# A Phase I Study of Intravenous Oncolytic Reovirus Type 3 Dearing in Patients with Advanced Cancer

Laura Vidal,[1,2] Hardev S. Pandha,[3] Timothy A. Yap,[1] Christine L. White,[2] Katie Twigger,[1] Richard G. Vile,[4,5] Alan Melcher,[5] Matt Coffey,[6] Kevin J. Harrington,[1,2] and Johann S. DeBono[1,2]

**Abstract**

**Purpose:** To determine the safety and feasibility of daily i.v. administration of wild-type oncolytic reovirus (type 3 Dearing) to patients with advanced cancer, assess viral excretion kinetics and antiviral immune responses, identify tumor localization and replication, and describe antitumor activity.

**Experimental Design:** Patients received escalating doses of reovirus up to $3 \times 10^{10}$ $TCID_{50}$ for 5 consecutive days every 4 weeks. Viral excretion was assessed by reverse transcription-PCR and antibody response by cytotoxicity neutralization assay. Pretreatment and post-treatment tumor biopsies were obtained to measure viral uptake and replication.

**Results:** Thirty-three patients received 76 courses of reovirus from $1 \times 10^8$ for 1 day up to $3 \times 10^{10}$ $TCID_{50}$ for 5 days, repeated every four weeks. Dose-limiting toxicity was not seen. Common grade 1 to 2 toxicities included fever, fatigue, and headache, which were dose and cycle independent. Viral excretion at day 15 was not detected by reverse transcription-PCR at 25 cycles and only in 5 patients at 35 cycles. Neutralizing antibodies were detected in all patients and peaked at 4 weeks. Viral localization and replication in tumor biopsies were confirmed in 3 patients. Antitumor activity was seen by radiologic and tumor marker (carcinoembryonic antigen, CA19.9, and prostate-specific antigen) evaluation.

**Conclusions:** Oncolytic reovirus can be safely and repeatedly administered by i.v. injection at doses up to $3 \times 10^{10}$ $TCID_{50}$ for 5 days every 4 weeks without evidence of severe toxicities. Productive reoviral infection of metastatic tumor deposits was confirmed. Reovirus is a safe agent that warrants further evaluation in phase II studies.

There is accumulating evidence for the potential clinical utility of oncolytic viruses capable of tumor-selective replication and cytolysis. Reovirus type 3 Dearing (Reolysin; Oncolytics Biotech), a wild-type member of the Reoviridae family, is nonpathogenic in humans, and infections are usually asymptomatic or associated with only minor respiratory/enteric symptoms (1). Reovirus is ubiquitous as witnessed by the high seropositivity rate in normal adult populations (2–4). Reovirus was initially shown to be oncolytic (tumor-selective) by virtue of its ability to replicate selectively in transformed, but not normal, cells (5). NIH-3T3 cells are naturally resistant to reovirus infection but become highly permissive when transformed with the epidermal growth factor receptor gene, *v-erbB*, *sos*, or *ras*, which are all activators of Ras signaling (6, 7). In normal cells, reovirus infection causes phosphorylation of cellular PKR (dsRNA-activated protein kinase) and arrest of viral protein translation and replication. In contrast, in Ras-activated cells, PKR remains inactive (unphosphorylated) and is incapable of aborting viral translation, replication, and cytolysis (7, 8). Ras-activating mutations promote tumor growth, angiogenesis, and metastasis and are found in ~30% of human cancers. However, two-thirds of cancers may be susceptible to reovirus therapy through Ras pathway targeting by virtue of activation or overexpression of elements upstream or downstream of Ras, such as epidermal growth factor receptor or platelet-derived growth factor receptor (9).

Reovirus therapy has yielded complete tumor regressions in murine models after intratumoral and systemic delivery (8, 10, 11). Reovirus was initially evaluated in humans via the intratumoral route (12). Toxicities were mild and consisted of transient flu-like symptoms and headache. As in the murine models, tumor regressions were observed in these studies in both injected and adjacent uninjected lesions.

This phase I, open-label, dose-escalation study of i.v. reovirus was conducted across two centers in the United Kingdom. The primary objective was to determine the safety and tolerability of i.v. reovirus and thereby to define the maximum tolerated dose

**Authors' Affiliations:** [1]The Royal Marsden NHS Foundation Trust, London and Sutton, United Kingdom; [2]The Institute of Cancer Research, London, United Kingdom; [3]University of Surrey, Guildford, United Kingdom; [4]Molecular Medicine Program, Mayo Clinic, Rochester, Minnesota; [5]University of Leeds, Leeds, United Kingdom; and [6]Oncolytics Biotech, Inc., Calgary, Alberta, Canada

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K.J. Harrington and J.S. DeBono contributed equally to this work.

**Requests for reprints:** Kevin J. Harrington, The Institute of Cancer Research, Cancer Research UK Center for Cell and Molecular Biology, Chester Beatty Laboratories, 237 Fulham Road, London SW3 6JB, United Kingdom. Phone: 44-207-808-2732; Fax: 44-207-808-2235; E-mail: Kevin.Harrington@icr.ac.uk.



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## Translational Relevance

There is significant interest in the development of oncolytic viruses for use as cancer therapeutics either alone or in combination with conventional modalities. In this article, we describe a first-in-man dose-escalation phase I trial of reovirus type 3 Dearing given by i.v. administration to patients with advanced cancers. The data reported here strongly support the future development of this agent for clinical use. Reovirus was safe and well tolerated, and DLT was not encountered. The recommended phase II dose for further study was $3 \times 10^{10}$ $TCID_{50}$ for days 1 to 5 every 4 weeks. Pharmacokinetic analyses confirmed the environmental biosafety of this agent with no evidence of viral shedding. As such, this agent is suitable for outpatient administration. Post-treatment biopsy specimens showed viral localization in disseminated tumor deposits after i.v. administration, the first time this has been reported for an oncolytic virus. However, we also showed that the main potential obstacle to clinical use of this agent will be the occurrence of significant neutralizing antireoviral antibody titers. Phase I/II studies of reovirus plus cytotoxic chemotherapy and immunomodulatory agents are currently open to recruitment.

(MTD). Secondary objectives included analysis of viral biodistribution, antiviral immune responses, and antitumor activity.

## Patients and Methods

Eligible patients with histologically or cytologically confirmed advanced-stage primary or metastatic solid tumors that were refractory to standard therapy were enrolled. Acute toxic effects of prior radiotherapy, chemotherapy, or surgical procedures had to have resolved to Common Terminology Criteria for Adverse Events (version 3.0) grade ≤1, with any surgery occurring ≥28 days before study enrollment. Patients were ages ≥18 years; using adequate birth control; had a life expectancy of >3 months; had measurable or assessable disease; an Eastern Cooperative Oncology Group performance status of 0 to 2; cardiac ejection fraction of ≥50% by multiple-gated acquisition scan; and adequate hepatic, renal, and bone marrow function (aspartate transaminase/alanine transaminase ≤2.5 × institutional upper limit of normal; total bilirubin ≤1.5 × institutional upper limit of normal; serum creatinine ≤1.5 × institutional upper limit of normal; hemoglobin ≥9.0 mg/dL; absolute neutrophil count ≥1,500/μL; platelet count ≥100,000/μL). Exclusion criteria included known brain metastases, pregnancy or breast-feeding, concurrent immunosuppressive therapy, known HIV and hepatitis B or C infections, clinically significant cardiac disease (New York Hearth Association class III or IV), and

