

Hepatic Arterial Infusion of a Replication-selective Oncolytic Adenovirus (*dl1520*): Phase II Viral, Immunologic, and Clinical Endpoints

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ABSTRACT

Replication-selective oncolytic adenoviruses are being developed for the treatment of cancer, but the safety and feasibility of repeated adenovirus delivery to tumors via the bloodstream was unknown, particularly in light of a patient death after hepatic artery infusion of a replication-defective adenovirus vector. We performed a Phase II trial of an oncolytic replication-selective adenovirus (*dl1520*, also known as Onyx-015) administered by hepatic artery infusion in patients with gastrointestinal carcinoma metastatic to the liver ($n = 27$). *dl1520* was infused into the hepatic artery (2×10^{12} particles) on days 1 and 8 as a single agent, and thereafter starting on day 22 in combination with i.v. 5-fluorouracil and leucovorin every 28 days. Repeated viral infusions were feasible, and no deaths occurred on study; reversible grade 3/4 hyperbilirubinemia occurred in 2 patients. Systemic inflammatory cytokine responses varied greatly between patients and even between cycles within a given patient. Proinflammatory cytokines [e.g., tumor necrosis factor, IFN- γ , and interleukin (IL) 6] typically rose within 3 h and were followed at 18 h by a rise in IL-10. However, in the single patient who suffered a severe but reversible systemic inflammatory response, a unique cytokine profile was detected: marked acute increases of IL-6 (20-fold higher than average for all of the patients) and inhibition of IL-10 production. Delayed secondary peaks of viremia were reproducibly detected 3–6 days after treatment, even in the presence of high level neutralizing antibody titers and antiviral cytokines. Mathematical modeling was used to calculate the number of virus particles produced and shed into the blood with each replication cycle. The combination of virotherapy and chemotherapy had antitumoral activity in some chemotherapy-resistant colorectal tumors. The intra-arterial infusion of oncolytic adenoviruses warrants additional study.

INTRODUCTION

Anticancer agents with novel mechanisms of action are needed. Viruses are being developed to treat cancer using two contrasting approaches. The initial approach of using replication-incompetent viruses as delivery agents for therapeutic genes to tumors has been limited by insufficient levels and distributions of gene expression (1, 2). A complementary strategy has now emerged to use replication-selective oncolytic viruses as therapeutic agents themselves (3–9). Superior efficacy may be achieved by combining these two strategies and arming replication-selective viruses with therapeutic transgenes (8, 10, 11). To date, the published clinical experience with these agents has been almost exclusively limited to intratumoral injection on Phase I and II studies. In addition, the well-publicized death of a patient on an adenoviral gene therapy trial for OTC deficiency at the University of Pennsylvania raised serious concerns about the safety of arterial adenovirus administration. As a result, numerous trials in the United States and Europe were put on hold (12–15). Data on the feasibility and safety of intra-arterial adenovirus administration is needed.

dl1520 (Onyx-015) is an oncolytic adenovirus type 2/5 chimera with a deletion in the *E1B-55kD* gene (16). E1B-55kD binds to and inactivates the p53 tumor suppressor gene product in complex with the E4ORF6 protein (17). As a result, it was predicted that this mutant would be unable to overcome the p53-mediated blockade of viral replication in cells with normal p53 function (18). In contrast, however, in a tumor cell lacking p53 function the E1B-55kD protein should be expendable (because p53 inhibition has already occurred during carcinogenesis), and replication should proceed (19). Publications from preclinical studies with different cell systems have reported conflicting data regarding the original McCormick hypothesis linking the selectivity of *dl1520* to p53 function, although most data are consistent with a role for p53 in modulating the replication of this virus (18–24). Nevertheless, numerous groups have confirmed that the replication and pathogenicity of this mutant in normal cells is reduced versus wild-type adenovirus, and that a therapeutic index exists between normal and cancerous cells (19, 20). During clinical trials of direct intratumoral injection into recurrent head and neck cancers, tumor-selective viral replication and necrosis in p53 mutant tumors has been demonstrated clearly with *dl1520* (25–28). The treatment was well tolerated without dose-limiting toxicities. Although durable responses were rare as a single agent in these advanced refractory tumors (27, 28), a potentially synergistic interaction was discovered subsequently between *dl1520* and chemotherapy (29–31). A randomized Phase III trial is under way in this indication.

Unfortunately, most cancers that recur after surgery and/or radiation are multifocal; therefore, intratumoral injection is not feasible. For viral agents to have significant benefit in these advanced cancer patients, regional or systemic delivery of viruses to tumors through the vasculature is necessary. Intra-arterial administration of genetically engineered replication-selective viruses in humans had not been described previously (4, 8). Potential hurdles to intravascular therapy included inflammatory hepatitis, antibody-mediated neutralization of the virus, and rapid clearance of the virus from the bloodstream by reticuloendothelial organs. i.v. treatment with *dl1520* in nude mouse-human tumor xenograft models led to infection and growth inhibition of s.c., and intrahepatic tumors in a dose-dependent fashion (19). The dose-limiting toxicity in C57/B6 mice was hepatic necrosis. However, clinical data on intra-arterial administration of adenoviruses was lacking.

Therefore, because *dl1520* has been well-tolerated with minimal toxicity after intratumoral injection, and preclinical efficacy resulted after intravascular administration, we performed a Phase I trial of *dl1520* administered via the hepatic artery to patients with metastatic gastrointestinal carcinomas (primarily colorectal) to the liver (32). These patients were selected for intra-arterial treatment for several reasons. Intrahepatic tumor progression is the cause of death in >80% of patients dying with colorectal carcinoma. Approximately 30–40% of patients dying of colorectal cancer may have macroscopic disease confined to the liver (33). In addition, preferential perfusion of tumor masses (versus normal liver tissue) can be achieved via hepatic artery infusions (33, 34). Therefore, experimental regional therapies using

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hepatic artery infusion have been developed for colorectal liver metastases (33). Treatment on the Phase I trial included two doses of *dl1520* by hepatic artery infusion as a single agent (days 1 and 8 to determine single agent safety) followed by combination treatment with i.v. 5-FU² and leucovorin (to determine safety and efficacy of the combination). Dose escalation was carried out from 2×10^9 particles to 2×10^{12} particles. No dose-limiting toxicity was identified either as a single agent or with chemotherapy, so the maximum dose was defined as the maximum feasible dose (Ref. 32; $n = 3$ at maximum dose). We subsequently carried out a Phase II trial of hepatic arterial *dl1520* (2×10^{12} particles) in combination with i.v. 5-FU and leucovorin in patients with gastrointestinal carcinoma metastatic to the liver.

MATERIALS AND METHODS

Objectives. The primary objectives of this study were as follows: (a) to determine the safety of repeated infusions of *dl1520* into the hepatic artery, alone and in combination with i.v. 5-FU and leucovorin; (b) to determine the antitumor efficacy of *dl1520* infusions in combination with 5-FU and leucovorin (as above) on metastatic colorectal tumors within the liver, specifically in patients with colorectal tumors refractory to 5-FU and leucovorin; (c) to determine viral shedding into the blood over time after initial clearance (secondary viremia consistent with replication). Secondary objectives included the following: (a) to determine the pharmacokinetic profile of the virus in the venous circulation; and (b) to determine the humoral and cytokine-mediated immune responses to hepatic artery infusions of *dl1520*.

