Activity of the Adenoviral E1A Deletion Mutant \( dl922-947 \) in Ovarian Cancer: Comparison with E1A Wild-type Viruses, Bioluminescence Monitoring, and Intraperitoneal Delivery in Icodextrin

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Abstract
The adenoviral mutant \( dl922-947 \) has potent activity in a variety of tumors. We investigated the efficacy of \( dl922-947 \) in ovarian carcinoma; compared its activity to wild-type adenovirus, \( dl309 \), and \( dl1520 \); and investigated the use of icodextrin to enhance activity in vivo. We also assessed the utility of luciferase bioluminescence imaging to quantify the response of human ovarian carcinoma xenografts to \( dl922-947 \). Ovarian carcinoma cell lines were transfected in vitro with \( dl922-947 \), adenovirus 5 wild-type (Ad5 WT), \( dl309 \), and \( dl1520 \) and monitored for S-phase induction, viral protein expression, replication, and overall survival. In vivo, the efficacy of \( dl922-947 \) when delivered in PBS or icodextrin to female nude mice bearing IGROV1 xenografts was determined. In vitro, \( dl922-947 \) induced lysis with greater efficacy than Ad5 WT, \( dl309 \), or \( dl1520 \) in all ovarian carcinoma cell lines tested, which was associated with earlier expression of viral proteins and S-phase induction. The lytic effect in immortalized ovarian surface epithelial cells confirmed that cellular retinoblastoma pathway status is a strong determinant of \( dl922-947 \) activity. In vivo, i.p. delivery of \( dl922-947 \) (5 × 10^9 particles daily × 5) increased median survival from 20 to 96 days (\( P < 0.0001 \)) and delivery in icodextrin-enhanced survival further. However, delayed hepatic toxicity was evident in some \( dl922-947 \)-treated mice, which was not dependent upon viral replication within tumor cells or the liver. \( dl922-947 \) has potency in ovarian carcinoma and i.p. delivery in icodextrin may enhance this activity. Immunocompetent models of ovarian carcinoma are required for further evaluation of hepatotoxicity. (Cancer Res 2006; 66(2): 989-98)

Introduction
In developed countries, ovarian cancer is the sixth most common cause of cancer death (1). Even with optimal treatment, the overall 5-year survival for those with advanced disease is only 30% (2), highlighting a need for more effective therapies. Ovarian cancer rarely metastasizes outside the abdominal cavity, and i.p. delivery of conventional chemotherapy may improve survival and reduce toxicity compared with i.v. administration (3). Early gene therapy approaches using i.p. delivery of nonreplicating viruses had encouraging preclinical results, but there was minimal therapeutic benefit in clinical trials and some unacceptable toxicity (4). A more promising approach harnesses the ability of viruses to infect cells, multiply within them, and cause cell death, with released mature viral particles infecting neighboring cells.

The first replication-competent adenoviral mutant, \( dl1520 \) (Onyx-015), contains a deletion of E1B-55K, which normally inhibits p53 to prevent premature cellular apoptosis (5). \( dl1520 \) was expected to replicate selectively in many human cancers, because >50% have functional abnormalities of the p53 pathway (6). However, \( dl1520 \) could also lyse cancer cells with normal p53 status in vitro, raising questions as to its mechanism of action. In clinical trials, some tumor-selective replication was seen, but durable responses to single-agent \( dl1520 \) were rare (7), especially in ovarian cancer (8). It has since been shown that loss of E1B-55K-mediated late-viral RNA transport restricts the replication of \( dl1520 \), making it selective only for tumor cells capable of taking over the RNA export function of E1B-55K (9). However, the toxicity profile of \( dl1520 \) in over 200 patients treated in phase I/II trials was promising, with little dose-limiting toxicity (7). This has encouraged the search for more effective replicating adenoviral agents.

A second generation adenoviral mutant, \( dl922-947 \), has a 24-hp deletion in E1A-CR2, which normally interacts with host cell retinoblastoma protein (pRb). E1A-CR2 dissociates pRb from E2F, thereby enabling S-phase entry and viral DNA replication. Because the Rb pathway is abnormal in nearly all human cancers (6), including ovarian cancer (10), \( dl922-947 \) should replicate in malignant cells but not quiescent normal cells. We have previously shown that, in a range of cancer cell lines that did not include ovarian cells, the \( dl922-947 \)-driven replication was significantly less than with Ad5 WT. Following both i.t. and i.v. injection in murine xenograft models, \( dl922-947 \) showed comparable potency to Ad5 WT, and both viruses improved survival and response rates compared with \( dl1520 \) (11). A similar adenovirus, \( \Delta \)24, with the same deletion in E1A-CR2, has shown activity in preclinical models of glioma (12).

