Activity of the Adenoviral E1A Deletion Mutant *dl*922-947 in Ovarian Cancer: Comparison with E1A Wild-type Viruses, Bioluminescence Monitoring, and Intraperitoneal Delivery in Icodextrin

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Abstract

The adenoviral mutant *dl*922-947 has potent activity in a variety of tumors. We investigated the efficacy of *dl*922-947 in ovarian carcinoma; compared its activity to wild-type adenovirus, *dl*309, and *dl*1520; 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 *dl*922-947. Ovarian carcinoma cell lines were transfected *in vitro* with *dl*922-947, adenovirus 5 wild-type (Ad5 WT), *dl*309, and *dl*1520 and monitored for S-phase induction, viral protein expression, replication, and overall survival. *In vivo*, the efficacy of *dl*922-947 when delivered in PBS or icodextrin to female nude mice bearing IGROV1 xenografts was determined. *In vitro, dl*922-947 induced lysis with greater efficacy than Ad5 WT, *dl*309, or *dl*1520 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 *dl*922-947 activity. *In vivo*, i.p. delivery of *dl*922-947 (5×10^9 particles daily $\times$ 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 *dl*922-947-treated mice, which was not dependent upon viral replication within tumor cells or the liver. *dl*922-947 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, *dl*1520 (Onyx-015), contains a deletion of E1B-55K, which normally inhibits p53 to prevent premature cellular apoptosis (5). *dl*1520 was expected to replicate selectively in many human cancers, because >50% have functional abnormalities of the p53 pathway (6). However, *dl*1520 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 *dl*1520 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 *dl*1520, making it selective only for tumor cells capable of taking over the RNA export function of E1B-55K (9). However, the toxicity profile of *dl*1520 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, *dl*922-947, has a 24-bp 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), *dl*922-947 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 *in vitro* efficacy of *dl*922-947 exceeded that of adenovirus 5 wild-type (Ad5 WT) and *dl*1520 (11). In contrast, in growth-arrested normal cells, *dl*922-947-driven replication was significantly less than with Ad5 WT. Following both i.t. and i.v. injection in murine xenograft models, *dl*922-947 showed comparable potency to Ad5 WT, and both viruses improved survival and response rates compared with *dl*1520 (11). A similar adenovirus, Δ24, 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. I.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

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doi:10.1158/0008-5472.CAN-05-2691

surface will come into contact with the fluid (14). Because icodextrin maintains its instilled volume for 48 hours (13), delivery of adenoviral agents in icodextrin should improve i.p. distribution and retention. Delivery of nonreplicating adenoviruses in icodextrin 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 icodextrin significantly prolonged survival compared with delivery in PBS (14).

Here, we describe superior *in vitro* activity of *dl*922-947 compared with viruses with wild-type E1A in cycling and growth-arrested ovarian cancer cell lines. In proliferating immortalized ovarian surface epithelial cells, we show that Rb pathway status is an important determinant of *dl*922-947 activity. In an i.p. ovarian cancer xenograft model, *dl*922-947 improves survival at least as well as Ad5 WT and *dl*309 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 icodextrin, 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% CO_2 in air, in DMEM plus 10% heat-inactivated fetal calf serum (IGROV1) or RPMI plus 10% FCS (OVCAR4). IGROV1 cells express no p16, whereas OVCAR4 cells overexpress cyclin D. Firefly luciferase–expressing IGROV1-LUC cells were generated following transfection of IGROV1 cells with pGL3-control (Promega, Madison, WI). The clone with the highest luciferase expression (B2) was employed in all experiments. *In vitro* luciferase activity was assayed in up to 1×10^6 IGROV1 or IGROV-LUC cells in groups of five or six according to manufacturer's instructions (Promega). IOSE80 cells were kindly provided by Dr. Nelly Auersperg (University of British Columbia, Canada) and maintained in DMEM plus 10% FCS supplemented with 50 μg/mL gentamicin. IOSE-C21 cells are hTERT-immortalized human ovarian surface epithelial cells, whose creation is described elsewhere (15) and which grow in NOSE-CM medium, supplemented with epidermal growth factor, hydrocortisone, insulin, bovine pituitary extract, and 15% FCS (16). *dl*922-947 is an Ad5 vector deleted in the region encoding amino acids 122 to 129 of the E1A-CR2 domain as well as in E3B (11). *dl*309 is an Ad5 vector with a wild-type E1A region but contains the same E3B deletion as *dl*922-947. The construction of the E1-deleted control adenoviral vector Ad LM-X has been described elsewhere (17). For cell viability assays, 2×10^4 cells were infected with adenovirus in serum-free medium. After 120 minutes with gentle rocking, cells were refed with medium + 5% FCS. Cell viability was assayed up to 168 hours later by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (18). All cell viability assays were done in triplicate, and experiments were all done at least twice. Representative results are shown unless stated. For viral replication assays, 1×10^5 cells were infected at a multiplicity of infection (MOI) of 10 plaque-forming units per cell (pfu/cell) and harvested up to 48 hours later into 0.5 mL of 0.1 mol/L Tris (pH 8). Cells were then subjected to three rounds of freeze/thawing (liquid N_2/37°C), after which they were centrifuged. The supernatant was then titered on JH293 cells by serial dilution.