**Table 2.** Patient demographics, tumor diagnoses, performance status, and prior therapies

| Patient characteristics | No. patients |
|---|---|
| Total no. | 33 |
| Age (y) | |
| Median | 59.5 |
| Range | 32-80 |
| Male/female | 23/10 |
| Primary tumor site | |
| Head and neck | 5 |
| Prostate | 5 |
| Colorectal | 5 |
| Pancreas | 3 |
| Upper gastrointestinal | 3 |
| Melanoma | 3 |
| Soft-tissue sarcoma | 3 |
| Bladder | 2 |
| Non-small cell lung cancer | 2 |
| Renal | 1 |
| Endometrial | 1 |
| Eastern Cooperative Oncology Group performance status | |
| 0 | 15 |
| 1 | 17 |
| 2 | 1 |
| No. prior chemotherapy regimens | |
| 0 | 6 |
| 1 | 9 |
| 2 | 8 |
| ≥3 | 10 |
| No. prior hormonal regimens | |
| 0 | 27 |
| 1 | 0 |
| 2 | 1 |
| ≥3 | 5 |
| No. prior immunotherapy regimens | |
| 0 | 31 |
| 1 | 2 |
| No. prior investigational agents | |
| 0 | 32 |
| 1 | 1 |
| Prior radiation therapy | 19 |
| Prior surgery | 22 |

**Table 1.** Dose-escalation scheme and the number of patients treated in each cohort

| Cohort no. | Dose ($TCID_{50}$) | Days of treatment per cycle | No. patients per cohort | Patient nos. |
|---|---|---|---|---|
| 1 | $1 \times 10^8$ | 1 | 3 | RL01-RL03 |
| 2 | $1 \times 10^8$ | 3 | 3 | RL04-RL06 |
| 3 | $1 \times 10^8$ | 5 | 4 | RL07-RL10 |
| 4 | $3 \times 10^8$ | 5 | 3 | RL11-RL13 |
| 5 | $1 \times 10^9$ | 5 | 3 | RL14-RL16 |
| 6 | $3 \times 10^9$ | 5 | 3 | RL17-RL19 |
| 7 | $1 \times 10^{10}$ | 5 | 6 | RL20-RL25 |
| 8 | $3 \times 10^{10}$ | 5 | 8 | RL26-RL33 |

NOTE: The duration of each treatment cycles was 4 wk. Patients are identified by study numbers RL01 to RL33 and their recruitment to individual dose cohorts is indicated. No attempt was made to escalate the dose beyond the $3 \times 10^{10}$ dose level, because this was considered to be the manufacturing limit of the virus.

**Table 3.** Details of primary tumor diagnoses and prior treatments for each individual

| Patient | Primary diagnosis | Surgery | Radiotherapy | Chemotherapy |
|---|---|---|---|---|
| RL01 | Transitional cell carcinoma (bladder) | 1. Transurethral resection of bladder tumor<br>2. Cystoprostatectomy | 1. Left neck (40 Gy) [P]<br>2. Lumbar spine [P] | Cisplatin (4 cycles) [A] |
| RL02 | Squamous cell carcinoma (head and neck) | | Oropharynx (70 Gy) and neck (bilateral) (60 Gy) [R] | 1. Cisplatin/5-fluorouracil (2 cycles) [C]<br>2. Cisplatin/5-fluorouracil (3 cycles) [P] |
| RL03 | Squamous cell carcinoma (head and neck) | 1. Neck dissection<br>2. Total laryngectomy | 1. Larynx (65 Gy) [R]<br>2. Trachea (20 Gy) [P] | Cisplatin*/5-fluorouracil/ gefitinib (2 cycles) [N] |
| RL04 | Adenoid cystic carcinoma (head and neck) | Partial maxillectomy | 1. Maxilla (62 Gy) [A] | |
| RL05 | Adenocarcinoma (lung) | | | 1. Docetaxel (4 cycles) [P]<br>2. Gefitinib (3 cycles) [P]<br>3. MVCarbo (4 cycles) [P] |
| RL06 | Sarcoma | 1. Partial pneumonectomy<br>2. Lung metastatectomy | Lung (20 Gy) [P] | 1. Doxorubicin/ifosfamide (6 cycles) [A]<br>2. ET-743 (4 cycles) [P]<br>3. BIBF (6 cycles) [P] |
| RL07 | Adenocarcinoma (pancreas) | | | |
| RL08 | Adenocarcinoma (prostate) | Transurethral resection of prostate (×4) | Pelvis (24 Gy) [P] | 1. Cyclophosphamide (6 cycles) [P]<br>2. Capecitabine (9 cycles) [P] |
| RL09 | Clear cell carcinoma (renal) | 1. Radical nephrectomy<br>2. Surgical bone fixation (×2) | Right leg (dose unknown) [P]<br>Left leg (dose unknown [P]) | Cyclophosphamide (6 cycles) [P] |
| RL10 | Squamous cell carcinoma (head and neck) | Neck dissection | Neck (50 Gy) [A] | Cisplatin/5-fluorouracil (4 cycles) [P] |
| RL11 | Adenocarcinoma (head and neck) | 1. Partial maxillectomy<br>2. Lung metastatectomy | Paranasal sinus (50 Gy) [A] | |
| RL12 | Adenocarcinoma (colon) | 1. Defunctioning colostomy<br>2. Hartmann's procedure | Pelvis (45 Gy) [A] | 1. Oxaliplatin/capecitabine (4 cycles) [N]<br>2. Irinotecan (4 cycles) [P]<br>3. Mitomycin/capecitabine (4 cycles) [P]<br>4. Capecitabine (4 cycles) [P] |
| RL13 | Melanoma (skin) | Wide local excision of skin and sentinel lymph node biopsy | | |
| RL14 | Adenocarcinoma (pancreas) | 1. Palliative bypass<br>2. Small bowel resection | | Gemcitabine (6 cycles) [P] |
| RL15 | Transitional cell carcinoma (bladder) | 1. Cystoureterectomy<br>2. Ureterectomy<br>3. Inguinal block dissection | | 1. MVAC (4 cycles) [N]<br>2. Gemcitabine/carboplatin (6 cycles) [P]<br>3. MVAC (3 cycles) [P] |
| RL16 | Melanoma (ocular) | | Eye (dose unknown) [R] | 1. Gemcitabine/treosulphan (3 cycles) [P]<br>2. ES-285 (2 cycles) [P] |
| RL17 | Adenocarcinoma (colon) | | | 1. Oxaliplatin/5-fluorouracil (12 cycles) [P]<br>2. Irinotecan (6 cycles) [P]<br>3. ZK304709 (4 cycles) [P] |
| RL18 | Adenocarcinoma (stomach) | Partial gastrectomy | | 1. ECF (6 cycles) [P]<br>2. Mito/Carbo/Cape (2 cycles) [P] |
| RL19 | Adenocarcinoma (prostate) | | Prostate (dose unknown) [R] | Cyclophosphamide (6 cycles) [P] |
| RL20 | Melanoma (skin) | 1. Wide local excision of skin | | 1. Mitomycin (cycles unknown) |

(*Continued on the following page*)