A potential contributing factor to the disappointing results in clinical trials of i.p. gene therapies is suboptimal viral delivery to malignant cells. L. P. gene therapy has previously been delivered in PBS, which is fully absorbed within 24 hours (13). Icodextrin is a high molecular weight α-1,4-linked glucose polymer with a long history of safe use in humans as peritoneal dialysis fluid. Its molecular weight prolongs intra-abdominal half-life, as absorption occurs slowly by lymphatics before systemic metabolism by α-amylase. It is estimated that, if 2 liters of fluid are maintained in the human peritoneal cavity for 24 hours, >90% of the peritoneal...
surface will come into contact with the fluid (14). Because iodex- 
drinate maintains its instilled volume for 48 hours (13), delivery of 
adenoviral agents in iodexdrinate should improve i.p. distribution and 
retention. Delivery of nonreplicating adenoviruses in iodexdrinate 
enhances transgene expression in rabbit peritoneum as well as 
murine tumor tissue and peritoneum compared with PBS (14). In 
addition, when replication-incompetent adenoviruses encoding 
p53 were administered to mice bearing p53-mutant ovarian (MDAH-2774) and p53-null prostate (PC-3) tumors, delivery in 
iodexdrinate significantly prolonged survival compared with delivery in 
PBS (14).

Here, we describe superior in vitro activity of dl922-947 compared with viruses with wild-type E1A in cycling and growth- 
arrested ovarian cancer cell lines. In proliferating immortalized 
ovidrinate surface epithelial cells, we show that Bb pathway status is 
an important determinant of dl922-947 activity. In an l.p. ovarian 
cancer xenograft model, dl922-947 improves survival at least as 
well as Ad5 WT and dl309 and has a marked survival benefit 
compared with an E1-deleted control adenovirus. In the first 
published report to date of replicating oncolytic viruses delivered in 
iodexdrinate, we show a possible survival benefit compared with 
delivery in PBS.

Ovarian cancer causes i.p. changes that are difficult to quantify, 
such as ascites formation and multiple nodules disseminated on 
the peritoneal surface. We have generated ovarian cancer cells that 
stably express firefly luciferase and emit light in the presence of 
firefly luciferin that can be quantified with an IVIS charge-coupled 
device. We have validated this bioluminescence imaging as a means of 
measuring tumor growth and response to viral agents in vivo.

Materials and Methods

Cell culture, adenoviral construction, cell viability and viral 
replication assays, and in vitro luciferase assay. IGROV1 and OVCAR4 
(obtained from R. Camalier, National Cancer Institute, Frederick, MD) were 
incubated at 37°C with 10% CO2 in air, in DMEM plus 10% heat-inactivated 
fetal calf serum (IGROV1) or RPMI plus 10% heat-inactivated 
bovine serum (OVCAR4). IGROV1 cells were kindly provided by Dr. Nelly Auersperg 
(Promega). IOSE80 cells were stably immortalized human ovarian surface epithelial cells, whose creation 
was described elsewhere (15) and which grow in NOSE-CM medium, 
and are presented as the sum of the photon counts from ventral and dorsal 
surfaces was imaged for 3 minutes (Xenogen IVIS Imaging System 100 
system). Data were analyzed using Living Image software (also Xenogen) 
and are presented as the sum of the photon counts from ventral and dorsal imaging.

Statistics. All dose-response curves, Kaplan-Meier survival curves, and statistical analyses were generated using GraphPad Prism version 3 
(GraphPad Software, San Diego, CA).