Flow cytometry. IGROV1 cells (3×10^5) or OVCAR4 cells (4×10^5) were trypsinized up to 96 hours after adenovirus infection, washed twice with cold PBS, and fixed in 70% ethanol at 4°C for at least 30 minutes. They were then washed twice with phosphate citrate buffer (192 μmol/L Na_2HPO_4, 40 μmol/L citric acid), treated with RNase A, and stained with propidium iodide. Cell cycle status was analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Oxford, United Kingdom). To assess cell infectibility by Ad5 vectors, 3×10^5 cells were infected with Ad green fluorescent protein (GFP), an E1-deleted Ad5 vector encoding GFP in the E1 region, at MOI 5 and 50 pfu/cell. Twenty-four hours later, cells were trypsinized, washed, fixed, and analyzed for GFP positivity by flow cytometry.

Western blots. Following adenoviral infection, 5×10^5 cells were scraped into 200 μL lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris (pH 7.5), 0.05% SDS, 1% Triton X-100] and sonicated on ice. Protein (10 μg) was electrophoresed on SDS-polyacrylamide gels and transferred onto a nitrocellulose filter by semidry blotting. Antibody binding was visualized using enhanced chemiluminescence (Amersham Pharmacia, Buckinghamshire, United Kingdom).

***In vivo* analyses and histopathology.** IGROV1 or IGROV1-LUC cells (3×10^6) were injected i.p. into adult female ICRF nude mice on day 1. I.P. virus injections (400 μL per injection) commenced on day 4. Mice were assessed daily for weight, general health, and accumulation of ascites. When judged to be terminally sick, they were sacrificed, and a full pathologic assessment was made. Tumors were dissected and weighed, ascites volumes were measured, and postmortem samples were snap frozen in isopentane in liquid nitrogen and processed for H&E staining. E1A expression was detected using a rabbit anti Ad2 E1A Ab (Santa Cruz Biotechnology, Santa Cruz, CA). All histopathology was viewed by one of us (Y.W.), who was blinded to the treatment groups. For the hepatotoxicity experiments, mice were injected either with 3×10^6 (tumor bearing) or 3×10^1 (nontumor bearing) cells on day 1 followed by 3×10^9 particles/d *dl*922-947 on days 4 to 8 inclusive as above. For luciferase imaging experiments, mice bearing i.p. IGROV1-LUC xenografts were anesthetized (2% halothane by inhalation) and injected i.p. with 100 μL of 50 mmol/L firefly luciferin (Xenogen, Alameda, CA). Five minutes later, while still under inhalational anesthetic, they were placed in a light-tight chamber on a warmed stage (37°C), and light emission from a defined region of interest on both 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

***dl*922-947 has superior *in vitro* activity to Ad5 WT and *dl*309 in ovarian cancer cells.** Human ovarian cancer cell lines were transfected at MOI 10 pfu/cell with *dl*922-947, 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 *dl*922-947 and Ad5 WT infection and continued to increase in both cells up to 96 hours (Fig. 1*A*). *dl*922-947 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. 1*B*), more rapid expression of early and late viral proteins was seen following infection with *dl*922-947 than with Ad5 WT. Ad5 WT viral protein expression