**Table 3.** Details of primary tumor diagnoses and prior treatments for each individual (Cont'd)

| Patient | Primary diagnosis | Surgery | Radiotherapy | Chemotherapy |
|---|---|---|---|---|
| | | 2. Pancreaticoduodenectomy<br>3. Transurethral resection of bladder tumor (×4) | | 2. Gemcitabine (cycles unknown) |
| RL21 | Adenocarcinoma (colon) | Hemicolectomy | | 1. Oxaliplatin/ 5-fluorouracil (14 cycles) [P]<br>2. Irinotecan (cycles unknown) [P]<br>3. Irinotecan/cetuximab (3 cycles) [P] |
| RL22 | Melanoma (skin) | Wide local excision of skin (×4) | | |
| RL23 | Adenocarcinoma (prostate) | | Thoracic spine (dose unknown)<br>Left femur (dose unknown)<br>Prostate (20 Gy)<br>Lumbar spine (8 Gy) | 1. Docetaxel (4 cycles) [P]<br>2. Mitoxantrone (5 cycles) [P] |
| RL24 | Adenocarcinoma (prostate) | | Prostate (64 Gy) | Cyclophosphamide (3 cycles) [P] |
| RL25 | Adenocarcinoma (prostate) | 1. Transurethral resection of prostate | Prostate (64 Gy) | 1. CP-751,871 (10 cycles) [P]<br>2. Docetaxel (8 cycles) [P] |
| RL26 | Adenocarcinoma (duodenum) | 1. Exploratory laparotomy | | |
| RL27 | Adenocarcinoma (endometrium) | | Pelvis (45 Gy)<br>Vaginal vault brachytherapy (12 Gy) | 1. Paclitaxel (6 cycles) [P]<br>2. Carboplatin (6 cycles) [P]<br>3. Doxorubicin (3 cycles) [P] |
| RL28 | Melanoma (skin) | 1. Wide local excision of skin | Thoracolumbar spine (20 Gy) | 1. Temozolomide (2 cycles) [P]<br>2. Carboplatin/paclitaxel (2 cycles) [P] |
| RL29 | Squamous cell carcinoma (esophagus) | | | 1. Cisplatin/epirubicin (8 cycles) [P]<br>2. Docetaxel (4 cycles) [P] |
| RL30 | Adenocarcinoma (colon) | 1. Anterior resection of colon<br>2. Partial hepatectomy | | 1. 5-Fluorouracil/folinic acid (7 cycles) [A]<br>2. Oxaliplatin/ capecitabine (9 cycles) [P] |
| RL31 | Sarcoma | | Abdomen (54 Gy)<br>Lumbar spine (25 Gy)<br>Chest wall (8 Gy) | Gemcitabine/docetaxel (4 cycles) [P] |
| RL32 | Melanoma (skin) | 1. Wide local excision of skin<br>2. Excision lymph node<br>3. Groin dissection (×3) | Pelvis (20 Gy) | 1. Dacarbazine (2 cycles) [P]<br>2. Sorafenib (2 cycles) [P] |
| RL33 | Adenocarcinoma (colon) | Sigmoid colectomy | | 1. Oxaliplatin/ capecitabine (7 cycles) [P]<br>2. Irinotecan (10 cycles) [P] |

NOTE: Numbered events for surgical, radiotherapeutic, and chemotherapeutic interventions indicate separate treatment episodes. The intent of treatment is indicated by letters in square parentheses: R (radical), N (neoadjuvant), C (concomitant during radiotherapy), and A (adjuvant) relate to treatment given with curative intent; P (palliative) relates to treatment (radiotherapy or chemotherapy) given for symptomatic benefit. Note that patient RL07 had not received any prior therapy before commencing treatment with the study drug.
Abbreviations: ECF, epirubicin, cisplatin, 5-fluorouracil; Mito/Carbo/Cape, mitomycin C, carboplatin, capecitabine; MVAC, methotrexate, vinblastine, doxorubicin, cisplatin; MVCarbo, mitomycin, vinblastine, carboplatin; *, carboplatin substituted for cisplatin after 1 cycle.

inability to give written informed consent. The study protocol was approved by the local ethics committees.

*Dose escalation.* In the first (or lowest dose) cohort, virus was administered at $1 \times 10^8$ $TCID_{50}$ as a single dose every 4 weeks. In the second cohort, virus was given at $1 \times 10^8$ $TCID_{50}$ for 3 consecutive days every 4 weeks. In the third cohort, virus was given at $1 \times 10^8$ $TCID_{50}$ for 5 consecutive days every 4 weeks, with 28 days defined as 1 cycle. Subsequent cohorts received 5 consecutive injections of escalating doses of virus every 4 weeks with the following dose-escalation scheme: cohort 4, $3 \times 10^8$ $TCID_{50}$: cohort 5, $1 \times 10^9$ $TCID_{50}$; cohort 6, $3 \times 10^9$ $TCID_{50}$; cohort 7, $1 \times 10^{10}$ $TCID_{50}$; and cohort 8, $3 \times 10^{10}$ $TCID_{50}$ (the manufacturing limit for dose escalation; Table 1). Cohorts of three were individually assessed for safety and dose-limiting toxicity (DLT). Subjects were "evaluable for dose-escalation decisions" if they received at least 1 cycle or withdrew from the study because of drug-related toxicity. Subjects withdrawn from the study without meeting these criteria were replaced. If one of three subjects in a cohort experienced a DLT during the first cycle, three more subjects were added to that dose group. If two or more subjects in a dose group experienced a DLT during the first cycle, the previous lower dose would be defined as the MTD. Intrasubject dose escalations were not permitted. Patients continued reovirus treatment as long as it was tolerated and there was no evidence of disease progression.

*Virus administration.* Reolysin was supplied by Oncolytics Biotech in single-use 1 mL glass vials containing a frozen viral suspension in PBS. Stock was stored at -70°C and thawed rapidly over 2 min, and the appropriate $TCID_{50}$ dose was diluted to 250 mL in 0.9% sodium chloride and infused over 60 min through a peripheral line. Patients were observed closely (including blood pressure, temperature, and heart rate monitoring) during and for at least 6 h after infusion. For the first five cohorts, patients remained as in-patients until it was confirmed that they were not shedding reovirus. For cohorts 6 to 8, hospital discharge occurred after the fifth dose of virus had been administered without analysis for viral shedding.

*Dose-limiting toxicity.* Toxicities were graded according to Common Terminology Criteria for Adverse Events version 3.0. DLT was defined

**Table 4.**

**(A) Nonhematologic toxicity**

**No. patients experiencing toxicity during first course***

| Cohort | Dose level ($TCID_{50}$) | No. patients | Fever | | Fatigue | | Headache | | Flu-like symptoms | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Grade 1-2 | Grade 3-4 | Grade 1-2 | Grade 3-4 | Grade 1-2 | Grade 3-4 | Grade 1-2 | Grade 3-4 |
| 1 | $1 \times 10^8$ (1 d) | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | $1 \times 10^8$ (3 d) | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| 3 | $1 \times 10^8$ (5 d) | 4 | 2 (3) | 0 | 4 (1) | 0 | 2 (1) | 0 | 1 (1) | 0 |
| 4 | $3 \times 10^8$ (5 d) | 3 | 4 | 0 | 2 (2) | 0 | 3 | 0 | 0 | 0 |
| 5 | $1 \times 10^9$ (5 d) | 3 | 2 | 0 | 1 | 0 | 2 | 0 | 1 | 0 |
| 6 | $3 \times 10^9$ (5 d) | 3 | 3 (3) | 0 | 1 (1) | 0 | 1 (1) | 0 | 1 (2) | 0 |
| 7 | $1 \times 10^{10}$ (5 d) | 6 | 5 (3) | 0 | 4 (2) | 0 | 0 (1) | 0 | 1 | 1 |
| 8 | $3 \times 10^{10}$ (5 d) | 8 | 5 (2) | 0 | 2 (6) | 0 | 7 (1) | 0 | 5 (2) | 0 |