Results

dl922-947 has superior in vitro activity to Ad5 WT and dl309 
in ovarian cancer cells. Human ovarian cancer cell lines were 
transfected at MOI 10 pfu/cell with dl922-947, Ad5 WT, or an 
E1-deleted, nonreplicating control adenovirus (Ad LM-X). Cells 
were harvested, and the percentage of cells in the S phase was 
evaluated by propidium iodide staining and fluorescence-activated 
cell sorting (FACS) analysis. An increase in the S-phase percentage 
above control was first seen in IGROV1 cells 48 hours after dl922- 
947 and Ad5 WT infection and continued to increase in both cells 
up to 96 hours (Fig. 1A). dl922-947 induced more S phase than Ad5 
WT in both cell lines at 72 hours (P < 0.001) and, in IGROV1, at 
48 hours also (P < 0.05), but both groups reached similar maximal 
levels by 96 hours, after which cell death prevented further analysis. In both IGROV1 and OVCAR4 cells (Fig. 1B), more rapid expression of 
early and late viral proteins was seen following infection with 
dl922-947 than with Ad5 WT. Ad5 WT viral protein expression
eventually equaled that of dl922-947. In IGROV1 cells, E1A expression was similar by 24 hours and penton by 48 hours, whereas in OVCAR4 cells, equality of expression was not reached until 72 hours.

Virus released from IGROV1 and OVCAR4 cells transfected with dl922-947, Ad5 WT, and/or dl309 was harvested 24 or 48 hours later and then titered on JH293 cells. In both cell lines, replication occurred to such an extent that by 48 hours, the amount of virus released exceeded 10 pfu/cell, the initial input dose (Fig. 1C). In IGROV1 cells, in keeping with more rapid induction of S phase and earlier expression of viral proteins, dl922-947 replicated to a greater degree than both Ad5 WT and dl309 at both 24 hours (P < 0.01) and 48 hours (P < 0.05).

dl922-947 has a greater cytopathic effect than dl309, Ad5 WT, and dl1520 in ovarian cancer cells. The cytotoxicity of dl922-947, as quantified by MTT assay, was compared with that of dl309, Ad5 WT and the E1B-55K mutant adenovirus dl1520 in IGROV1 (Fig. 2A) and OVCAR4 cells (Fig. 2B) following infection at a range of MOI from 0.001 to 1,000 pfu/cell. The E1-deleted control virus Ad LM-X produced no reduction in survival at the same range of MOI (data not shown). In IGROV1 cells, dl922-947 had a significantly greater cytopathic effect than all three other viruses; the IC50 for dl922-947 120 hours after infection was 0.2 pfu/cell compared with 1.76 pfu/cell for dl309, 2.81 pfu/cell for Ad5 WT, and 23.6 pfu/cell for dl1520 (P < 0.001 for all compared with dl922-947). In OVCAR4 cells, a similar pattern was seen, with the IC50 for dl922-947 again significantly lower than for dl309, Ad5 WT, and dl1520. Similar patterns of cell survival were seen following infection of other human ovarian carcinoma cells, including A2780, OVCAR3, OVCAR5, and SKOV3ip1 (data not shown).

dl922-947 superiority is only seen in cells with disrupted Rb pathway. IOSE80 cells are human ovarian surface epithelial cells that have been immortalized by SV40 large T antigen, which binds to and inactivates the p53 and pRb tumor suppressor proteins. In contrast, IOSE-C21 are human ovarian surface epithelial cells derived from a separate patient that have been immortalized by overexpression of the human telomerase reverse transcriptase gene and have a demonstrably intact Rb pathway (15). Infectivity assays