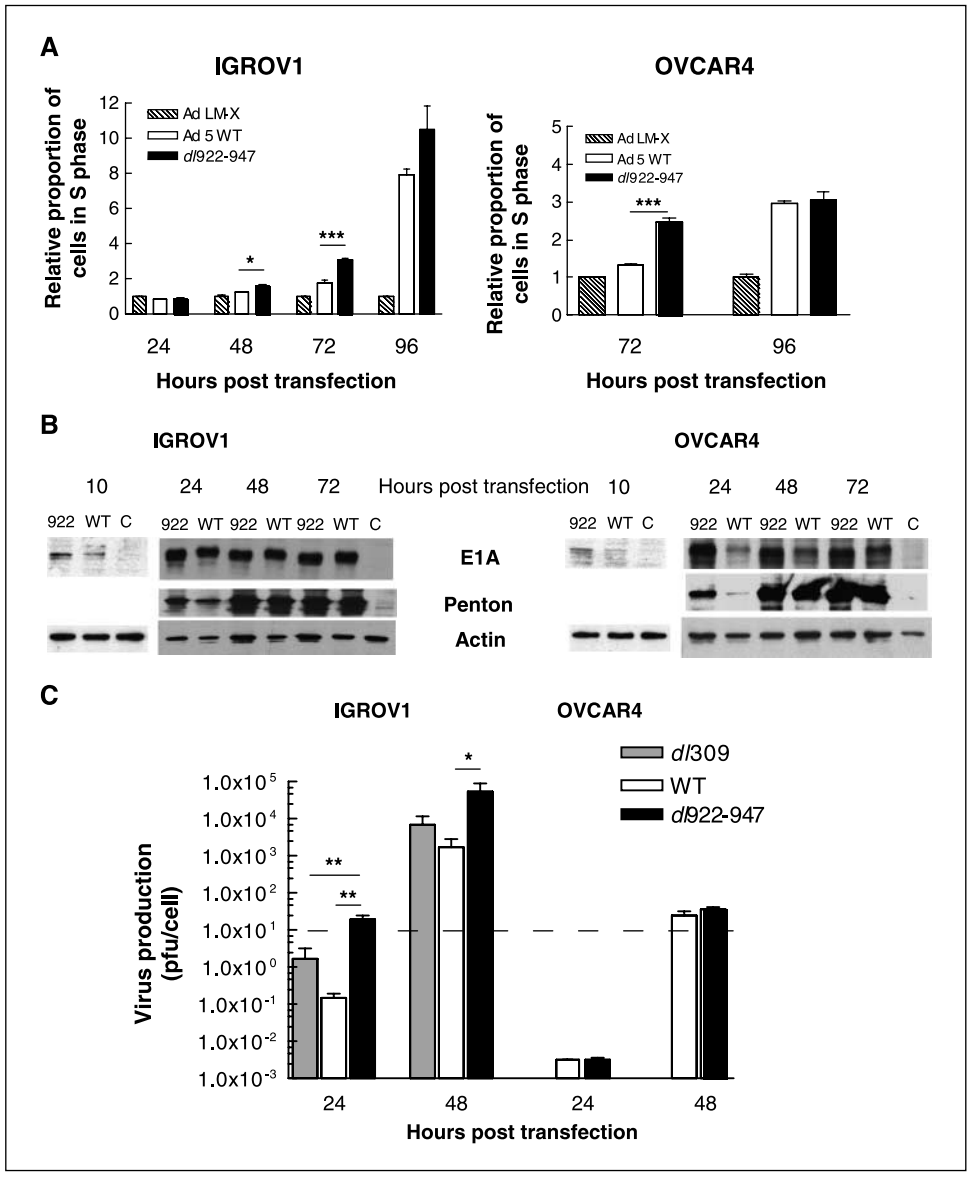


Figure 1. Activity of *dl*922-947 and Ad5 WT in ovarian carcinoma cells *in vitro*. *A,* S-phase induction. S-phase induction in IGROV1 and OVCAR4 cells was assessed up to 96 hours after viral infection (MOI 10 pfu/cell) by cell cycle analysis following propidium iodide staining. Proportion of cells 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 (5×10^5) were transfected with *dl*922-947 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 (1×10^5) were transfected with *dl*922-947, Ad5 WT, and/or *dl*309 (all MOI 10) and harvested up to 48 hours later. Virus production in infected cells was assessed by $TCID_{50}$ 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).