**No. patients experiencing toxicity during first course***

| Cohort | Dose level ($TCID_{50}$) | No. patients | Nausea | | Vomiting | | Hyperhidrosis | | Chills | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Grade 1-2 | Grade 3-4 | Grade 1-2 | Grade 3-4 | Grade 1-2 | Grade 3-4 | Grade 1-2 | Grade 3-4 |
| 1 | $1 \times 10^8$ (1 d) | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | $1 \times 10^8$ (3 d) | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | $1 \times 10^8$ (5 d) | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | $3 \times 10^8$ (5 d) | 3 | 0 | 0 | 0 | 0 | 1 | 0 | 2 | 0 |
| 5 | $1 \times 10^9$ (5 d) | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | $3 \times 10^9$ (5 d) | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | $1 \times 10^{10}$ (5 d) | 6 | 0 | 0 | 0 | 0 | 1 | 0 | 1 (1) | 0 |
| 8 | $3 \times 10^{10}$ (5 d) | 8 | 3 (3) | 0 | 1 (1) | 0 | 4 | 0 | 0 | 0 |

**(B) Hematologic toxicity**

**No. patients experiencing toxicity during first course***

| Cohort | Dose level ($TCID_{50}$) | No. patients | Lymphopenia | | Thrombocytopenia | | Anemia | | Neutropenia | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Grade 1-2 | Grade 3-4 | Grade 1-2 | Grade 3-4 | Grade 1-2 | Grade 3-4 | Grade 1-2 | Grade 3-4 |
| 1 | $1 \times 10^8$ (1 d) | 3 | 0 | 1 (1) | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | $1 \times 10^8$ (3 d) | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | $1 \times 10^8$ (5 d) | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | $3 \times 10^8$ (5 d) | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| 5 | $1 \times 10^9$ (5 d) | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | $3 \times 10^9$ (5 d) | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | $1 \times 10^{10}$ (5 d) | 6 | 1 (2) | 1 | 1 (3) | 0 | 0 (2) | 0 | 0 (7) | 1 |
| 8 | $3 \times 10^{10}$ (5 d) | 8 | 3 (5) | 3 (3) | 0 | 0 | 0 | 0 | 3 (3) | 2 |

NOTE: Figures represent the number of patients experiencing either grade 1/2 or 3/4 in each cohort. Figures in parentheses represent the toxicity rates in all subsequent treatment courses.
*Numbers in parentheses represent all subsequent courses.

**Table 5.** Analysis for the presence of reovirus in serum, urine, saliva, and anal swab using 35 cycles of RT-PCR as described in Materials and Methods

| Patient | Day 5/8 | | | | Day 15 | | | |
|---|---|---|---|---|---|---|---|---|
| | Serum | Urine | Saliva | Anal swab | Serum | Urine | Saliva | Anal swab |
| RL1 | - | - | - | - | - | - | - | - |
| RL2 | - | - | - | - | - | - | - | - |
| RL3 | - | - | - | - | - | - | - | - |
| RL4 | ND | ND | ND | ND | ND | ND | ND | ND |
| RL5 | ND | ND | ND | ND | - | - | - | - |
| RL6 | - | - | - | - | - | - | - | - |
| RL7 | ND | ND | ND | ND | - | - | - | - |
| RL8 | - | - | - | - | ND | ND | ND | ND |
| RL9 | - | - | - | - | - | - | - | - |
| RL10 | - | - | - | - | - | - | - | - |
| RL11 | - | - | - | - | - | - | - | - |
| RL12 | - | - | - | - | - | - | - | - |
| RL13 | - | - | - | - | - | - | - | - |
| RL14 | - | - | - | - | - | - | - | - |
| RL15 | ND | ND | ND | ND | - | - | - | - |
| RL16 | ND | ND | ND | ND | - | - | - | - |
| RL17 | ND | ND | ND | ND | + | - | - | - |
| RL18 | ND | ND | ND | ND | - | - | - | - |
| RL19 | ND | ND | ND | ND | - | - | - | - |
| RL20 | - | - | - | - | - | + | + | + |
| RL21 | - | - | - | - | - | - | - | - |
| RL22 | - | - | - | - | - | - | - | - |
| RL23 | ND | ND | ND | ND | - | - | - | - |
| RL24 | ND | - | - | - | ND | - | - | + |
| RL25 | + | - | - | - | - | - | - | - |
| RL26 | - | - | - | - | - | - | - | - |
| RL27 | ++ | + | + | ++ | - | + | + | ++ |
| RL28 | - | - | - | - | - | - | - | - |
| RL29 | ND | ND | ND | ND | ND | ND | ND | ND |
| RL30 | ND | ND | ND | ND | ND | ND | ND | ND |
| RL31 | - | - | - | - | ND | ND | ND | ND |
| RL32 | - | + | + | + | - | + | - | ++ |
| RL33 | - | - | - | - | - | - | - | - |

Abbreviation: ND, not done.

during the first cycle of treatment and included absolute neutrophil count $<0.5 \times 10^9$ with sepsis, platelet count $<25 \times 10^9$/L, grade 2 neurotoxicity or cardiotoxicity, inability to tolerate one course of therapy due to toxicity, and any other drug-related nonhematologic grade 3 or 4 toxicity (except flu-like symptoms and nausea and vomiting if appropriate prophylactic or therapeutic measures had not been administered).

***Pretreatment and follow-up studies.*** Safety was assessed by evaluating the type, frequency, and severity of adverse events; changes in clinical laboratory tests (including hematology, clinical chemistry, and urinalysis); immunogenicity; and physical examination. Multiple-gated acquisition scan (or echocardiography) and radiologic studies of assessable disease were done at baseline and after *alternate* cycles. Patients continued treatment in the absence of progressive disease or intolerable toxicity.

***Response evaluation.*** Response was measured radiologically by Response Evaluation Criteria in Solid Tumors and by densitometric analysis of computed tomography imaging (13). For this latter analysis, the computed tomography attenuation coefficient (density) of the target lesion in Hounsfield units (HU) was measured on a commercially available workstation (AGFA 4.0) by drawing a region of interest circumscribing the margin of the tumor. The tumor HU measurements of all lesions in a patient were combined to calculate an average HU for each patient. The percentage of change in tumor density from the pretreatment evaluation to the post-treatment evaluation was calculated for each lesion, and the average percentage of change was then estimated for the patient. The percentage of change in tumor enhancement was graded on a scale of 1 to 4 as described previously (13). The tumor density was graded as follows: grade 4, decrease of >30%; grade 3, decrease of 11% to 30%; grade 2, decrease of ≤10% or increase of ≤10%; and grade 1, increase of >10%. Tumor response was also followed, where possible, by measurement of carcinoembryonic antigen (CEA), prostate-specific antigen (PSA), and CA19.9 tumor markers.

***Analysis of viral shedding by reverse transcription-PCR.*** Initial evaluation of the detection limit of reovirus RNA by 25 or 35 cycles of reverse transcription-PCR (RT-PCR) was done. Viral RNA was extracted from 140 μL stock using the QIAamp viral RNA mini-kit (Qiagen) and serially diluted and 5 μL was assayed directly by RT-PCR using the One-Step RT-PCR Enzyme Mix Kit (Qiagen). Reovirus s3 cDNA targeted primers used were forward 5′-GGGCTGCACATTACCACTGA and reverse 5′-CTCCTCGCAATACAACTCGT. PCR conditions were 50°C for 30 min (for reverse transcription); 95°C for 15 min; and 25 (or 35) cycles of 95°C for 30 s, 62°C for 45 s, and 72°C for 45 s followed by 72°C for 7 min (Supplementary Fig. S1). For the clinical assays, blood samples were taken at baseline, post-treatment dose and weekly during the first cycle. Blood from the contralateral arm was collected into EDTA tubes, centrifuged at 1,200 × *g* for 10 min at 4°C, and stored at -70°C. Urine, sputum, and fecal swab (after PBS elution) samples were also stored at -70°C. Samples were analyzed after the last treatment dose in each cycle and weekly using the 25- and 35-cycle RT-PCR. Reovirus RNA (300-bp PCR product) and water were included in all experiments as positive and negative controls, respectively.