Eligibility Criteria. Inclusion criteria included the following: histologically or cytologically confirmed carcinoma of gastrointestinal origin; cancer that was not considered resectable for potential cure; confirmed hepatic artery perfusion of both liver lobes and >50% of all tumor mass(es); Karnofsky performance status of $\geq 70\%$; life expectancy of ≥ 3 months; ≥ 18 years of age; consent form for study participation signed; must have been using a reliable method of contraception if sexually active or of reproductive potential; creatinine <2.0 mg/dl; AST and ALT <3.0-fold upper limit of normal; total bilirubin <2.0 mg/dl; PT/INR <2.0 and PTT within normal limits; and neutrophils >1,500/ml, hemoglobin >9 g/dl, and platelets >100,000/ml. Exclusion criteria were as follows: known chronic liver dysfunction before the development of metastatic cancer (e.g., cirrhosis, chronic hepatitis), which in the estimation of the Principal Investigator put the patient at high risk for liver complications; >50% liver replacement by tumor (estimated radiographically); history of esophageal variceal bleeding within the preceding 8 weeks; active infection, including documented HIV; any viral syndrome diagnosed within the previous 2 weeks; chemotherapy within the previous 3 weeks (6 weeks for nitrosoureas or mitomycin-C); radiotherapy to the target tumor site within the last 4 weeks; concomitant hematological malignancy; chronic immunosuppressive medication; pregnant or lactating females; prior participation in any research protocol that involved administration of adenovirus vectors; and treatment with any other investigational therapy within the last 4 weeks. Because no single gold-standard test for p53 function within a tumor existed, p53 tumor status did not affect enrollment. However, the p53 gene status of the tumor was assessed by gene sequencing when sufficient amounts of tissue could be obtained (exons 2–11).

Test Article. *dl1520* (Onyx-015) is a chimeric human group C adenovirus (Ad2 and Ad5) that does not express the 55 kD product of the *E1B* gene; the virus was constructed in the laboratory of Barker and Berk (16). The virus contains a deletion between nucleotides 2496 and 3323 in the *E1B* region encoding the 55-kD protein. In addition, a C to T transition at position 2022 in *E1B* generates a stop codon at the third codon position of the protein. These alterations eliminate expression of the *E1B* 55-kD gene in *dl1520*-infected

cells. *dl1520* was grown and titered on the human embryonic kidney cell line HEK293 as described previously (19).

Treatment Schedule. The treatment schedule is summarized in Fig. 1. The safety of single agent *dl1520* (2×10^{12} particles) administered via the hepatic artery was determined after single infusions on days 1 and 8 (cycles 1 and 2). Starting on day 22, treatment cycles were 28 days and consisted of *dl1520* infusions followed by i.v. chemotherapy within 6 h after virus infusion (see below). After completion of cycle 4, up to four additional cycles were optional based on toxicity and tumor response (see below).

Treatment Procedures. A standard hepatic artery catheter was introduced via the femoral artery for the infusion, unless an indwelling hepatic arterial port was already in place. Patient treatment by percutaneous femoral artery catheterization was performed in interventional radiology suites. Selective catheterization of the proper hepatic artery was performed using standard diagnostic catheters and fluoroscopic guidance through a percutaneous femoral arterial approach. Patients were sedated with short-acting opiates and benzodiazepines as necessary for the procedure, and provided analgesics and antiemetics as necessary afterward. Patients underwent diagnostic hepatic arteriograms immediately before the initial virus solution infusion to define arterial anatomy and to confirm hepatic arterial supply to intrahepatic tumor(s). Images were obtained by digital subtraction angiography. The existence of anomalous arterial anatomy, which may occur in up to 40% of the normal patient population, was established and documented. If there was a single branched hepatic artery, the infusion was given into the proper hepatic artery (not the common). In the event of multiple arterial supply, the proportion of the liver supplied by each artery was estimated from the pretreatment CT scan and the arteriogram. After optimal positioning of the catheter to ensure minimal reflux into arterial branches not supplying the liver, a single infusion of 10 ml of viral solution was administered over ~5 min followed by a 10-ml normal saline flush given at the same rate and site. The total viral dose administered was specified for the cohort. When multiple hepatic arteries were identified, the total dose was divided and administered into each artery according to the proportion of the liver supplied by that artery to provide even and complete distribution throughout the entire liver. After completion of the infusion, the femoral catheter was removed.

Frozen vial virus solution was warmed and diluted with normal saline to the appropriate titer for each patient dose level. Vials of ONYX-015 were opened and diluted in biological safety level 2 cabinets. Virus was maintained at 2–8°C during dilution and handling, except for warming to room temperature immediately before administration. The virus solution was then additionally diluted to a final volume of 10 ml. Dilutions were performed immediately before tumor injection using plastic syringes. ONYX-015 infusions were given via intra-arterial catheter over 3–5 min. This infusion was followed immediately by a 10-ml D5W/Electrolyte 48 solution flush given in identical fashion. Vital sign assessments were taken at baseline, at the conclusion of the infusion, and then every 30 min for a total of 2 h. Patients were observed overnight in the hospital after injection at the discretion of the Principal Investigator.

For cycles 3 and 4 (starting on days 22 and 50), chemotherapy was given for 5 consecutive days starting within 6 h after the *dl1520* infusion. Leucovorin (LCV) 20 mg/m² i.v. was followed by 5-FU 425 mg/m²/day by i.v. bolus. Chemotherapy dose modifications because of toxicity were made according to

	(Study day)	Pre	1	4	8	22	50	78+
Treatment								
• ONYX-015 h.a.i.			X		X	X	X	X
• 5-FU/leucovorin i.v.						X	X	X
Assessment								
• Pharmacokinetics			X			X		
• Viral replication, shedding	X			X*				
• Cytokine assessment	X		X					
• Neutralizing antibodies	X					X	X	X
• Efficacy (CT scan, serologic)	X					X	X	X

Fig. 1. Treatment and assessment schema. The treatment and assessment schema is outlined below by study day. *h.a.i.*, hepatic artery infusion; *pre*, pretreatment. *, day 4 assessment of replication and shedding was based on quantitative PCR of the blood (genomes/ml) ~72 h (± 24 h) after the first injection. Patients with evidence of antitumor activity on day 78 were eligible to continue treatment every 28 days for up to four additional cycles (day 78+).

² The abbreviations used are: 5-FU, 5-fluorouracil; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PT, prothrombin time; PTT, partial thromboplastin time; IL, interleukin; TNF, tumor necrosis factor; CT, computed tomography; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; CEA, carcinoembryonic antigen; OTC, ornithine transcarbamylase; ARDS, adult respiratory distress syndrome.

guidelines at each study center. Up to four additional cycles of combination *dI1520* and chemotherapy were allowable if treatment was well tolerated and symptomatic tumor progression had not been documented.

Toxicity Assessment on Study. The schedule of activities for patients on study is outlined in Fig. 1. Blood was drawn on day 8 of each cycle for serum chemistry, complete blood count, PT/PTT, liver function tests (AST, ALT, total and direct bilirubin, and alkaline phosphatase), and lactate dehydrogenase. Toxicity, including reports of adverse events, was assessed throughout treatment and for at least 28 days after treatment completion. The NCIC-Common Toxicity Criteria were used to categorize and grade toxicities.

Quantitative PCR Testing of Blood for Viral Genomes. The first 5 patients had pharmacokinetic blood draws taken on cycle 1 (day 1) and cycle 3 (day 22) at the following time points after *dI1520* infusion: 5, 10, 30, 60, 90, 120, 180, and 360 min. Plasma was tested for virus by quantitative PCR. Blood was tested for the presence of *dI1520* by quantitative PCR on day 1 (pharmacokinetic draws, described above) and on day 4 (± 1) of cycle 1 in patients consenting to this extra blood draw. PCR for *dI1520* was performed using the TaqMan assay, which quantitates the number of ONYX-015 genomes in human plasma (the amplicon overlaps the E1B region deletion and does not detect wild-type adenovirus sequences). PCR details are available (35). In brief, viral DNA is extracted from patient samples, standards, and controls using QIAmp DNA mini kit. The lower limit of detection is 1.05×10^4 particles of ONYX-015 per ml of plasma. The presence of PCR inhibitors in the sample is monitored using an independent PCR reaction.