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Figure 1. Activity of dl922-947 and Ad5 WT in ovarian carcinoma cells in vitro. A, S-phase induction. S-phase induction in IGROV1 and OVCAR4 cells was assessed up to 96 hours after viral infection (MOI 10 pfu/cell) by cell cycle analysis following propidium iodide staining. Proportion of cells in the S phase relative to those transfected with control adenovirus Ad LM-X (also MOI 10). Columns, means (n = 3); bars, SD. *, P < 0.001 (two tailed t test). B, expression of viral proteins. IGROV1 and OVCAR4 cells (5 × 105) were transfected with dl922-947 or Ad5 WT (both MOI 10) or mock transfected and harvested up to 72 hours later. Protein (10 µg) was separated on 10% SDS-PAGE gels and analyzed by immunoblot for expression of E1A and Penton. C, viral replication. IGROV1 and OVCAR4 cells (1 × 105) were transfected with dl922-947, Ad5 WT, and/or dl309 (all MOI 10) and harvested up to 48 hours later. Virus production in infected cells was assessed by TCID50 assay, as detailed in Materials and Methods. Columns, mean (n = 3; pfu virus produced per cell); bars, SD. Dotted line, input dose of virus. *, P < 0.05; **, P < 0.01 (two tailed t test).
indicated that IOSE80 cells were significantly easier to infect with Ad5-based vectors than IOSE-C21 (Fig. 2C), although infection rates were in excess of 80% for both cell lines at MOI 50. Following infection with dl922-947 and dl309, different patterns of cytopathic effect are seen in the two cell lines. In IOSE80 cells, as with the cancer cell lines, dl922-947 is significantly more toxic (IC50 at 168 hours, 0.014 pfu/cell for dl922-947 versus 0.19 pfu/cell for dl309), giving a d1309/dl922-947 IC50 ratio of 14.1. In contrast, although IOSE-C21 cells are intrinsically less sensitive to adenovirus-mediated cell death, this ratio is reversed (IC50 at 168 hours, 26.8 pfu/cell for dl922-947 versus 11.1 pfu/cell for dl309), giving a dl309/dl922-947 IC50 ratio of 0.414 (Fig. 2D).

dl922-947 can induce S phase in and lyse G0-G1–arrested IGROV1 ovarian cancer cells. Because as few as 10% of cells within a solid tumor may be replicating at any given time (19), we investigated whether dl922-947 could induce S phase in and lyse noncycling ovarian cancer cells. IGROV1 cells were arrested in G0-G1 by serum starvation for 72 hours (Fig. 3A) and then infected in serum-free medium with dl922-947, Ad5 WT, or Ad LM-X (MOI 10). Cells remained in serum-free conditions and were harvested up to 72 hours later for cell cycle analysis. A large increase in S-phase induction above control was seen by 24 hours in cells transfected with both Ad5 WT and dl922-947 and increased steadily up to 72 hours after infection. Ad5 WT and dl922-947 produced similar cytopathic effect in these cells (Fig. 3B), although, at all time points, dl922-947-induced toxicity exceeded that of Ad5 WT. However, the difference was less than in cycling cells.

dl922-947 significantly prolongs median survival of mice bearing IGROV1 xenografts. In two identical experiments, female nude mice were injected i.p. with 3 × 10^6 IGROV1 cells on day 1. LP, treatment was administered on days four to eight inclusive in 400 µL vehicle (PBS or 20% icodextrin). In each experiment, 8 to 16 mice per group were treated with vehicle alone, Ad LM-X (5 × 10^9 viral particles per injection [ppi]), or dl922-947 at two dose levels (5 × 10^8 or 5 × 10^9 ppi). Mice were monitored for signs of tumor or ascites formation and general well being. The results of a combined analysis are presented in Fig. 4A. In this aggressive model of ovarian cancer in which median survival for vehicle-treated
Survival of mice in these two experiments was then analyzed according to whether the virus was delivered in 20% icodextrin or PBS. In the vehicle-only and Ad LM-X cohorts, the nature of the treatment schedule, 10 mice per group were treated with Ad LM-X, Ad5 WT, or Ad 922-947 (all MOI 10) in serum-free conditions. Survival was assessed by MTT assay up to 168 hours later. Percentage cell survival compared with those transfected with control virus, Ad LM-X. Points, means (n = 3); bars, SD.

In separate experiments, using the same xenograft model and treatment schedule, 10 mice per group were treated with Ad LM-X, Ad5 WT, or Ad 922-947 (all 5 x 10^9 pppi) delivered in 20% icodextrin or PBS (Fig. 4B, right). At the higher dose of 922-947, median survival in PBS was 80.5 days, with 7 of 16 mice surviving long term. In icodextrin, the results were even more impressive with 11 of 16 mice surviving long term and the median survival not being reached (P < 0.001), with 18 mice still alive at day 125.