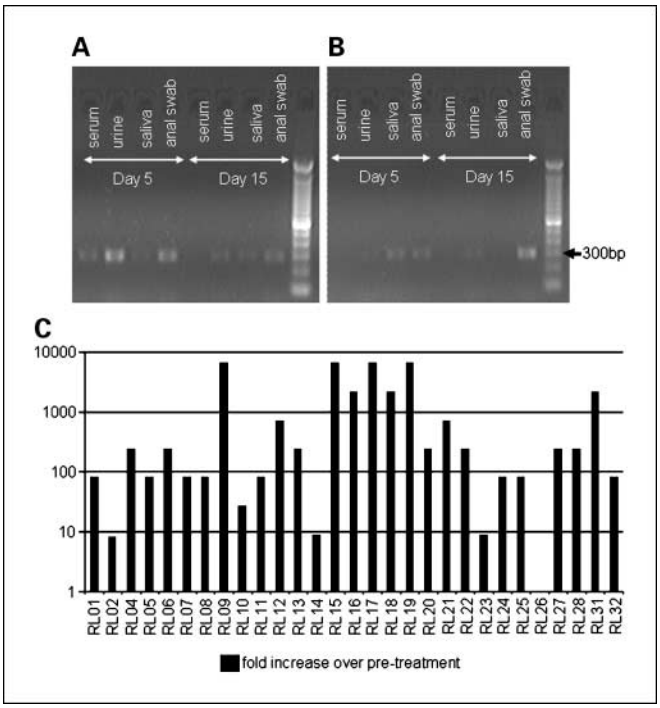


**Fig. 1.** RT-PCR analysis (35 cycles) of serum, urine, saliva, and anal swab for reovirus from patients RL27 (*A*) and RL32 (*B*). Reovirus RNA (300-bp PCR product) and water were included as positive and negative controls, respectively. Reovirus was detected at day 5 in 2 patients and at day 15 in 5 patients (see Table 5). *C,* fold increase in reoviral titer in 29 patients for whom data were available. Note patient RL26 had high level neutralizing antibody titer pretreatment and therefore had a 1-fold increase in titer.

***Detection of neutralizing antireoviral antibodies.*** A modified neutralizing antibody assay was used to detect antibody titers at baseline and weekly during the first 2 cycles of treatment by measuring the effect of patient serum samples on the ability of reovirus to kill a monolayer of target mouse L929 cells (14). The neutralizing antireoviral antibodies (NARA) titer of serum samples was expressed as the last dilution causing <80% cell killing as described previously (15).

***Estimation of virus titer from tumor samples.*** Paired pretreatment and post-treatment tumor biopsy samples were obtained in 3 patients and stored at -80°C until the time of analysis. At this time, the biopsies were thawed and macerated in 1 mL DMEM and centrifuged at 3,600 rpm for 5 min. Supernatant was taken, serially diluted (1:10), and placed on to L929 cells in quadruplicates in a 96-well plate. Titer was calculated using the Kärber statistical method for a standard $TCID_{50}$ assay. In addition, photomicrographs were taken of the plates at the time of calculating the viral titer.

## Results

***Patients.*** Thirty-three patients with a variety of underlying malignant diagnoses were enrolled and treated over 8 dose levels with a total of 76 cycles (median, 2; range, 1-6). Patients received the following numbers of treatment cycles: 1 cycle, RL02, RL03, RL08, RL13, RL16, RL23, RL29, and RL32; 2 cycles, RL01, RL04, RL07, RL10, RL11, RL14, RL18, RL21, RL22, RL24, RL25, RL26, RL28, RL30, and RL33; 3 cycles, RL06, RL09, RL17, RL19, and RL31; 4 cycles, RL05, RL15, and RL20; 5 cycles, RL27; and 6 cycles, RL12. Patient demographics are displayed in Table 2. Tumor diagnoses and exposure to prior therapies are detailed in Table 3.

***Safety.*** Treatment was well tolerated in all cohorts with no grade 4 toxicities. Grade 3 toxicity was restricted to flu-like symptoms (Table 4A) and uncomplicated lymphopenia and neutropenia, which were more prominent in cohort 8 (Table 4B). The most common toxicities observed were grade 2 (or lower) flu-like symptoms (fever, chills, fatigue, nausea/vomiting, and headache) that primarily occurred 2 to 6 h after reovirus administration and resolved fully with acetaminophen and nonsteroidal anti-inflammatory therapy. These symptoms were more frequent and pronounced on cycle 1. There was no

**Table 6.** Objective radiologic assessment of tumor response in 10 patients who had clinical evidence of stable disease

| Patient | Target lesion(s) | Pre-ΣD (cm) | Interval (d) | Post-ΣD (cm) | % Change vs baseline | Overall response |
|---|---|---|---|---|---|---|
| RL05 | Liver | 22.9 | 61 | 24.2 | +5.7 | SD |
| | | | 124 | 21.8 | -4.8 | SD |
| RL06 | Lung, liver, peritoneum | 303.5 | 74 | 335.2 | +10.5 | SD |
| RL12 | Lymph node | 68.7 | 60 | 81.4 | +18.5 | PD |
| | | | 123 | 72.3 | +5.2 | PD |
| | | | 179 | 82.4 | +19.9 | PD |
| RL15 | Lymph node | 26.0 | 56 | 27.9 | +7.3 | SD |
| | | | 126 | 27.8 | +6.9 | SD |
| RL17 | Liver | 235.4 | 57 | 228.9 | -2.8 | SD |
| RL19 | Liver | 98.2 | 56 | 91.5 | -6.8 | SD |
| | Lymph node | | 84 | 82.1 | -16.4 | SD |
| RL20 | Lymph node | 33.5 | 53 | 34.0 | +1.5 | SD |
| | | | 111 | 37 | +10.4 | SD |
| RL25 | Lymph node | 73.0 | 54 | 102.4 | +40.3 | PD |
| RL27 | Liver, pelvis | 346.1 | 73 | 315.1 | -9.0 | SD |
| | | | 143 | 276.8 | -20.0 | SD |
| RL31 | Liver | 210.2 | 71 | 216.8 | +3.1 | SD |
| | Lymph node | | 123 | 256.9 | +22.2 | PD |

NOTE: Minor responses were seen at 124, 84, and 143 d in patients RL05, RL19, and RL27, respectively.
Abbreviations: Pre-ΣD, sum of longest diameters of target lesions before start of reovirus treatment; Post-ΣD, sum of longest diameters of target lesions after reovirus treatment; SD, stable disease; PD, progressive disease.

relationship between the reovirus dose level and the incidence and grade of these symptoms. Patients who developed grade 2 flu-like symptoms on day 1 received prophylactic nonsteroidal anti-inflammatory drugs on other treatment days. One patient with pancreatic cancer presented with disease-related pulmonary embolism during cycle 1 at the $1 \times 10^8$ $TCID_{50}$ dose level. Following anticoagulation treatment, he was rechallenged uneventfully with the same dose. During the first cycle, the second patient treated at $1 \times 10^{10}$ $TCID_{50}$ had an asymptomatic increase in liver transaminases (grade 2), CK-MB (grade 3), and troponin-I levels (grade 1 at baseline to grade 3 on day 3). Electrocardiogram and echocardiogram were normal. These biochemical toxicities resolved to baseline on day 15 and the patient was rechallenged with a lower dose ($3 \times 10^9$) with no further sequelae. To characterize further the safety at the $1 \times 10^{10}$ $TCID_{50}$ dose level, 4 more patients were included. No similar or other significant toxicities were observed and dose escalation continued to the $3 \times 10^{10}$ dose level where 8 patients were treated. No DLT was observed at this level and this dose was recommended for phase II evaluation.