Mathematical Modeling of Viral Replication and Shedding into the Blood. The following data were used to calculate the estimated number of viral genomes produced and shed into the blood on cycle 4 in patient 3016: viral load was below the level of detection at 24 h after infusion ($<10^4$ particles/ml); 10^5 particles/ml at 48 h; 1.5×10^5 particles/ml at 72 h; 4×10^4 particles/ml at 96 h; below the level of detection at 120 h. We broke time into three periods: 0–48, 48–72, and >72 h. From 0–48 h, we assume the virus grows exponentially starting at $v_0 = 1$ g/ml. Similarly for $t > 72$ h, we assume the virus decays exponentially; note that $v_{120} = 2859$ g/ml, which is less than the limit of detection and, therefore, is consistent with the data. From 48–72 h, we fit a smooth function, using the values and derivatives (from the exponential functions) at 48 and 72 h. This gives us four pieces of data allowing us to uniquely fit the cubic equation $v_t = at^3 + bt^2 + ct + d$. The resulting function is $v_t = (11/4)t^3 + (49/2)t^2 + (283/4)t - (129/2)$; all times 10^5 . The clearance rate from $t_{1/2} = 12$ min (see pharmacokinetic results) is $(\ln 2/0.2)/h$.

Neutralizing Antibody Level Assessment. Neutralizing antibodies to *dI1520* were assessed at baseline, day 22, and day 50. Titers against *dI1520* were determined on blood samples as follows. Patient and control samples were incubated at 55°C for 30 min to inactivate complement. Clinical plasma samples determined previously to produce high, midrange, and negative titers were designated as plasma controls. Each dilution was mixed with adenovirus stock at a titer prequalified to produce 15–20 plaques/well of a 12-well dish in DMEM growth medium. The patient samples and controls were inoculated for 1 h at room temperature, and applied to 70–80% confluent JH293 cells in 12-well dishes. After 2 h of incubation at 37°C, 5% CO₂ plasma-virus mix was removed, and 2 ml of 1.5% agarose in DMEM was added to each well. Plates were read on day 7 after inoculation by counting the number of plaque forming units per well. The titer of neutralizing antibody for each sample was reported as the dilution of plasma that reduced the number of plaques to 60% of the number of plaques in the virus control without antibody.

Cytokine Assessment. Cytokine ELISAs (R&D Quantikine kits) were performed on patient serum as described (35); the following cytokines were assessed: IL-1, -6, -10, TNF, and IFN- γ . In brief, patient peripheral blood was collected by venipuncture into Vacutainer tubes without anticoagulant. Serum samples were extracted from clotted samples and stored at -80°C . Multiple serum samples collected over the treatment course of each patient were analyzed simultaneously (in triplicates), using cytokine-specific immunoassay reagents according to manufacturer's protocols. The colorimetric reaction was quantified as a function of absorbance at 540 nm (SpectraMax 340; Molecular Devices, Sunnyvale, CA). Cytokine concentration was calculated according to a reference standard curve generated with four parameter logistic curve fit and absorbance values of known, graded concentrations of recombinant cytokine.

p53 Gene Status Determination. *p53* gene sequence (exons 2–11) was determined as described (27) from the tumor to be injected whenever possible

($n = 14$). In brief, DNA was extracted directly from sections of formalin-fixed, paraffin-embedded needle biopsies. If necessary, tumor cells were microdissected from sections of paraffin-embedded tissue. DNA was isolated from tumor cells using phenol-chloroform extraction and ethanol precipitation, and the *p53* gene was amplified in several fragments by PCR. The amplicons were purified by gel electrophoresis and analyzed using either the DNA Sequencing kit (Boehringer Mannheim, Indianapolis, IN) or the ABI Model 310 automated sequencer. Accurate reading of the sequencing gels and the sequencer printout was confirmed by National Biosciences, Inc. (Plymouth, MN).

Tumor Response Assessment. The antitumoral activity of single agent *dI1520* was determined after cycles 1 and 2. Combination therapy efficacy was determined after cycle 4 and every two cycles thereafter. Tumor size (cross-sectional area) was determined by contrast-enhanced CT scans. Response was assessed separately on the intrahepatic and extrahepatic tumor foci (if present). All of the intrahepatic masses were measured and included in the response assessment. CR was defined as complete disappearance of all of the tumor at the assessed site; PR as regression of the overall tumor mass by $\geq 50\%$ but $< 100\%$; SD as tumor decrease or increase in size by $< 25\%$; PD as $\geq 25\%$ increase in overall tumor cross-sectional area.

Regulatory Approvals and Conflict of Interest Issues. This trial was carried out under oversight by the United States Food and Drug Administration and the Institutional Review Boards of each participating institution. None of the Principle Investigators had any financial interest (*e.g.*, stock, stock options) in the sponsoring company during the trial. Clinical toxicity and efficacy data were monitored by an independent clinical research organization. Guidelines of the Declaration of Helsinki and Good Clinical Practice were followed.

RESULTS

Baseline Patient Characteristics. Baseline patient characteristics are described in Table 1. The tumor type was colorectal in 88% and pancreatic in 12%. The median age of patients on study was 59 years old (range, 39–78), and 28% were female. Prior chemotherapy had been received in 88% of patients. Most patients had a Karnofsky Performance Status (KPS) of 90–100%. *p53* gene status of the tumor could be obtained in approximately half of patients; 7 tumors had mutant sequences and 7 had wild-type gene sequences (exons 2–11).

Treatment Characteristics. Twenty-five patients received at least one cycle of *dI1520*. Eighteen patients subsequently received at least

Table 1 Baseline patient characteristics by dose group

		($n = 27$)
Age (years)	Mean \pm SD	59.0 \pm 10.06
	Median	59.0
	Range	39–78
Gender	Female	8 (30%)
	Male	19 (70%)
	Baseline KPS	14 (52%)
Baseline KPS	100	3 (11%)
	90	8 (30%)
	80	2 (7%)
	70	0
	60	0
	Unknown	0
	Ethnic group	Caucasian
Black		0
Asian		3 (11%)
Other		0
Prior therapy	Surgery and chemo	19 (70%)
	Surgery alone	3 (11%)
	Chemo alone	1 (4%)
	Surgery/chemo/other	4 (15%)
Colorectal histology	Yes	24 (89%)
	No	3 (11%)
Months since diagnosis of liver metastases	Median	15.2
	Range	0.03–53.09
Total bilirubin (baseline)	< 0.7	11 (44%)
	0.7–2.0	14 (56%)
Tumor area (baseline)	Median	48.8
	Range	5.6–385.66

one cycle of *dl1520* plus 5-FU and leucovorin (mean 2.9 ± 1.65 ; median three cycles, maximum six).

Viral Pharmacokinetics. Quantitative PCR testing of the blood for ONYX-015 was performed at predetermined time points over the first 6 h after injection on cycles 1 and 3 ($n = 5$ patients; Fig. 2). The virus was rapidly cleared from the blood over 6 h. The pharmacokinetic parameters were nearly identical during cycles 1 and 3 (after high-level antibody titer increases): $t_{1/2} \alpha$ (10 versus 14 min, respectively) and $t_{1/2} \beta$ (113 versus 135 min, respectively) were therefore not demonstrably affected by neutralizing antibody levels.

