mice developed lethargy and chills; several mice had edema with organs full of fluid and one of nine was found dead.

effective against lung metastases; three injections of cyclophosphamide separated by 6 days each cured all the mice of disease and the addition of i.v. reovirus was not, therefore, able to add efficacy (not shown). However, in the s.c. tumor model, the metronomic dosing of cyclophosphamide + reovirus generated significantly increased therapy relative to either reovirus alone or cyclophosphamide alone ($P < 0.02$) along with no obvious systemic toxicity and no animals requiring euthanasia due to treatment-related toxicity [Fig. 4B; 3(cyclophosphamide + reovirus)]. The experiment of Fig. 4B was repeated using changes in body weights as an additional objective measure of systemic toxicity in these treatment groups. Mice treated with PBS alone, or with PBS and reovirus, showed a gradual gain of body weight over the 20- to 30-day period after initiation of treatment, at which time they needed to be euthanized due to tumor growth (Fig. 4C and D). Mice treated with metronomic cyclophosphamide alone did not gain weight as in the control groups but only lost weight toward the end of the experiment at which time tumor burdens were large (Fig. 4E). Finally, mice treated with metronomic cyclophosphamide + reovirus also maintained body weights with a single animal in this experiment losing weight sharply at the end of the experiment coincident with tumor growth and euthanasia due to unacceptable tumor burden (Fig. 4F). Consistent with these data, although the metronomic regimen of cyclophosphamide + reovirus still achieved high levels of access of the circulating virus to the tumor, and subsequent replication within it at levels of $\sim 10^7$ pfu/mg (Fig. 4G), much less virus was recovered from the heart ($\sim 10^3$ pfu/mg) compared with the toxic levels in that organ generated by the high-dose cyclophosphamide + reovirus regimen ($\sim 10^7$ pfu/mg; Fig. 3C). Finally, consistent with these lower levels of virus detected in the hearts of the mice treated with metronomic cyclophosphamide + reovirus,

pathology reports indicated that most of the heart tissue was normal with some mice showing an isolated area of mild myocarditis (Fig. 4H). Significantly, serum from mice treated with the metronomic cyclophosphamide + reovirus regimen typically contained low but readily detectable NAb levels (Table 1). These data show that metronomic dosing of cyclophosphamide with reovirus *in vivo* preserves low, but significant, levels of NAb to the virus compared with the high-dose cyclophosphamide + reovirus regimen that essentially ablates NAb (Table 1). Therefore, this modified high-dose reovirus/cyclophosphamide regimen (*a*) permits therapeutically high levels of virus to access the tumors; (*b*) is significantly less toxic than the previous high-dose regimen; and (*c*) is associated with significantly lower levels of virus in the heart that is probably correlated with preservation of low, but detectable, titers of systemic NAb.

Discussion

We show here, for the first time to our knowledge, that coadministration of cyclophosphamide with i.v. delivery of oncolytic reovirus leads to high levels of viral replication within established s.c. tumors in a fully immunocompetent host. When used in our high-dose regimen, significant antitumor effects were observed, which were not seen when the virus was administered by itself. However, this protocol also induced very severe toxicities consistent with systemic distribution of virus leading to replication in a series of normal organs. There were very close similarities between both B-cell knockout mice treated with i.v. reovirus and mice treated with high-dose cyclophosphamide + reovirus. These included the absence of detectable NAb induced by either lack of B cells or high-dose cyclophosphamide; the levels of virus that reached, and

subsequently replicated within, the s.c. tumors in both models; the systemic, cardiac-based toxicities in both sets of mice and the similar levels of virus recovered from systemic organs.

By modulating the timing of cyclophosphamide and virus administration to a series of iterative injections (metronomic dosing) of 3 mg once every 6 days (31–33, 35), significant antitumor efficacy against s.c. tumor could be retained while substantially ameliorating the systemic toxicity of the high-dose cyclophosphamide + reovirus regimen. Importantly, metronomic dosing with cyclophosphamide induced an incomplete depression of NAb levels—in contrast to high-dose cyclophosphamide that ablated detectable NAb over several weeks. Taken together, these data suggest that both the