eventually equaled that of *dl*922-947. 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 *dl*922-947, Ad5 WT, and/or *dl*309 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. 1*C*). In IGROV1 cells, in keeping with more rapid induction of S phase and earlier expression of viral proteins, *dl*922-947 replicated to a greater degree than both Ad5 WT and *dl*309 at both 24 hours ($P < 0.01$) and 48 hours ($P < 0.05$).

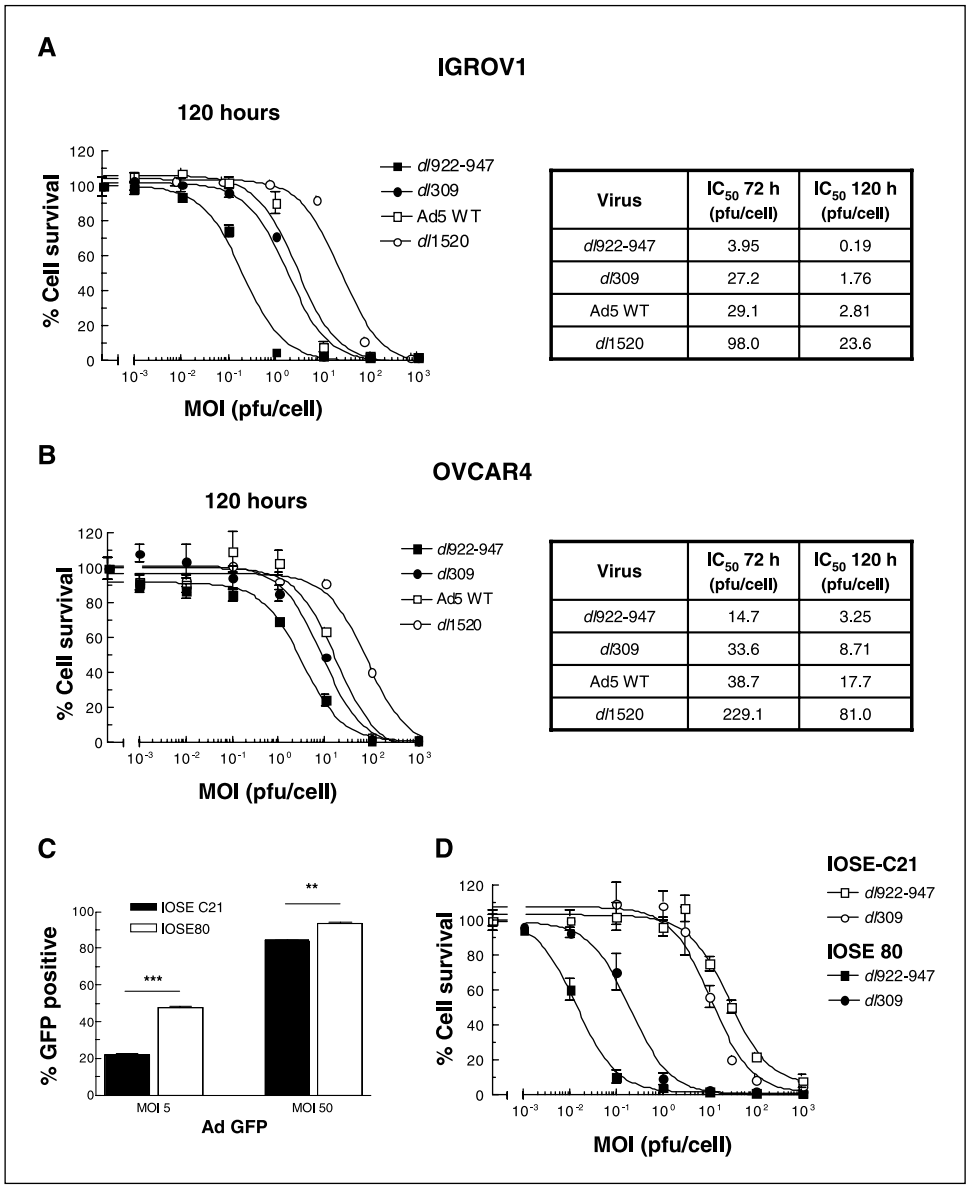
***dl*922-947 has a greater cytopathic effect than *dl*309, Ad5 WT, and *dl*1520 in ovarian cancer cells.** The cytotoxicity of *dl*922-947, as quantified by MTT assay, was compared with that of *dl*309, Ad5 WT and the E1B-55K mutant adenovirus *dl*1520 in IGROV1 (Fig. 2*A*) and OVCAR4 cells (Fig. 2*B*) 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, *dl*922-947 had a significantly greater cytopathic effect than all three other viruses: the IC_{50} for *dl*922-947 120 hours after infection was 0.2 pfu/cell compared with 1.76 pfu/cell for *dl*309, 2.81 pfu/cell for Ad5 WT, and 23.6 pfu/cell for *dl*1520 ($P < 0.001$ for all compared with *dl*922-947). In OVCAR4 cells, a similar pattern was seen, with the IC_{50} for *dl*922-947 again significantly lower than for *dl*309, Ad5 WT, and *dl*1520. Similar patterns of cell survival were seen following infection of other human ovarian carcinoma cells, including A2780, OVCAR3, OVCAR5, and SKOV3ip1 (data not shown).