Adverse Events. *dl1520* was generally well tolerated both as a single agent (cycles 1 and 2) and in combination with 5-FU/LV (cycles 3 and higher). No deaths occurred on study; after withdrawal from study, patients have died only as a result of tumor progression. Adverse events during cycles 1 and 2 of *dl1520* (single agent) are reported by grade in Table 2. Nearly all of the patients reported flu-like symptoms, including fever, myalgias, asthenia, and/or chills. Chills, myalgias, and flu-like symptoms were mild to moderate (grade 1–2) in most cases, and the duration of these symptoms was typically short (<72 h). Cyclical fevers occurred in some patients at a period of ~72 h. No patients discontinued therapy on the basis of flu-like

Table 2 Incidence of adverse events by toxicity grade for cycles 1 and 2: *dl1520* alone ($\geq 18\%$ incidence)

Adverse events	Life-threatening				Total
	Mild/moderate	Severe	Fatal	Unknown	
Fever	22	3	0	0	25 (93%)
Chills	19	0	1	0	20 (74%)
Alkaline phosphate increase	13	3	0	0	16 (59%)
AST increased	13	1	1	0	15 (56%)
Nausea	12	1	0	0	13 (48%)
Lymphopenia	8	4	0	0	12 (44%)
LDH increased	11	0	0	0	11 (41%)
Bilirubinemia	6	1	3 ^a	0	10 (37%)
ALT increased	8	2	0	0	10 (37%)
Abdominal pain	7	2	0	0	9 (33%)
Hyperglycemia	7	1	0	1	9 (33%)
Asthenia	9	0	0	0	9 (33%)
Hypochromic anemia	8	0	0	0	8 (30%)
Granulocytosis	8	0	0	0	8 (30%)
Vomiting	6	2	0	0	8 (30%)
Myalgia	5	1	0	0	6 (22%)
Diarrhea	6	0	0	0	6 (22%)
Tachycardia	6	0	0	0	6 (22%)
Anemia	5	1	0	0	6 (22%)

^a Hyperbilirubinemia was caused by intrahepatic tumor progression in two of these cases; in 1 patient the adverse event was categorized as “definitely” related. The bilirubin in this patient decreased back down to grade one within 7–10 days on both cycles 1 and 2.

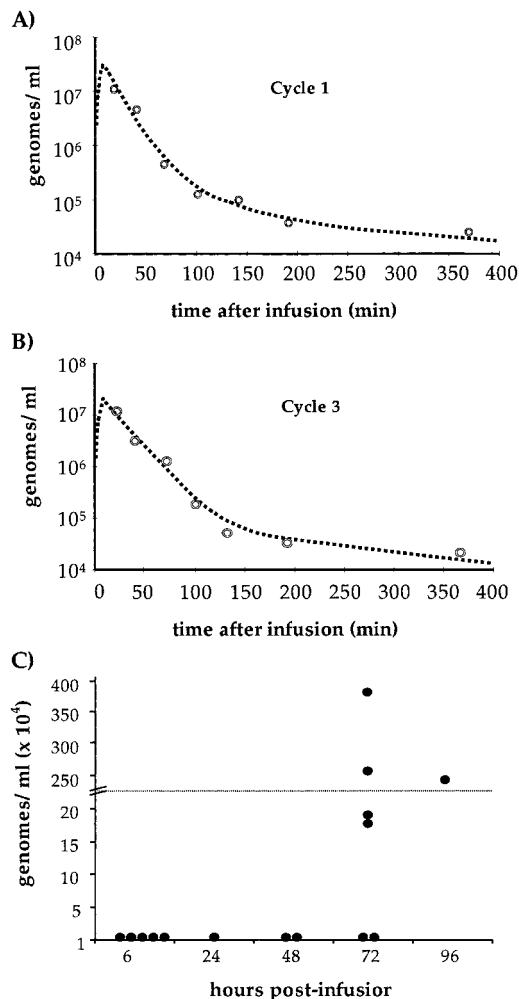


Fig. 2. Pharmacokinetics, clearance, and reappearance of ONYX-015 in plasma after hepatic arterial infusion. The first 5 patients treated in Phase II (2×10^{12} particles) had pharmacokinetic blood draws taken on cycle 1 (day 1, A) and cycle 3 (day 22, B) at the following time points after ONYX-015 infusion: 5, 10, 30, 60, 90, 120, 180, and 360 min. The genomes of virus per ml of plasma were determined by quantitative-PCR. C, genomes/ml in plasma for all patients with samples tested between 6 h and 96 h after virus infusion (pooled data on 7 patients).

symptoms. A detailed description of chemotherapy-related toxicity will be the subject of another manuscript.³ The frequency and severity of chemotherapy-related toxicities were within the range expected when these agents are administered without *dl1520*.

No treatment-emergent clinical hepatotoxicity occurred during treatment, despite preexisting liver abnormalities because of intrahepatic metastases in more than half of the patients at baseline. Transient grade 1–2 hyperbilirubinemia was detected in approximately one-third of patients. In addition, two patients with grade 1–2 hyperbilirubinemia before treatment experienced reversible grade 3/4 hyperbilirubinemia categorized as “probably” or “definitely” related to the virus. The liver toxicity resolved within 7–10 days. These patients received two or more additional cycles without complication. Transient low-grade (1–2) transaminitis was documented in several patients (after single agent virus) and was classified by the investigator as “possibly attributable” to *dl1520*; the laboratory abnormalities resolved within 7–12 days and did not reoccur after subsequent treatments. In light of the treatment-associated death because of ARDS on trial with a replication-defective adenovirus in a patient with OTC deficiency, it is notable that no coagulation abnormalities/disseminated intravascular coagulation were noted in any patients.

A single patient developed a systemic inflammatory reaction after treatment with cycle 4. This patient had tolerated three previous cycles of therapy without significant toxicity. Within the first 48 h after cycle 4 the patient developed a temperature of 104°F along with a significant lactic acidosis and localized vascular leak at sites of metastases in the chest. Within 5 days after treatment the patient was back to clinical and laboratory baseline, and was discharged soon thereafter without long-term sequelae. The patient serum cytokine profile after this cycle of treatment was highly unusual compared with cycles 3 or 4 for other patients (Fig. 5; $P < 0.001$ for baseline versus 3 h and/or 18 h) and to cycle 1 for this patient (Fig. 6).

Viral Genomes in Blood after Initial Clearance. Quantitative PCR of the blood was performed on day 1 (6 or 24 h) and on day 4 (± 1) to assess viremia and surrogate evidence suggestive of viral

³ T. Reid, A Phase II trial of 5-fluorouracil, leucovorin and Onyx-015 in patients with colorectal metastases to the liver, manuscript in preparation.