***Viral biodistribution.*** All pretreatment and post-treatment blood, urine, saliva, and rectal samples were negative for reovirus detection using RT-PCR screening based on 25 cycles of amplification. This assay was used for decision-making for discharge from the hospital. As a result, all patients were discharged on schedule and no patient was kept in hospital because of viral persistence in screened samples. However, in recognition of the fact that the initial 25-cycle assay for viral excretion may have missed relatively low levels of viral RNA in the samples, we conducted further RT-PCR analysis based on 35 cycles of amplification (where the detection limit was 200 $TCID_{50}$; Supplementary Fig. S1). In these assays, positive signal was detected in cycle 1 in a small number of patients at days 5 and 15 after viral administration (Table 5; Fig. 1A and B).

***NARA response.*** Data were available for the fold increase in neutralizing antibody titers for 29 patients. After treatment, 28 patients showed an increase in NARA titers to a maximum at 4 weeks (median increase, 250-fold; range, 9-6,437; Fig. 1C). This NARA titer remained constant during subsequent cycles (15).

***Response assessment.*** No objective radiologic responses were observed in terms of Response Evaluation Criteria in Solid Tumors. Eight of the patients who were radiologically evaluable showed disease stabilization or minor response. Data on the sum of the diameters of target lesions before and after reovirus therapy are provided for 10 patients who had clinical evidence of disease stability (Table 6). There was no clear correlation between clinical stabilization and dose or duration of reovirus therapy. In patient RL19, a computed tomography scan done after 2 cycles of therapy showed bilateral necrosis of involved iliac lymph nodes and stable disease by Response Evaluation Criteria in Solid Tumors for 3 months (Fig. 2A-C; Supplementary Fig. S2). Response Evaluation Criteria in Solid Tumors analysis recorded a 16.4% reduction in the sum of the longest diameters of predefined target lesions at 84 days. Densitometric evaluation of the change in contrast enhancement in tumor tissue revealed a grade 3 (12%) reduction from a baseline level of 75.5 to 66 HU. The iliac lymph nodes were biopsied and histologic evaluation revealed extensive necrosis in keeping with the imaging appearances (Fig. 3). Patient RL27 had a 20% reduction in the sum of the longest diameters of predefined target lesions at 143 days (Fig. 4A-C; Supplementary Fig. S3). Densitometric evaluation of the change in contrast enhancement in tumor tissue revealed a grade 2 (<10%) reduction from a baseline level of 65 to 61 HU. Sequential data were not available for similar lesions without any treatment or after chemotherapy treatment.

Reductions in tumor markers (CEA, CA19.9, and PSA) were detected in 3 patients (Fig. 5A-D). Two patients (RL12 and RL17) with colorectal cancer had reductions in CEA levels of 68% and 27% (Fig. 5A and B) and stable disease for 3 and 6 months, respectively. Patient RL17 also had a detectable level of CA19.9 and this fell by 18% (Fig. 5C). Patient RL19 showed a 51% fall in PSA from 100.9 to 49.2 ng/mL over 4 weeks after completion of cycle 2 (Fig. 5D).

***Viral replication in tumor biopsies.*** Biopsy analysis confirmed the presence of viable virus in post-treatment (but not

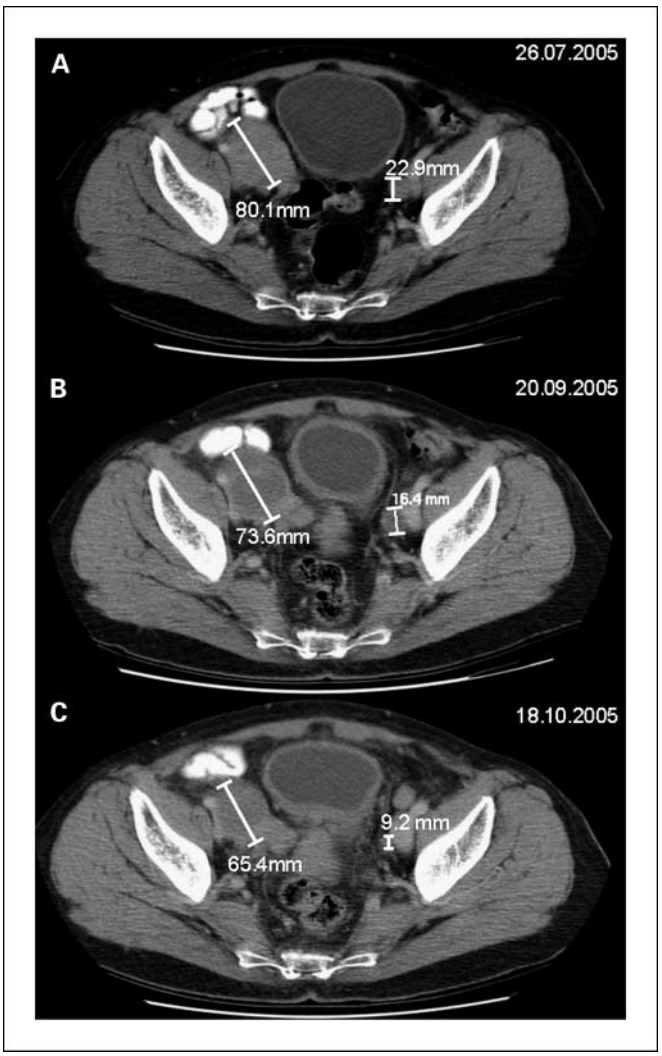


**Fig. 2.** Serial computed tomography imaging data for patient RL19. *A,* pretreatment baseline image showing bilateral iliac lymph node enlargement measuring 80.1 and 22.9 mm, respectively. *B,* repeat scan after 2 cycles of treatment showing reduction of lymph node enlargement. Densitometric evaluation of the change in contrast enhancement in tumor tissue revealed a grade 3 (12%) reduction from a baseline level of 75.5 to 66 HU. *C,* repeat imaging after 3 cycles showing further reduction in lymphadenopathy.

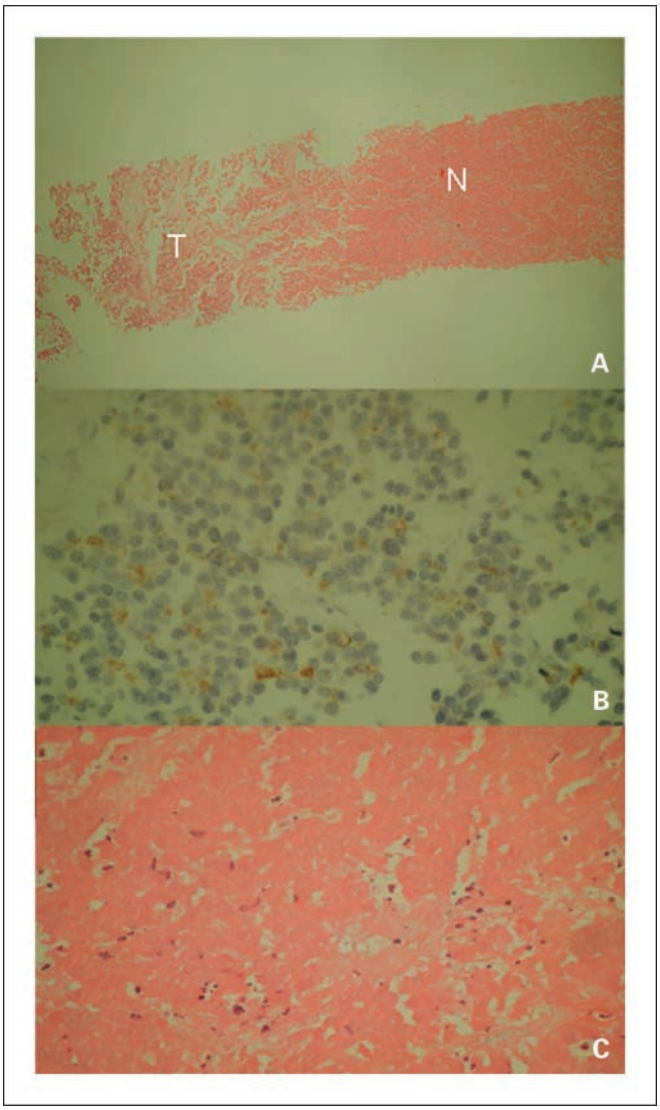


**Fig. 3.** Biopsy sample from patient RL19. *A,* H&E-stained core biopsy showing areas of viable tumor (*T*) and necrosis (*N*). *B,* area of viable tumor tissue stained immunohistochemically for PSA confirming the presence of metastatic prostate cancer in iliac lymph node biopsy. *C,* high-power view of area of tumor necrosis.