In the experiment comparing 922-947 with Ad5 WT, a similar reduction in tumor mass at sacrifice was again seen in the 922-947-treated mice (median mass 922-947, 0.71 g range, 0.12-2.64 g; median mass Ad LM-X, 2.40 g range, 1.04-3.83 g; P < 0.05). With Ad5 WT, this was even more marked, with four of the six mice having no evidence of residual tumor after sacrifice due to clinical deterioration. Macroscopically, many of the 922-947- and Ad5 WT–treated mice had thickening of the liver capsule and evidence of diffuse adhesion formation. Although much less marked, similar findings were evident in the Ad LM-X-treated animals. Microscopically, the livers of Ad LM-X-treated mice were generally grossly normal, although some showed evidence of patchy necrosis (Fig. 5A, I). By contrast, the Ad5 WT livers were grossly necrotic and showed diffuse eosinophilic degeneration (Fig. 5A, 2). The livers of 922-947-treated mice that had minimal visible tumor were also necrotic and vacuolated with areas of eosinophilic degeneration, but the extent was less than with Ad5 WT (Fig. 5A, 3). By contrast, in 922-947-treated mice that died with significant tumor burdens, liver architecture was grossly normal (Fig. 5A, 4). The Ad5 WT– and 922-947-treated animals alive at the termination of the experiment had grossly normal livers (images not shown).

We wished to investigate whether replication of 922-947 within tumor cells contributed to hepatotoxicity. Therefore, tumorbearing or non–tumor-bearing mice were treated with 922-947 (5 x 10^9 particles per injection on days 4-8 following tumor inoculation) and sacrificed at weekly intervals (days 11, 18, 25, 32, and 39 after inoculation; i.e., 3, 10, 17, 24, and 31 days after the final virus injection) in groups of six. Blood was taken at time of sacrifice, and full pathologic assessments were again made. Areas

**Figure 3.** Effect on G0-G_1–arrested ovarian carcinoma cells. A, S-phase induction. IGROV1 cells (3 x 10^5) were arrested in G0-G_1 by serum starvation for 72 hours and then transfected with dl922-947, Ad5 WT, or Ad LM-X (all MOI 10) in serum-free conditions. G0-G_1 arrest was confirmed by cell cycle analysis following propidium iodide staining. S-phase induction was then assessed up to 72 hours after viral infection by 72 hours of serum starvation, 2 x 10^4 IGROV1 cells were transfected with dl922-947, Ad5 WT, or Ad LM-X (MOI 10) in serum-free conditions. Survival was assessed by MTT assay up to 168 hours later. Percentage cell survival compared with those transfected with control virus, Ad LM-X. Points, means (n = 3); bars, SD.

Ten mice treated with high-dose dl922-947 died with <1.00 g residual tumor, two of whom had no evidence of any disease, indicating that their clinical deterioration was due to a factor other than tumor progression. The median time of sacrifice of these 10 mice was day 43 (range, 22-81 days), and seven of the mice received virus in PBS, three in icodextrin.

In the experiment comparing dl922-947 with Ad5 WT, a similar reduction in tumor mass at sacrifice was again seen in the dl922-947-treated mice (median mass dl922-947, 0.71 g; range, 0.12-2.64 g; median mass Ad LM-X, 2.40 g; range, 1.04-3.83 g; P < 0.05). With Ad5 WT, this was even more marked, with four of the six mice having no evidence of residual tumor after sacrifice due to clinical deterioration. Macroscopically, many of the dl922-947- and Ad5 WT–treated mice had thickening of the liver capsule and evidence of diffuse adhesion formation. Although much less marked, similar findings were evident in the Ad LM-X-treated animals. Microscopically, the livers of Ad LM-X-treated mice were generally grossly normal, although some showed evidence of patchy necrosis (Fig. 5A, I). By contrast, the Ad5 WT livers were grossly necrotic and showed diffuse eosinophilic degeneration (Fig. 5A, 2). The livers of dl922-947-treated mice that had minimal visible tumor were also necrotic and vacuolated with areas of eosinophilic degeneration, but the extent was less than with Ad5 WT (Fig. 5A, 3). By contrast, in dl922-947-treated mice that died with significant tumor burdens, liver architecture was grossly normal (Fig. 5A, 4). The Ad5 WT– and 922-947-treated animals alive at the termination of the experiment had grossly normal livers (images not shown).