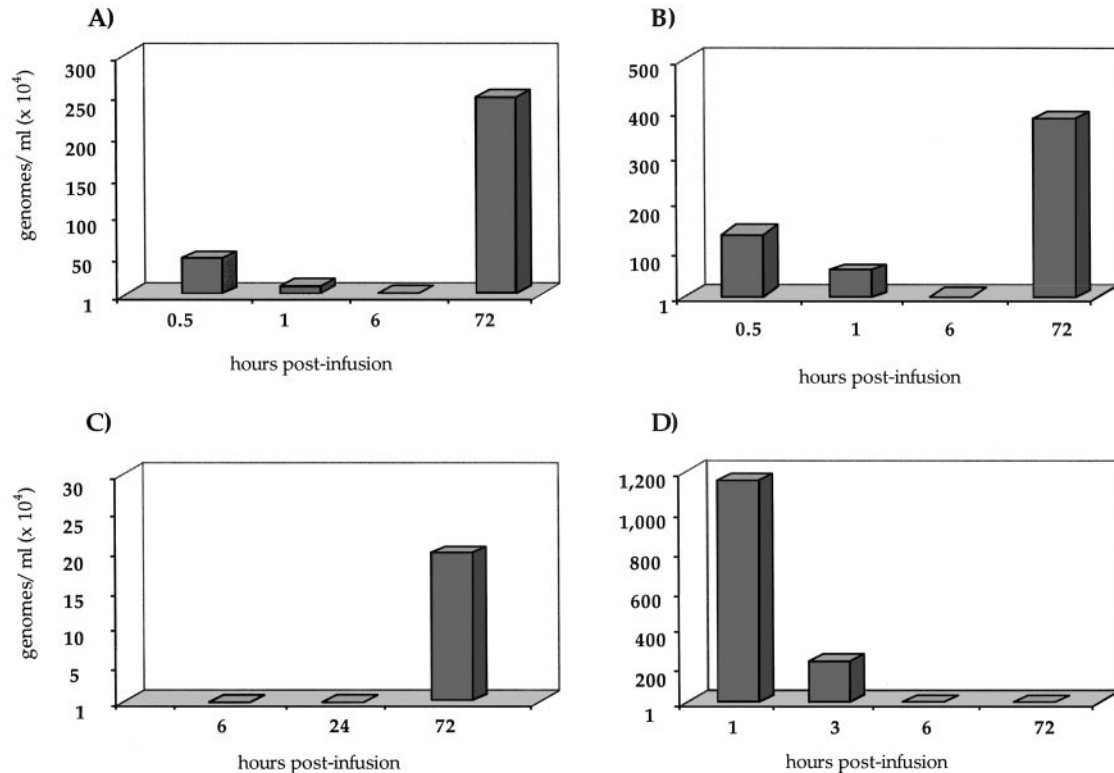


Fig. 3. Viral genomes shed into the blood. Quantitative PCR of the plasma was performed for quantitation of Onyx-015 (genomes/ml) on day 1 (0.5–6 h after treatment) and on day 3 (72 h after treatment) of cycle 1 ($n = 4$ patients with multiple samples tested). After Onyx-015 clearance from the blood within the first 6 h after infusion (A–D), viral genomes are detectable again at 72 h in 3 of 4 patients at levels that are 18–396-fold above the lower limit-of-detection for the assay (A–C).

replication (Fig. 3). Input virus was rapidly cleared within 6 h after the infusion to levels at or below the level of detection ($<10^4$ genomes/ml) in all 7 of the patients. Of these seven patients, 5 had viremia on day 4–5 at levels 17–400-fold greater than the limit of detection (Fig. 3; $P < 0.001$ for 6 versus 72 h). All 5 of the patients had colorectal carcinomas.

One patient had blood samples obtained daily after the cycle 4 infusion for 9 days (Fig. 4). Two cycles of viral shedding are demonstrated; interestingly, this is despite high level neutralizing antibodies and antiviral cytokines. Mathematical modeling of the viral replication cycle allowed calculations of the estimated peak concentration in blood: 2.2×10^5 genomes/ml estimated to occur at 60 h after infusion; the area under the curve = 3.2×10^5 genomes/ml \times days; and the total number of viral genomes produced, shed into, and detected within the blood during the replication cycle = 1.3×10^{11} genomes. Of note, the estimated number of genomes produced and shed is a fraction of the total virus produced within the tumor and, therefore, this is a determination of the minimum amount produced.

Immune Response. Neutralizing antibody titers to *d11520* (Ad5 protein coat) were positive in ~50% of patients before treatment. Titers increased and/or became positive in all of the patients after intravascular administration with *d11520*. The median antibody titer after a single cycle was ~1:10,000. Titers continued to rise after a second cycle of treatment (median ~1:25,000).

Acute inflammatory cytokine expression was assessed in circulating WBCs (RNA expression) and serum (ELISA) of 9 patients at baseline, 3 h and 18 h after treatment cycles (Fig. 5). The following cytokines were measured: TNF, IFN- γ , IL-1, -6, and -10. IL-1 levels were not detectable at baseline, rose significantly by 3 h, and returned to near baseline by 18 h. TNF, IL-6, and IFN- γ all increased markedly by 3 h after treatment. Levels decreased between 3 and 18 h, but

remained above baseline in most patients. The magnitude of changes in cytokine levels varied greatly between individual patients (Fig. 5) and from cycle to cycle in a given patient (Fig. 6).

Antitumoral Activity. Antitumoral activity was demonstrated with *d11520* in combination with 5-FU and leucovorin (Fig. 7). Overall tumor responses were as follows: 3 PR (11%), 4 MR (15%), 9 SD (33%), and 11 PD (41%). The antitumoral effects of *d11520* cannot be adequately assessed in patients who had not received previously this chemotherapy regimen; responses are conceivably because of chemotherapy alone. However, the three minor responses (30–48% shrinkage) in 5-FU/LCV-refractory patients are evidence for *d11520*-associated antitumoral activity. Patient 3013 presented with a rapidly progressive CEA level and 5-FU-refractory colon cancer within the liver after three prior 5-FU-based chemotherapy regimens, including bolus and continuous infusions of 5-FU. After two cycles of single agent *d11520*, the CEA continued to increase (from 350 to 450). After the second cycle of combination treatment with *d11520* plus 5-FU/leucovorin (cycle 4), a sudden spike in the CEA level was demonstrated from 450 to 750 over several days; a significant increase in intratumoral necrosis was noted on CT scan at this time. The CEA subsequently decreased significantly over time down to 77 ng/ml (83% reduction) over ~20 weeks (Fig. 7G). An interim CT scan demonstrated significant shrinkage (~30%), but unfortunately the patient refused a CT scan after combination therapy, so the final radiographic response is not known. A second 5-FU-refractory patient had PD on virus alone, followed by significant shrinkage on combination therapy. Tumor shrinkage was 35% from baseline and 50% from the time of combination therapy initiation.

Predictors of antitumoral efficacy cannot be definitively assessed in a study of this size with different tumor histologies, differing pretreatment characteristics, and varied prior treatments. However, several observations were of note and may generate hypotheses to be tested in

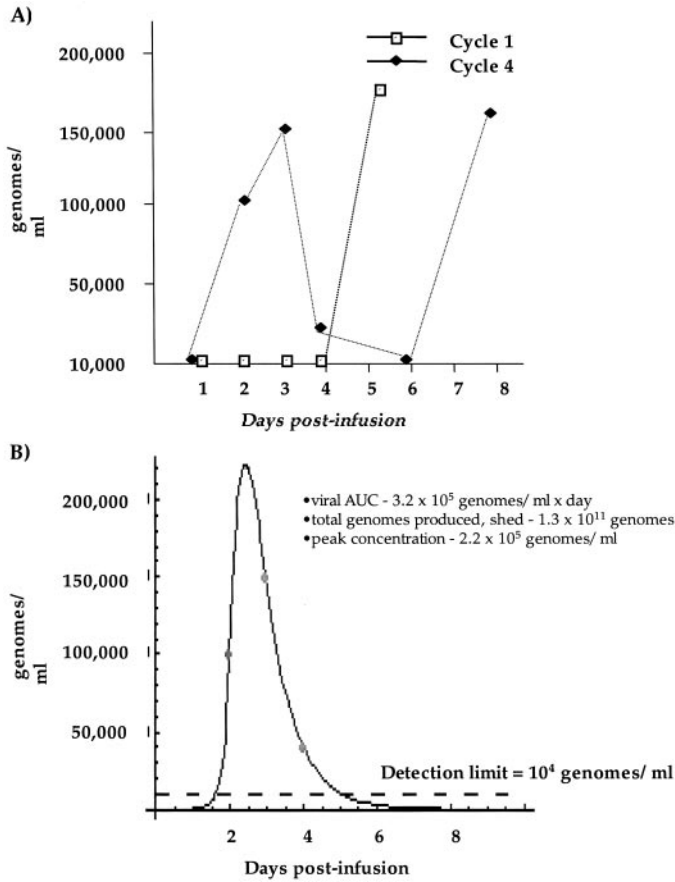


Fig. 4. Posttreatment viral genome concentrations for cycle 1 versus cycle 4 (A) and mathematical modeling of replication cycle (B). To determine whether viral replication could occur on cycle 4 after marked neutralizing antibody development and inflammatory cytokine generation, we performed daily quantitative PCR of plasma from patient 3016 for both cycles (A). Viral replication and shedding is suggested during both cycles. Cycle 4 viremia occurs earlier than on cycle 1. Note evidence for a second cycle of replication and shedding during cycle 4. For cycle 4 data, mathematical smooth-fit modeling of the replication cycle is shown (B), demonstrating the predicted timing and magnitude of the replication cycle. AUC, area under the curve. Total viral genomes produced and shed over the course of one replication cycle is calculated based on AUC and clearance rate.