therapeutic effects and the toxicity associated with high-dose cyclophosphamide + reovirus might be explained in part by the effects of cyclophosphamide on modulating levels of NAb. Thus, in the fully immunocompetent mouse, NAb prevent repeat administrations of virus from accessing the tumor, thereby blocking antitumor efficacy. However, in the NAb-depleted mouse (B-cell knockout or high-dose cyclophosphamide-treated), these same NAb are no longer available to neutralize large amounts of virus that are released from the tumor, which now acts as a source of ongoing virus production and dissemination throughout the body. In the metronomic schedule of cyclophosphamide + reovirus, high levels of systemic virus were still able to reach, and replicate within, s.c. tumors ($\sim 2.1 \times 10^7$ pfu/mg tumor). However,
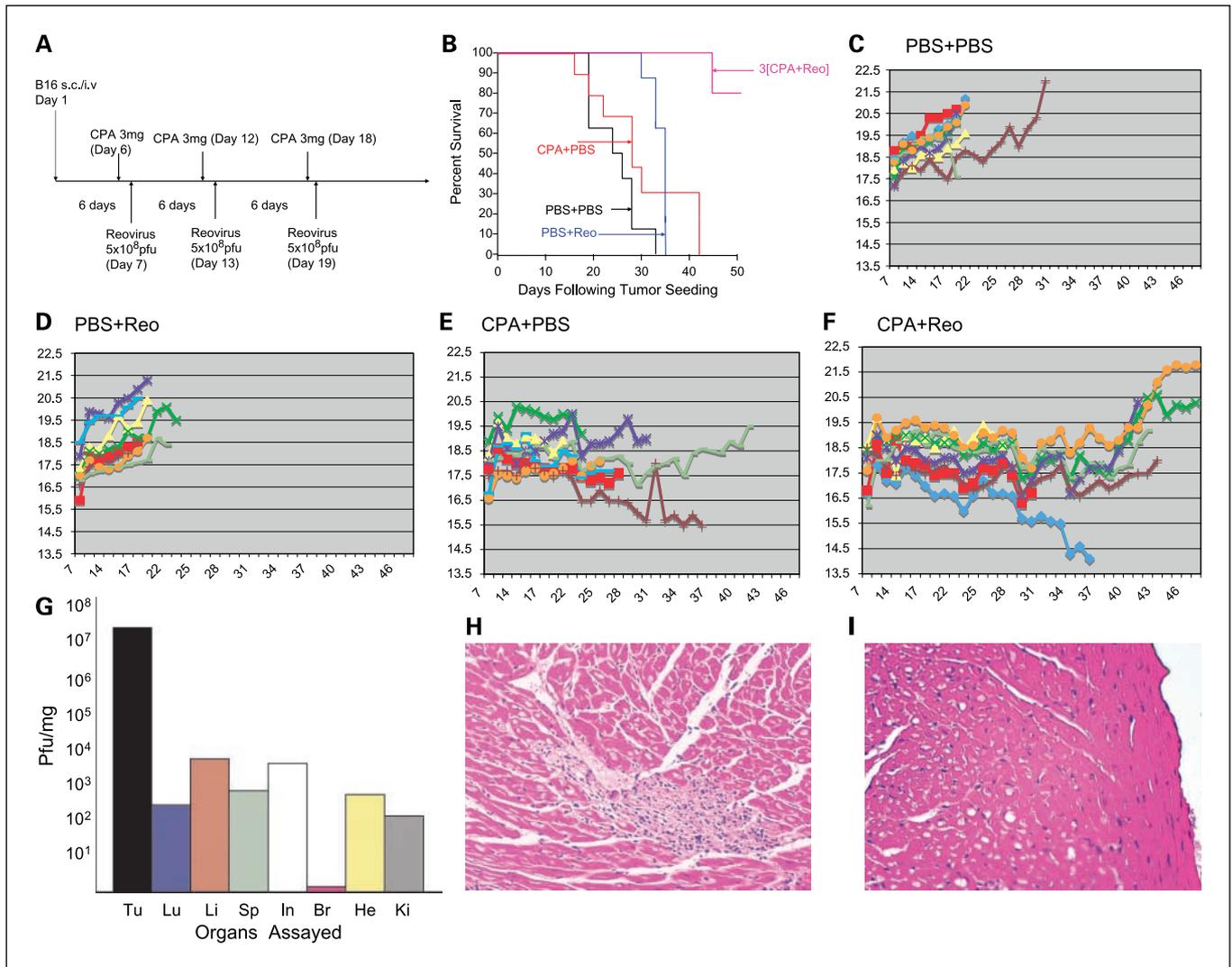