We wished to investigate whether replication of dl922-947 within tumor cells contributed to hepatotoxicity. Therefore, tumor-bearing or non–tumor-bearing mice were treated with dl922-947 (5 x 10^9 particles per injection on days 4-8 following tumor inoculation) and sacrificed at weekly intervals (days 11, 18, 25, 32, and 39 after inoculation; i.e., 3, 10, 17, 24, and 31 days after the final virus injection) in groups of six. Blood was taken at time of sacrifice, and full pathologic assessments were again made. Areas
of necrosis were seen in both groups as early as day 11 (Fig. 5B, 1 and 2). There was no deterioration on days 18 and 25 (not shown), but by day 32, obvious coalescing necrosis was evident in both groups, but was more extensive in tumor-bearing animals (Fig. 5B, 3 and 4). By day 39, some improvement was seen (Fig. 5B, 5 and 6). PCR of serum indicated that viral DNA had been cleared by day 11 (72 hours after last virus injection) and did not reappear, and viral titer in the serum was always below the limit of detection (4 × 10^2 pfu/mL). Diffuse expression of E1A was detected by immunohistochemistry in day 11 livers (Fig. 5C, 1). By day 32, a few E1A-positive cells were still detected, but this did not correlate with areas or extent of necrosis (Fig. 5C, 2).

Light emitted by IGROV-LUC cells has been validated as a means of measuring tumor cell growth and response to viral agents in vitro and in vivo. IGROV-LUC cells are a clone of IGROV1 cells that stably express firefly luciferase and emit light when exposed to firefly luciferin. In vitro bioluminescence, as quantified by a bioluminometer, correlated linearly with cell number over two log scales (Fig. 6A). IGROV-LUC cells (3 × 10^6) were injected i.p. in female nude mice on day 1, and bioluminescence was measured on nine occasions with a Xenogen IVIS camera as detailed in Materials and Methods. A logarithmic increase in light output was observed for >50 days (Fig. 6A). In vitro, there was a steady increase in light emission over 7 days following infection of IGROV-LUC cells with Ad LM-X or mock infection (Fig. 6B). In contrast, infection with d922-947 (MOI 10) caused an initial increase in bioluminescence that was maximal between 72 and 96 hours, after which light output began to decrease. By 168 hours, it was significantly below starting levels (P < 0.005).

When mice bearing i.p. IGROV-LUC xenografts were treated i.p. with vehicle alone or Ad LM-X (5 × 10^8 particles daily for 5 days), a steady increase in bioluminescence was observed over time.
(Fig. 6C), consistent with tumor progression. With i.p. dl922-947 (5 \times 10^7 particles daily for 5 days), there was a small, transient increase in light output at day 8, after which bioluminescence was maintained at or below baseline levels for over 2 months (comparison with Ad LM-X, \( P < 0.05 \)).

**Discussion**

In our previous study, dl922-947 was shown to have greater activity than Ad5 WT and dl1520 against a range of human cancers *in vitro* and *in vivo*, but its use in ovarian cancer was not evaluated (11). Although coat-modified viruses with the same E1A-CR2 deletion have shown efficacy in preclinical models of ovarian cancer (20, 21), this is the first comprehensive report of the use of dl922-947 in this setting. In human ovarian cancer cell lines, the *in vitro* efficacy of dl922-947 consistently exceeded that of both Ad5 WT and dl309. The inclusion of dl309 as a second control indicated that the E3B deletion in dl922-947 is not the cause of its superior activity compared with Ad5 WT. The objective of replication-competent viral therapy is to achieve targeted lysis of malignant...
cells as a direct result of intracellular viral replication. Because the infected cell must be driven into the S phase for viral replication to take place, the observation that dl922-947 induced S phase more rapidly than Ad5 WT could explain its superior oncolytic potency. Both cell lines expressed early (E1A) and late (penton) proteins more quickly following infection with dl922-947 than with Ad5 WT and replication of dl922-947 in IGROV1 cells also exceeded that of Ad5 WT and dl309. dl922-947 also produced greater cytopathic effect than dl309, Ad5 WT, and dl1520 in both cell lines at all time points tested. In keeping with reduced viral replication, higher doses of each virus were required to kill OVCAR4 cells than IGROV1. It is also noticeable that viral replication occurs before that of the host cell DNA, as detected by FACS analysis, and this was corroborated by bromodeoxyuridine incorporation (data not shown). This implies that viral DNA synthesis occurs preferentially or at least more rapidly than that of the host cell genome.