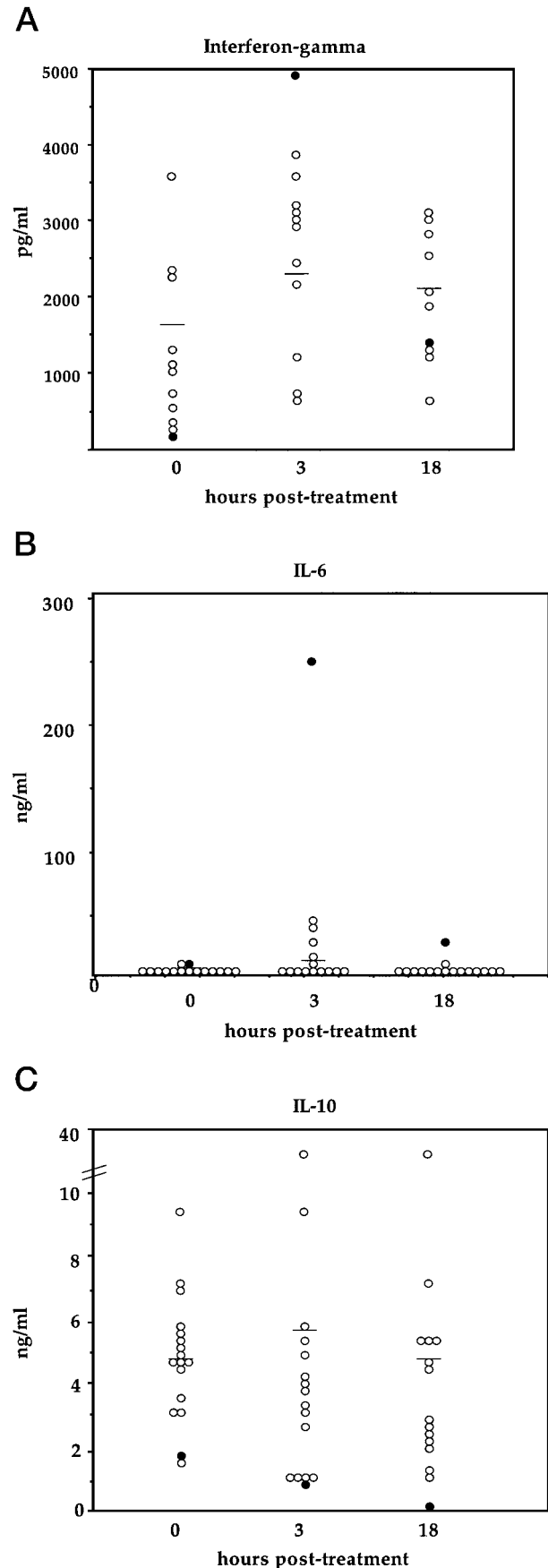


Fig. 5. Acute inflammatory cytokine levels after viral infusion. Cytokines were measured by ELISA assay on serum at the time points listed (cycles 3–6). Note that the single patient with a severe systemic inflammatory reaction (3016) is represented by ●.

future trials. Of the 3 patients with 5-FU-refractory tumors who had minor responses to 5-FU plus *dl1520*, all had delayed viremia. Two were *p53* gene sequence mutant and one was *p53* wild-type. The neutralizing antibody titers were positive (>1:20) at baseline in 2 of the 3 patients (approximately 1:10,000–1:30,000), and all 3 were positive after cycles 1 and 2.

DISCUSSION

We performed a Phase II trial of the replication-selective adenovirus *dl1520* administered by hepatic arterial infusion, in combination with i.v. 5-FU and leucovorin, in patients with liver metastases from gastrointestinal (primarily colorectal) carcinomas. This is the first Phase II trial describing the intravascular administration of genetically engineered, replication-selective virus in patients. This study has major implications regarding the safety and feasibility of adenovirus administration into the blood, and into the hepatic artery specifically. This is particularly true given the report of a patient death after the hepatic artery administration of a replication-incompetent adenovirus into the hepatic artery for OTC deficiency. We report here that adenovirus administration into the blood can be performed with acceptable toxicity in patients with metastatic cancer. Importantly, the virus was well tolerated at doses that resulted in significant cyclical/