Fig. 4. Metronomic dosing of cyclophosphamide and reovirus balances antitumor efficacy and systemic toxicity. *A,* the timing of cyclophosphamide injections was modified relative to that in Fig. 2A, to precede each of the three injections of reovirus by 24 h. *B,* s.c. tumor bearing C57Bl/6 mice (8-10 per group) were treated according to the metronomic dosing regimen of *A* with three injections of PBS (days 6, 12, and 18) + 5 × 10⁸ pfu of reovirus (days 7, 13, and 19); or with similar regimens of PBS/PBS; cyclophosphamide/PBS; or with cyclophosphamide/reovirus (as shown). Survival of mice in each group is shown with time with euthanasia being required only due to tumor size rather than treatment-associated toxicity. *C* to *F,* the treatment regimen of *B* above was repeated in additional groups of mice and systemic toxicity was assessed through changes of body weight as shown. *G,* s.c. tumor-bearing C57Bl/6 mice were treated according to the metronomic dosing regimen of *A* with three injections of cyclophosphamide (days 6, 12, and 18) + 5 × 10⁸ pfu of reovirus (days 7, 13, and 19) 50 d after tumor seeding, tumor and organs (lungs, liver, spleen, intestines, brain, heart, and kidney) were harvested, weighed, and lysed by freeze thawing. Virus titers recovered were determined on L929 cells and are shown as pfu of reovirus per milligram of tissue. *H* and *I,* hearts harvested from mice treated as in *G* with metronomic cyclophosphamide alone (*I*) showed normal pathology; those harvested from mice treated with metronomic cyclophosphamide + i.v. reovirus (*H*) showed predominantly normal pathology but with sporadic and isolated areas of myocarditis.

sufficient levels of NAb were also retained within the mouse to capture virus emerging from the tumor, thereby restricting the amount of virus that could access, and replicate within, other systemic organs such as the heart (3×10^3 pfu/mg).

Throughout our studies, we observed significant differences between treatment of s.c. and lung tumors in the C57Bl/6 mice. For example, high-dose cyclophosphamide alone had significant therapeutic efficacy against lung metastases that i.v. reovirus was unable to augment (Fig. 2B). However, the addition of i.v. reovirus in this protocol did not add significant levels of toxicity. Both low- and high-dose cyclophosphamide alone had significant therapeutic effects against s.c. B16 tumors (at day 11 for low-dose cyclophosphamide and from days 11-18 for high-dose cyclophosphamide; Fig. 2G and I); however, combination with i.v. reovirus had dramatic antitumor effects in combination with high-dose cyclophosphamide (Fig. 2H and I), consistent with very high levels of virus reaching, and replicating in, the tumor (Fig. 3C). Similarly, metronomic cyclophosphamide alone was a very effective chemotherapy against metastatic lung B16 tumors but had only moderate effects against s.c. tumor (Fig. 4B). However, once again, addition of i.v. reovirus significantly improved therapy of these s.c. tumors relative to either cyclophosphamide or virus alone (Fig. 4B), again consistent with high levels of virus replication in the tumor (Fig. 4G) but this time without dramatic toxicity represented by high levels of virus replication in other organs (Fig. 4G and H).

These data are also consistent with the hypothesis that cyclophosphamide enhances the ability of systemically delivered virus to access tumors, at least in part by its effects on modifying levels of NAb that would otherwise block repeated administrations from reaching the tumor. In the case of lung metastases, the chemotherapeutic effects of cyclophosphamide alone are probably sufficient to reduce the tumor burden in the lungs so that viral replication within the tumors there is likely to be relatively modest compared with that from s.c. tumors. Indeed, we could recover no more than $\sim 10^4$ pfu of reovirus/mg of lung tissue from mice bearing B16 tumors treated with metronomic cyclophosphamide + reovirus. This level of virus production probably does not serve as an effective source of virus for further systemic distribution through the animal, hence the lack of systemic toxicity associated with high-dose cyclophosphamide + reovirus in the lung metastatic model (Fig. 2B). In contrast, because cyclophosphamide enhances the levels of virus that can access the s.c. tumors, without reducing the tumor load to great extents, these s.c. tumors can then serve as very effective viral factories, generating up to 10^8 pfu of reovirus per milligram of tumor tissue (Fig. 3C). We believe that this viral production then serves as the source of extensive systemic distribution of virus to other organs, including the heart (Fig. 3E). In the presence of cyclophosphamide, these organs may also be much more susceptible to viral entry and possible replication, probably because of the multifaceted effects that cyclophosphamide exerts on the vasculature, and on innate and adaptive immune effectors, which would otherwise provide very effective barriers to infection and replication of the virus in normal, nontumor organs (6–10, 29, 30). Significantly, we only observed the dramatic toxicity associated with high-dose cyclophosphamide + reovirus in mice that have a s.c. tumor, supporting the hypothesis that it is tumor-derived virus that

serves as the source of the disseminating virus that causes systemic toxicity as opposed to the input dose of virus.