***dl*922-947 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

indicated that IOSE80 cells were significantly easier to infect with Ad5-based vectors than IOSE-C21 (Fig. 2*C*), although infection rates were in excess of 80% for both cell lines at MOI 50. Following infection with *dl*922-947 and *dl*309, different patterns of cytopathic effect are seen in the two cell lines. In IOSE80 cells, as with the cancer cell lines, *dl*922-947 is significantly more toxic (IC_{50} at 168 hours, 0.014 pfu/cell for *dl*922-947 versus 0.19 pfu/cell for *dl*309), giving a *dl*309/*dl*922-947 IC_{50} ratio of 14.1. In contrast, although IOSE-C21 cells are intrinsically less sensitive to adenovirus-mediated cell death, this ratio is reversed (IC_{50} at 168 hours, 26.8 pfu/cell for *dl*922-947 versus 11.1 pfu/cell for *dl*309), giving a *dl*309/*dl*922-947 IC_{50} ratio of 0.414 (Fig. 2*D*).

***dl*922-947 can induce S phase in and lyse G_0-G_1–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 *dl*922-947 could induce S phase in and lyse noncycling ovarian cancer cells. IGROV1 cells were arrested in G_0-G_1 by serum starvation for 72 hours (Fig. 3*A*) and then infected in serum-free medium with *dl*922-947, 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 *dl*922-947 and increased steadily up to 72 hours after infection. Ad5 WT and *dl*922-947 produced similar cytopathic effect in these cells (Fig. 3*B*), although, at all time points, *dl*922-947-induced toxicity exceeded that of Ad5 WT. However, the difference was less than in cycling cells.

***dl*922-947 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. I.P. 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 *dl*922-947 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. 4*A*. In this aggressive model of ovarian cancer in which median survival for vehicle-treated



Virus	IC_{50} 72 h (pfu/cell)	IC_{50} 120 h (pfu/cell)
*dl*922-947	3.95	0.19
*dl*309	27.2	1.76
Ad5 WT	29.1	2.81
*dl*1520	98.0	23.6

Virus	IC_{50} 72 h (pfu/cell)	IC_{50} 120 h (pfu/cell)
*dl*922-947	14.7	3.25
*dl*309	33.6	8.71
Ad5 WT	38.7	17.7
*dl*1520	229.1	81.0