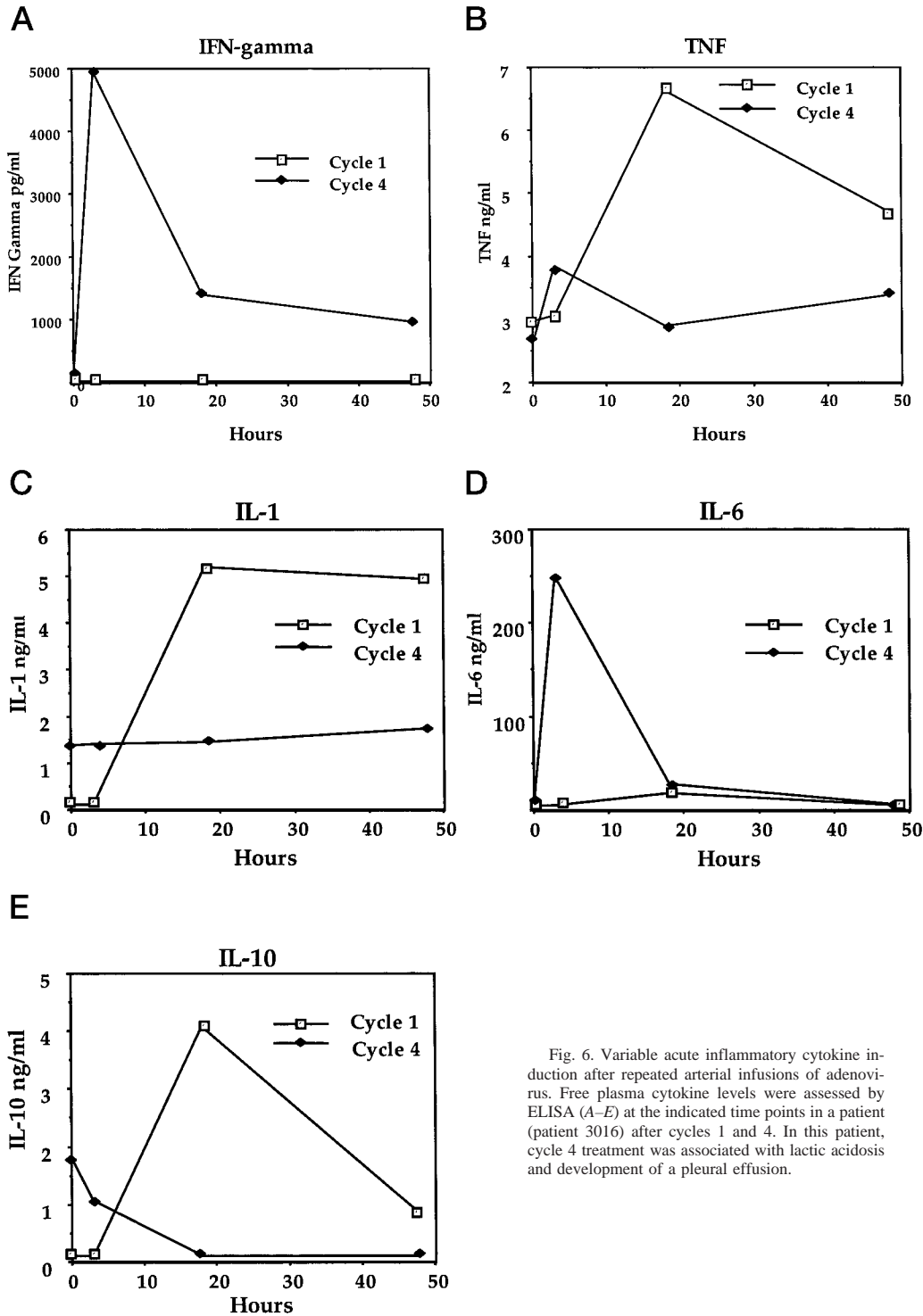


Fig. 6. Variable acute inflammatory cytokine induction after repeated arterial infusions of adenovirus. Free plasma cytokine levels were assessed by ELISA (A–E) at the indicated time points in a patient (patient 3016) after cycles 1 and 4. In this patient, cycle 4 treatment was associated with lactic acidosis and development of a pleural effusion.

recurrent viremia consistent with viral replication, proinflammatory cytokine induction, and evidence for chemosensitization of refractory tumors.

Concerns were raised about the safety of hepatic arterial adenovirus after the patient death on a clinical trial for patients with OTC deficiency (12–15). This patient received a dose of $\sim 4 \times 10^{13}$ particles with a replication-deficient adenovirus expressing the OTC gene. By report, in <24 h the patient experienced hyperammonemia, ARDS, and disseminated intravascular coagulation; this was followed over the next few days by multiorgan system failure and death (12).

These complications were not encountered on this study. Despite being well tolerated overall in the vast majority of patients, significant idiosyncratic toxic events did occur in several patients. Of note, reversible grade 3/4 hyperbilirubinemia (direct) attributable to virus treatment occurred in 2 patients. In addition, reversible grade 4 dyspnea occurred in 1 patient with a systemic proinflammatory response associated with profound lactic acidosis and peritumoral vascular leak; this event was associated with extreme elevations of IL-6 and undetectable IL-10 within the first 18 h after cycle 4 (see below). Although liver toxicity was dose-limiting in mice (32), liver toxicity

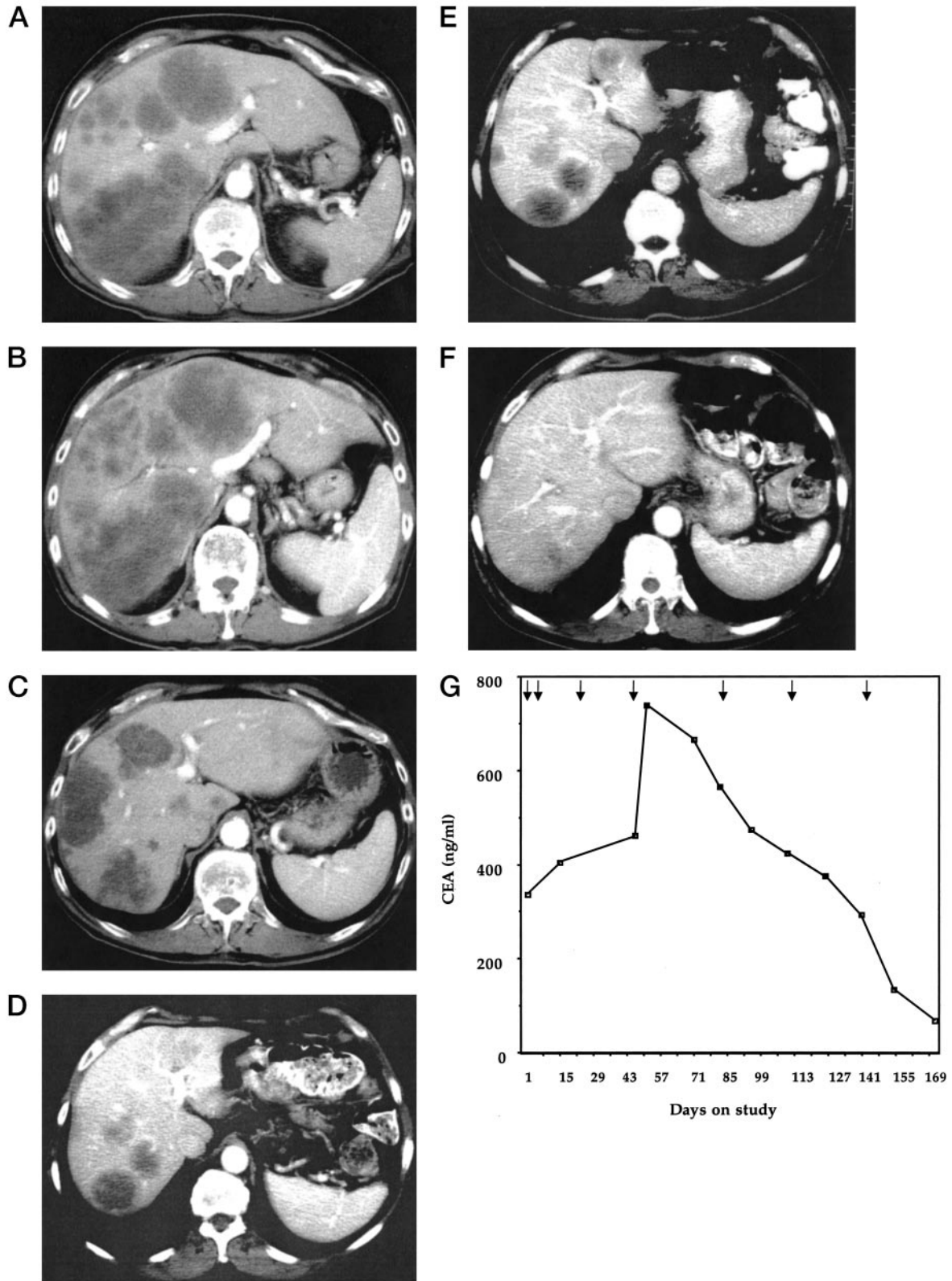


Fig. 7. Early and delayed radiographic and tumor marker changes in patients with metastatic colon cancer. Serial contrast-enhanced CT scans at the level of the left portal vein from 3 patients are shown. Patient 3006 had previously untreated metastatic colon cancer to the liver at the time of study entry (A). After two cycles of single agent *dl1520* the tumor masses were notable for acute swelling (B). After combination treatment with *dl1520* plus chemotherapy (C), the overall tumor cross-section has decreased by >50% and the vascularity of the residual masses is markedly reduced (consistent with necrosis). Patient 3005 had metastatic colon cancer that was treated previously with three cycles of 5-FU/leucovorin. Nine months later the patient presented with tumor progression (D). After two cycles of single agent *dl1520* (E), the largest tumor mass was notable for acute swelling and decreased vascularity (consistent with necrosis). After combination treatment with *dl1520* plus 5-FU/leucovorin (F), near complete disappearance of the tumor masses was demonstrated. G, patient 3013 presented with a rapidly progressive CEA level and 5-FU-refractory colon cancer within the liver after three prior 5-FU-based chemotherapy regimens, including bolus and continuous infusions of 5-FU. After two cycles of single agent *dl1520*, the CEA continued to increase (downward arrows delineate treatment cycles). After the second cycle of combination treatment with *dl1520* plus 5-FU/leucovorin (fourth arrow), a sudden spike in the CEA level was demonstrated; a significant increase in intratumoral necrosis was noted on CT scan at this time. The CEA subsequently decreased significantly over time. Significant necrosis and tumor shrinkage was noted on CT scan during this interval.

did not lead to treatment discontinuation in patients at this dose despite replacement of up to 50% of patient livers by tumor. In addition to the 2 cases of hyperbilirubinemia described above, transient mild to moderate transaminitis was documented in approximately one-third of patients. No disseminated intravascular coagulation was detected at the time points assessed. Mild to moderate fever and rigors were the most common adverse events; these were transient and did not lead to treatment discontinuation. Therefore, given that these patients had terminal cancer, hepatic arterial infusion of this E1B-55kD gene-deleted adenovirus was associated with acceptable toxicity both as a single agent and in combination with 5-FU-based chemotherapy.