Indeed, the toxicities we observed in immunocompetent, tumor-bearing mice treated with high-dose cyclophosphamide, and in tumor-bearing B-cell knockout mice, were associated with dramatic manifestations, including heart failure and the "black foot syndrome," both of which have been reported previously in SCID or SCID/NOD mice treated with reovirus intratumorally (48–50). Black foot syndrome was described as the discoloration and necrosis of feet, tails, distal legs, and ears in SCID/NOD mice several weeks after injection of reovirus intratumorally (48), again suggesting that virus replicating within the tumor serves as a source for systemic dissemination (49). The pathogenesis of black foot syndrome was characterized as due to venous vasculitis secondary to reovirus infection along with reovirus-induced myocarditis and heart failure (48, 50) and typically, these symptoms developed weeks or months after the reovirus therapy into the tumor (48, 49). We observed a variation of this syndrome in which tails of cyclophosphamide-treated mice became swollen, very sensitive, and, in a minority of cases, detached from the animal. This is the first time, to our knowledge, that black foot syndrome has been observed in wild-type mice; moreover, onset of both reovirus-induced myocarditis and black foot syndrome was more rapid under the influence of cyclophosphamide treatment than has been reported in SCID or SCID/NOD mice (48, 49), indicating that cyclophosphamide is having multiple effects *in vivo*, which can resemble the induction of a severely immunocompromised state.

In this respect, it seems highly likely that activities of cyclophosphamide, in addition to the suppression of NAb to the virus, are important to facilitate the high levels of intratumoral viral replication, systemic survival, and access to both tumor and normal tissues that we observed. In particular, we are currently investigating what other effects cyclophosphamide exerts in our studies through suppression of both innate immune responses (natural killer cell, macrophages, and IFN-γ), which may inhibit intravascular delivery, and intratumoral replication, of virus (6–10, 29, 30), as well as through effects on adaptive T-cell responses (34–41).

Significantly, the metronomic dosing regimen induced only a few isolated areas of mild myocarditis, which was not seen in control animals receiving either reovirus or cyclophosphamide alone, but which did not seem to manifest in any detectable clinical way in these mice. The potential risk of development of mild myocarditis, or calcifications, during combination therapy with reovirus plus cyclophosphamide will remain unclear until clinical experience of this approach accrues. However, existing clinical data suggest that cardiac effects of i.v. reovirus do not represent a significant problem. In our recently completed phase I clinical trial, reovirus was administered by i.v. infusion at doses between 1×10^8 on day 1, to 3×10^{10} on days 1 to 5 of a 4-weekly cycle to 33 patients with advanced cancers.[7] Careful assessment of the cardiac effects of i.v. reovirus administration formed part of the design of this phase I trial. Patients underwent electrocardiogram, radioisotopic multiple uptake gated acquisition scan, and creatine kinase MB isoenzyme screening and troponin I estimation at baseline. Electrocardiogram and creatine kinase MB isoenzyme and troponin I were repeated on the days of reovirus administration and weekly between cycles. Multiple uptake gated

acquisition was repeated every two cycles. In regard to cardiac toxic events, a single patient treated at the 1×10^{10} dose level experienced grade 3 elevation in creatine kinase MB isoenzyme and troponin I on day 3 of the first treatment cycle. At this time, the patient was found to have a normal electrocardiogram and echocardiogram and all biochemical disturbance resolved to normal at day 15. The patient was subsequently rechallenged at the 3×10^{9} dose level with no further sequelae. No other cardiac event was seen in the study. The preclinical data derived from the current murine studies and the existing clinical data provide a clear framework for cardiac monitoring in future trials of reovirus in combination with cyclophosphamide. Such safety assessments will be a key determinant in decisions regarding dose escalations between patient cohorts.

In summary, we have shown that cyclophosphamide can be used to facilitate the intratumoral access, and replication, of an oncolytic virus to s.c. tumors when delivered i.v. in a fully immunocompetent host. The balance between intratumoral virus replication facilitated by cyclophosphamide and systemic toxicity generated by viral spread to normal organs can be manipulated by instigating a metronomic dosing of virus and cyclophosphamide, whereas this allowed sufficient depression of the levels of NAb to the virus to maintain high levels of intratumoral virus access/replication, it also maintained sufficient levels of NAb to neutralize virus released from the tumors to prevent access of high levels of virus to normal organs. These data support the development of clinical protocols in which cyclophosphamide is combined with systemically administered oncolytic viruses. Such studies will necessitate careful analysis of toxicity (especially cardiac) end points and retreatment and dose-escalation decisions will need to be based on real-time analysis of the levels of NAb in the patients' circulation. Under these circumstances, it should be possible to test the clinical hypothesis that judicious combination of cyclophosphamide and reovirus will enhance systemic delivery of the virus to the tumor but retain the ability of low-level NAb responses to protect the patient against toxicity induced by systemic dissemination of the virus.

Acknowledgments

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