Previously, we showed the tumor selectivity of dl922-947 by showing that S-phase induction and replication are impaired in growth-arrested normal endothelial and epithelial cells compared with cancer cell lines (11). By showing that dl922-947 induces less cytotoxicity than dl309 in ovarian surface epithelial cells with an intact Rb pathway (IOSE-C21) but far greater toxicity than dl309 in those immortalized with pRb inactivation (IOSE-80), we confirm that cellular Rb pathway status is an important determinant of dl922-947 activity. However, it remains unclear quite why dl922-947 activity is so superior to E1A wild-type viruses in cells with an abnormal Rb pathway. Although IOSE-C21 are somewhat less susceptible to infection with Ad5 vectors than IOSE80, this alone

Figure 6. Bioluminescence monitoring. A, light emission from IGROV-LUC cells correlates with cell number in vitro and increases with time in vivo. Between 1 × 10⁶ and 1 × 10⁸ IGROV1-LUC cells were plated in six-well plates in groups of six. After overnight incubation, cells were lysed and in vitro firefly luciferase assays were done according to manufacturer’s instructions. Points, means (n = 6); bars, SD. IGROV1-LUC cells (3 × 10⁶) were injected i.p. into nude female mice. On nine occasions thereafter, mice were injected i.p. with 100 μL of 50 mmol/L firefly luciferin and imaged on a Xenogen IVIS CCD device (as detailed in Materials and Methods). Light emission from defined regions of interest was quantified using Living Image software version 2.11. Points, means (n = 7); bars, SE. Representative images. B, change in light emission by IGROV-LUC cells in vitro in response to viral infection. IGROV-LUC cells (3 × 10⁶) were either mock transfected or transfected with dl922-947 or Ad LM-X at MOI 10. Cells were lysed, and in vitro luciferase assays were done up to 168 hours later according to manufacturer’s instructions. Points, mean (n = 5); bars, SD. C, change in light emission by IGROV-LUC cells over time, in response to viral infection. IGROV1-LUC cells (3 × 10⁶) were injected i.p. into nude female mice on day 1. On days 4 to 8, mice received daily i.p. injections of dl922-947, Ad LM-X (both 5 × 10⁶ ppi), or 20% icodextrin. Nine bioluminescence images were obtained from each mouse as detailed in Materials and Methods. Points, means (n = 4); bars, SD. Representative images. *, P < 0.05 (two tailed t test).
may not explain their much reduced sensitivity to dl309-mediated lysis, which suggests that other factors are influencing overall viral cytotoxicity. These two cell lines derive from separate patients and are thus not isogenic: further evaluation of these mutants in isogenic lines immunolylized by either SV40 large T or hTERT would be illuminating. Although most malignant cells have Rb pathway abnormalities (6), the proportion of cells within a tumor that are replicating at any given time may be only 10% (19). Therefore, we assessed ovarian cancer cells in G1 to investigate their response to viral infection. dl922-947 and Ad5 WT were equally capable of inducing S phase in G1-arrested cells, implying that replicating viruses actively induce G1-S transition in infected cells rather than simply taking advantage of cells already in the S phase. The cytolytic advantage of dl922-947 over Ad5 WT in cycling cells was, however, partially diminished in cells arrested in G1, presumably due to loss of E1A-CR2 function.

We have shown that the promising in vitro results seen with dl922-947 translate into a dramatic in vivo survival advantage, with many mice surviving long term following dl922-947 treatment. This is at least comparable with treatment with Ad5 WT or dl309, but the large advantage that existed between dl922-947 and the other viruses in vitro is not seen in vivo. This may occur as a result of toxicity to normal tissue (see below), thereby reducing any therapeutic advantage, while little is known about the kinetics of human adenovirus clearance from the peritoneal cavity of mice, especially nude mice. Interestingly, the E1-deleted nonreplicating control virus, Ad LM-X, also increased median survival by 7 days over the vehicle-treated group. This observation, which has been made previously (22), implies either a nonspecific inflammatory effect of adenoviral proteins or possible low level replication. The latter has been shown in tumor cells infected with E1-deleted viruses (23), which may be due to abnormalities in G2-M cell cycle checkpoint in malignant cells (23) or genes present in the remainder of the genome that partially compensate for the loss of E1, such as E4orf6/7, which can also disrupt the interaction between pRb and E2F (24).