Several aspects of the single idiosyncratic systemic proinflammatory response to a virus infusion are worth discussing. First, this patient had tolerated three prior infusions of *dl1520* without significant toxicity. However, after cycle 4 the patient developed localized vascular leak at the site of metastatic tumors in the chest. This reaction was associated with an increased temperature and respiratory rate, decreased serum HCO_3^- and an elevated lactic acid. Despite high level proinflammatory cytokines, viral shedding into the bloodstream consistent with replication was detectable. This patient had markedly elevated levels of IL-6 acutely compared with all of the other patients (20-fold higher than average) and compared with levels after his first three treatment cycles. In addition, unlike all of the other patients and/or treatment cycles, his IL-10 levels decreased after cycle 4 acutely and were undetectable after 18 h. Therefore, this reaction could not have been predicted. It is possible that such a reaction contributed to the patient death on the OTC trial. Future studies may define predictive factors that can be used to either avoid treating high-risk patients or to identify them soon after treatment (*e.g.*, by measuring their cytokine response) and thus allow rapid intervention.

Several important differences exist between this trial and the OTC trial. First, the viral dose tested on this study was ~10-fold lower. However, viral replication on this study almost certainly led to longer-term adenoviral exposure and shedding into the bloodstream than on the OTC trial with a replication-deficient adenovirus. In addition, 5 patients on a separate trial have now been treated *i.v.* with *dl1520* at doses of 2×10^{12} to 2×10^{13} particles (35); these doses are up to 10-fold higher than on this trial and are close to those used before the fatal event in the OTC trial. None of the complications that occurred in the OTC patient occurred in these patients. It is probable that OTC patients have a greater sensitivity to viral exposure and any ensuing systemic inflammatory reaction than did the patients on this trial. Patients with OTC deficiency have a heightened sensitivity to viral and bacterial infections compared with the general population, and hyperammonemia, ARDS, and death can result (36). A proinflammatory reaction such as was demonstrated on this trial might not be tolerated in OTC patients. Of note, the protein coats of the viral particles were identical on these two studies. On the basis of this study, it is clear that adenoviruses, including replication-selective adenoviruses, can be administered into the bloodstream, and in particular into the hepatic artery, with toxicity that is acceptable in cancer patients. Future trials of intravascular adenovirus in patients with advanced, refractory cancers are indicated.

This study is the first to clearly report data consistent with the replication of a therapeutic virus after intra-arterial administration in humans. The virus was cleared rapidly from the peripheral blood (half-life ~12 min); by 6 h, patients had blood levels at or below the limit of detection for the assay ($<10^4$ genomes/ml). Viral replication was then assessed by after the viral concentration in plasma over time. Previous clinical trials have demonstrated a strong correlation between *in situ* evidence of replication and detectable viral genomes in the blood at the same time (3). However, posttreatment tumor biopsies

in our previous trials with liver metastasis patients were usually invaluable because of necrosis. In addition, biopsies are generally feasible at only a single time point, at best, whereas blood for genome assessment can easily be drawn at numerous time points. After clearance of virus to undetectable levels within 6 h, viral concentrations of 1.7×10^5 to 4×10^6 genomes/ml were detected 72 h later; 72 h is the approximate duration of the viral replication and cell lysis cycle. This pattern of rapid and complete viral clearance followed by re-emergence of detectable viral genomes is consistent with viral replication both in mouse models and in previous human trials. Mathematical modeling based on viral genome concentrations over time in the blood documented that at least 10^{11} genomes were produced and shed into the blood over the 72 h replication cycle on cycle 4 in 1 patient who had daily genome quantitation. However, detectable virus production may still be less than the virus input. Even by assuming a relatively low virus production per cell, it is estimated that only 10^8 tumor cells were infected initially. This would represent $<0.1\%$ of tumor cells in these large masses. Therefore, future research must focus on improving the efficiency of vascular delivery and on improving the replication and potency of the therapeutic virus. For example, an E1A mutant adenovirus (*dl922-947*) has been described that is significantly more potent than *dl1520* both *in vitro* and *in vivo*, and significant efficacy was demonstrated after *i.v.* administration (31). Such viruses hold promise as intravascular treatments for cancer. Although antibodies didn't prevent efficacy or replication on this trial, if future clinical trials demonstrate that viral replication is significantly inhibited by neutralizing antibodies the utility of antibody inhibition before and during intravascular viral therapy should be explored (37). In addition, strategies to minimize antibody binding (*e.g.*, modification of coat with polyethylene glycol) or serotype switching should be explored (38).

The combination of *dl1520* plus 5-FU and leucovorin was associated with antitumoral activity. Of note, 3 patients whose tumors were refractory to 5-FU plus leucovorin chemotherapy had evidence of antitumoral effects. These findings are consistent with evidence of potential synergy after intratumoral injection of *dl1520* in head and neck cancer clinical trials (29). It is encouraging that high neutralizing antibody titers and antiviral cytokines did not prevent viral replication or efficacy. The treatment regimen on this study was designed to allow for safety assessment with virus as a single agent before cycles of combination therapy; this regimen may not be optimal for efficacy. Novel regimens should be explored in future studies.

Combination therapy with replication-selective adenoviruses and chemotherapy may hold promise based on data reported here and elsewhere. Tumor cross-resistance is unlikely with agents working through such radically different mechanisms (5), and the combination has been well tolerated to date because of a lack of overlapping toxicities (29). Additional clinical trials exploring the efficacy of this combination therapy approach are indicated. The mechanism for this chemosensitization has not been proven and it may be multifactorial (39, 40). High levels of chemosensitizing cytokines including TNF and IFN- γ were induced in many of these patients. These cytokines synergize with each other, with chemotherapy, and with hyperthermia (at temperatures recorded in most patients on trial) to destroy tumor cells. In addition, E1A gene expression itself is able to sensitize tumor cells to p53-dependent and p53-independent apoptosis. Studies are under way to determine the role of inflammatory cytokines and specific viral gene products (*e.g.*, E1A; Refs. 41, 42) in this interaction.

These findings have significant implications for the fields of viral and gene therapies, although future trials are clearly needed. Arterial delivery to tumors is now apparently feasible with human adenovirus, and such treatment can be administered with an acceptable toxicity

profile in combination with chemotherapy. This opens up the possibility of treating many tumors using this approach. The chemosensitization demonstrated by *dl1520* on trials of head and neck cancer patients has now been documented with another tumor type and another route of administration. In addition, based on these clinical results, clinical testing of replication-selective adenoviruses as i.v. therapies for metastatic tumors is feasible; a Phase I trial has been completed recently (35). To achieve equivalent levels of virus within tumors using the i.v. route, however, higher doses may be necessary. Neutralizing antibodies will almost certainly play a larger role after i.v. administration, and novel methods to inhibit antibody production may be needed.

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