pretreatment) tumor samples in 3 patients (RL03, RL04, and RL11). Supernatant from freeze-thawed and macerated tumor biopsies that were taken after the initiation of reovirus therapy caused cytopathic effect in L929 target cells (Fig. 6A, *vi-x*). Pretreatment samples did not cause cytopathic effect (Fig. 6A, *i-v*). For the 3 patients analyzed, the titer of reovirus recovered correlated directly with the dose of reovirus administered (Fig. 6B).

## Discussion

Oncolytic reovirus offers significant advantages over recently described virotherapies in that it has activity against a wide range of tumor types with activated Ras signaling pathways (16–18). The lack of a requirement for genetic modification retains the antitumor activity of wild-type reovirus (in contrast to other genetically modified viruses that sacrifice potency in achieving selectivity). Intratumoral delivery of reovirus has been shown to be safe in humans, with antitumor activity reported in patients with high-grade gliomas (12). This study has extended this approach to systemic delivery of reovirus to target disseminated disease.

The purpose of this trial was to determine the safety, DLT, and MTD of i.v. reovirus in patients with refractory solid malignancies, with an aim of defining the recommended phase II dose. Importantly, no DLT was observed and $3 \times 10^{10}$ $TCID_{50}$ was defined as the MTD by virtue of the fact that this was the highest dose available for administration. The activation status of the ras pathway was not measured in this study, but we do not believe that this variable would have affected the MTD or recommended phase II dose. We have shown previously that viral replication in tumors in immunocompetent mice is not associated with systemic toxicity. Therefore, in a clinical situation in which the immune system is operating, we believe that the MTD or recommended phase II dose will be independent of the Ras pathway status in the tumor. It is also significant

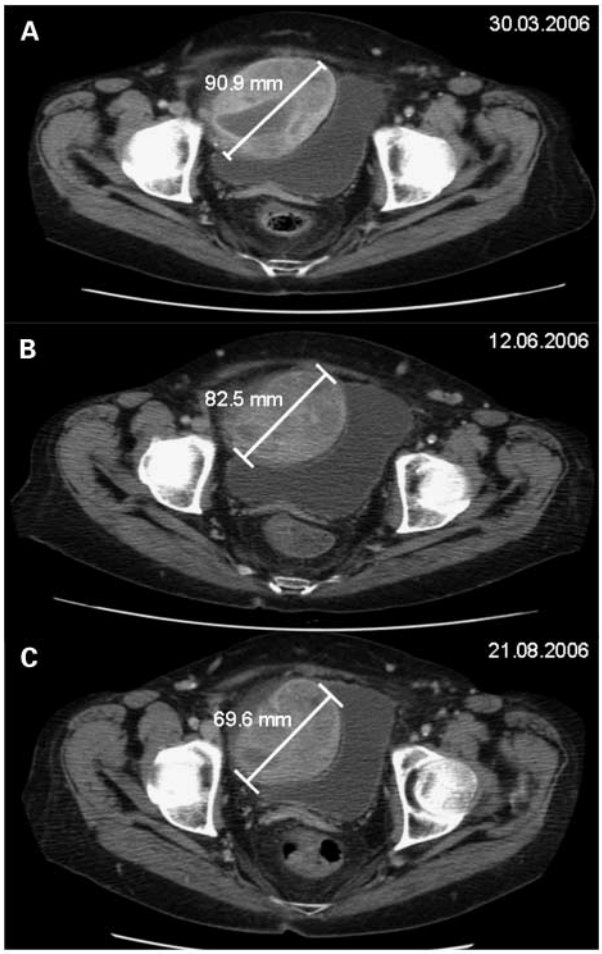


**Fig. 4.** Serial computed tomography imaging data for patient RL27. *A,* pretreatment baseline image showing pelvic tumor measuring 90.9 mm. *B,* repeat scan after 2 cycles of treatment showing reduction of mass to 82.5 mm. Densitometric evaluation of the change in contrast enhancement in tumor tissue revealed a grade 2 reduction from a baseline level of 65 to 61 HU. *C,* repeat imaging after 5 cycles showing further reduction in pelvic mass to 69.6 mm.

that there was no evidence of viral shedding in any patient using the RT-PCR assay designed for safety analysis. This finding suggests that this agent is potentially suitable for office-based administration. Further viral biodistribution studies using a more sensitive assay based on 35 cycles of RT-PCR showed detectable virus at 5 or 15 days in a small minority of patients. Although these data offer reassurance about patient and environmental safety, they may also represent evidence of relatively rapid clearance of virus from the systemic circulation.

As expected, a significant number of patients had measurable NARA titers pretreatment. All patients showed an early increase in NARA titers that remained essentially constant during subsequent treatment cycles. This may account for the relatively lower toxicity scores despite much higher doses of reovirus in later treatment cycles. Clearly, this NARA response may limit the effect of repeated therapy by rapid neutralization of virus before it reaches tumor sites. However, despite this, we found evidence of viable virus in biopsies taken after 2 cycles of treatment from 3 patients by quantitative assay. This suggests that reovirus is able to access tumor tissue in patients and persists there even in the face of a rapidly evolving NARA response.

Although evaluation of antitumor activity was not a primary endpoint of the study, patients were monitored for evidence of antitumor efficacy. There were no objective responses by Response Evaluation Criteria in Solid Tumors, but there were several indications of activity. Eight patients had radiologic evidence of stable disease, including 2 patients (RL19 and RL27) with 16.4% and 20% reductions in the sum of the longest diameters of target lesions. Densitometric analysis of the radiologic data revealed a grade 3 reduction in patient RL19 and a grade 2 reduction in patient RL27. These data must be considered in light of the fact that we do not have data from similar untreated or drug-treated lesions. However, they are of sufficient interest to lead us to include densitometric analyses in ongoing studies in which we are combining reovirus and chemotherapy. In addition, there were reductions in tumor marker levels (CEA, CA19.9, and PSA) in 3 patients and biopsy-confirmed evidence of tumor necrosis in metastatic lymph nodes in 1 patient. Clearly, in the absence of data on the Ras pathway activation status in the individual tumors, it is not possible to attribute the changes in tumor sizes and marker levels to reovirus treatment. Future studies that include molecular analyses of the Ras pathway may clarify this area.