We compared the delivery of viral agents in icodextrin to delivery in the conventional carrier vehicle PBS in an attempt to optimize viral delivery. Previous human experience with icodextrin has been with the 4% solution in peritoneal dialysis. Because icodextrin is metabolized by α-amylase, which is absent in the peritoneal cavity of humans but present in rodents (14), we used 20% icodextrin to deliver viruses i.p. to mice. Delivery of dl922-947 at two dose levels in icodextrin prolonged survival compared with delivery in PBS, and although this difference did not quite reach statistical significance, a similar trend to improved survival was observed in repeated experiments. The survival advantage seen with icodextrin was not due to a therapeutic effect of the delivery fluid itself because the nature of the carrier vehicle did not influence survival in the control groups. Similarly, icodextrin produced no increase in toxicity.

Following sacrifice due to clinical deterioration, postmortem examination of the mice treated with dl922-947 and Ad5 WT revealed no evidence of residual tumor in some. However, several were found to have evidence of hepatic necrosis. Generally, a spectrum in the response to dl922-947 was observed: some mice received little therapeutic benefit but did not suffer liver damage, whereas the tumors in others responded to the treatment, but liver toxicity contributed to their demise. Although those mice treated with Ad5 WT and dl922-947 that survived until the end of the experiment had grossly normal livers, the occurrence of significant liver toxicity is clearly of concern. We studied livers prospectively over 40 days in both tumor-bearing and non–tumor-bearing mice treated with dl922-947. In both cohorts, areas of hepatic necrosis were seen 3 days after the final virus injection (day 11). However, by day 32, more severe changes were seen, especially in the tumor-bearing animals. Although there was evidence of diffuse E1A expression on day 11, this had largely disappeared by day 32, and there was no correlation between E1A expression and the site or extent of necrosis. Acute hepatotoxicity is well described following i.v. administration of adenoviral vectors (25), and the etiology is likely to be multifactorial (26, 27). Data regarding hepatic damage following i.p. administration are much more limited. No liver toxicity was reported in either the preclinical (28) or clinical (8) evaluation of dl520 in ovarian cancer, but i.p. delivery of 107 particles of a fiber-modified E1A-CR2-deleted virus Ad5/3−Δ24 was shown to induce acute hepatic necrosis in severe combined immunodeficient (SCID) mice (29). We observed changes in both tumor-bearing and tumor-free animals, suggesting that tumor-specific viral replication is not the only determinant of this response. Our data were obtained from nude mice, and it is known that the immune status of mice is a determinant of toxicity following i.v. administration (30). This may explain why Raki et al. found liver damage at relatively low viral doses in SCID animals (29) and underlines the need for an immunocompetent model of ovarian cancer that supports adenoviral replication. These data also emphasize the need for prolonged and careful examination of toxicity in xenograft models.

We used bioluminescence to evaluate the response of ovarian cancer cells to dl922-947. This is a convenient method of measuring tumors in individual mice, and there are some reports of its use in assessing responses to viral gene therapy (21, 31). Our bioluminescence data show a correlation between in vitro and in vivo responses to oncolytic viral agents and suggest that this technique can show early changes in tumor cell behavior following viral delivery. In vitro, light emission increased following infection with dl922-947 and was maximal at 72 to 96 hours, corresponding to the time of peak S-phase induction. The transient increase in bioluminescence following administration of dl922-947 to murine IGROV-LUC xenograft models may be the in vivo equivalent of these in vitro changes. These short-lived increases may result from activation of the cytomegalovirus promoter controlling luciferase expression by adenoviral promoters, thereby allowing an indirect visual measurement of virally induced changes in cellular activity. Most importantly, the maintenance of light emission at baseline levels for >2 months in dl922-947-treated mice correlates with the powerful antitumor effect in mice treated with this virus.

In summary, the E1A-CR2 mutant dl922-947 has impressive activity in ovarian carcinoma. Although i.p. delivery in murine models was associated with some delayed hepatotoxicity, dl922-947 was still able to produce some long-term survivors in an aggressive xenograft model, suggesting that this mutant may have clinical potential in the treatment of ovarian carcinoma.

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References
Activity of the Adenoviral E1A Deletion Mutant dl922-947 in Ovarian Cancer: Comparison with E1A Wild-type Viruses, Bioluminescence Monitoring, and Intraperitoneal Delivery in Icodextrin

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