Development of reovirus as a cancer therapeutic is likely to depend on an improved understanding of the mechanisms of reovirus-induced cell death, circumvention of humoral and cellular immune responses, and concomitant use with other anticancer agents. Reovirus may be a relevant therapeutic approach in both tumors with oncogenic Ras mutations

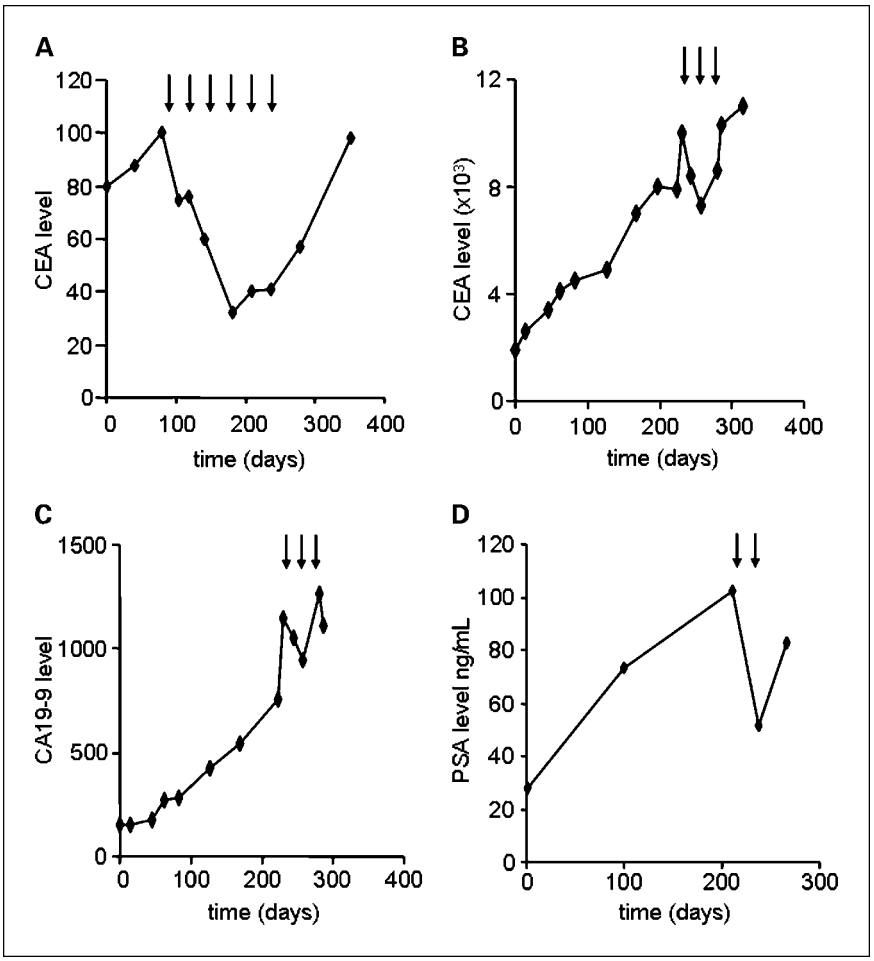


**Fig. 5.** Tumor marker response data for 3 evaluable patients. *Arrows,* timing of administration of cycles of reovirus. *A,* CEA levels in patient RL12 showing steadily rising levels before institution of reovirus therapy followed by a sustained fall (by a maximum of 68%). *B,* CEA levels ($\times 10^3$) in patient RL17 showing a progressive increase before the start of reovirus treatment followed by a short-term reduction (by a maximum of 27%). *C,* CA19.9 levels in patient RL17 showing a similar pattern to those seen for CEA in *B. D,* PSA level in patient RL19 showing a progressive increase in PSA level before the start of reovirus administration followed by a short-term reduction (by a maximum of 51%).

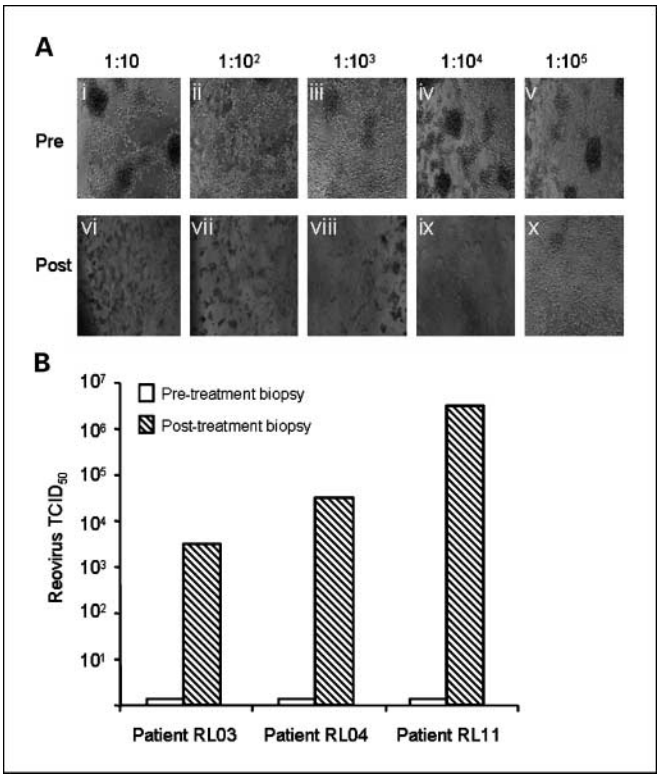


**Fig. 6.** Recovery of reovirus from tumor biopsy samples. *A,* photomicrographs of L929 cells exposed to serial dilutions (1:10) of supernatant obtained from pretreatment (*i-v*) or post-treatment (*vi-x*) biopsy samples from patient RL11. L929 cells exposed to supernatant from the pretreatment biopsy show no evidence of cytopathic effect (*CPE*) and form typical heaped colonies (*i-v*). In contrast, L929 cells exposed to supernatant from the post-treatment biopsy show almost complete CPE for dilutions 1:10 to $1:10^4$ (*vi-ix*). Heaped colonies are first seen at the $1:10^5$ dilution (*x*). *B,* quantitative analysis of reovirus titer in three paired (pretreatment and post-treatment) biopsy specimens. A dose-dependent increase in the titer of reovirus recovered was seen. No titerable virus was recovered from the pretreatment biopsy specimens.

and those with upstream or downstream Ras pathway activation. This opens up the prospect of using reovirus in a wide range of common tumor types. In certain tumor models, reoviral cytotoxicity may be mediated through activation of apoptosis rather than simply as a result of viral replication and lysis (19). Patients were not selected for this study based on the Ras status of their tumors, as this was primarily a safety evaluation. Future phase II studies, using the recommended phase II dose, will be enriched for patients with tumors that have known activation of the Ras pathway. Moreover, preclinical studies have confirmed that modulation of the immune response by concomitant administration of either cyclosporine A or anti-CD4/CD8 antibodies results in improved antitumor activity of i.v. administered reovirus in immunocompetent hosts (11, 20). Such immune modulation, by agents such as cyclophosphamide (21) or rituximab, may maximize antitumor activity and is now being pursued for this oncolytic virus in clinical trials. Furthermore, combinations of reovirus and standard anticancer agents, such as radiotherapy and cytotoxic drugs, are being pursued (22–24).

In conclusion, this trial confirms the feasibility of i.v. delivery of repeated high doses of reovirus. The recommended phase II dose is $3 \times 10^{10}$ for 5 consecutive days every 4 weeks. This study has shown successful intratumoral localization of reovirus after systemic administration. These data provide the essential background for subsequent studies of reovirus either as a single agent or in combination with standard anticancer therapies and immune modulating agents.

## Disclosure of Potential Conflicts of Interest

M. Coffey is employed by Oncolytics Biotech and has an ownership interest in that company; K. Harrington, A. Melcher, and R. Vite have received research grants from Oncolytics Biotech; J. de Bono has received research support from Oncolytics Biotech.

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# A Phase I Study of Intravenous Oncolytic Reovirus Type 3 Dearing in Patients with Advanced Cancer

Laura Vidal, Hardev S. Pandha, Timothy A. Yap, et al.

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