Figure 2. Viral cytotoxicity *in vitro*. *A* and *B*, comparison of *dl*922-947 with *dl*309, Ad5 WT, and *dl*1520 in ovarian carcinoma cells. IGROV1 (*A*) and OVCAR4 (*B*) cells were infected with *dl*922-947, *dl*309, Ad5 WT, or *dl*1520 (MOI, 0.001-1,000 pfu/cell). Cell survival was assessed by MTT assay up to 120 hours later. Percentage cell survival compared with mock-transfected cells from 120 hours after infection. *Points,* means ($n = 3$); *bars,* SD. IC_{50} results from 72 and 120 hours after infection (table). *C* and *D,* IOSE cells. *C,* infectivity of 3×10^5 SV40 large T-immortalized IOSE80 and hTERT-immortalized IOSE-C21 cells was assessed by flow cytometry following infection with Ad GFP at MOI 5 and 50 pfu/cells (*C*). *Columns,* means ($n = 3$, with 10,000 counts per sample); *bars,* SD. **, $P < 0.01$; ***, $P < 0.001$ (two-tailed *t* test). *D,* IOSE80 and IOSE-C21 cells (2×10^4) were infected with *dl*922-947 and *dl*309 (MOI, 0.001-1,000), and cell survival was assessed up to 168 hours later by MTT assay. Survival (168 hours after infection) relative to mock-transfected cells. *Points,* means ($n = 3$); *bars,* SD.

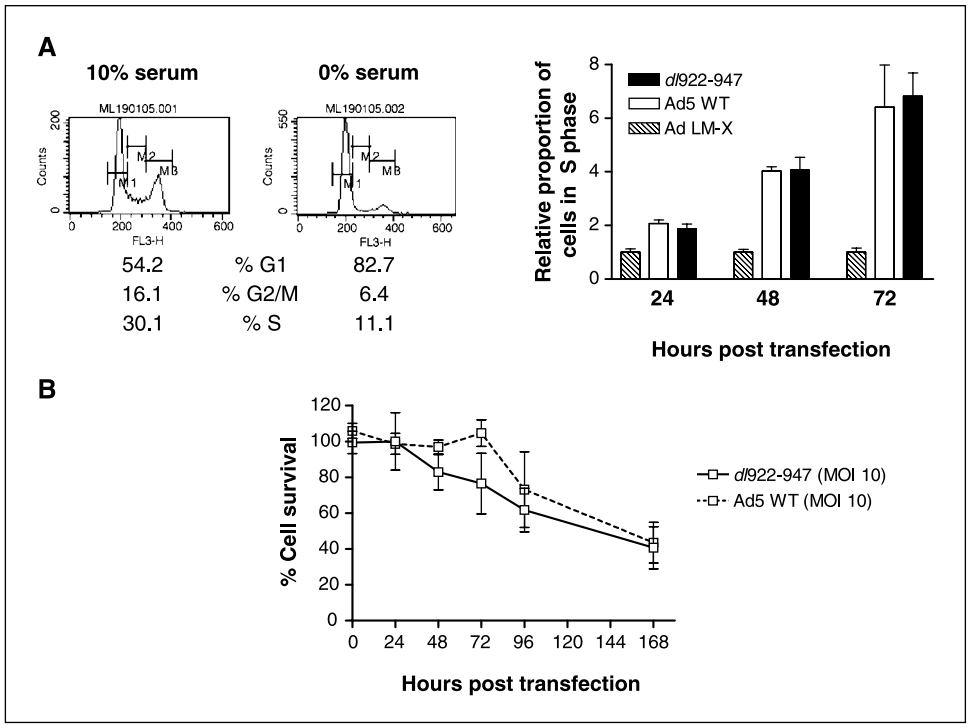


Figure 3. Effect on G_0-G_1–arrested ovarian carcinoma cells. *A,* S-phase induction. IGROV1 cells (3×10^5) were arrested in G_0-G_1 by serum starvation for 72 hours and then transfected with *dl*922-947, Ad5 WT, or Ad LM-X (all MOI 10) in serum-free conditions. G_0-G_1 arrest was confirmed by cell cycle analysis following propidium iodide staining. S-phase induction was then assessed up to 72 hours after viral infection again by propidium iodide staining. Proportion of cells in the S phase relative to those transfected with control adenovirus Ad LM-X. *Columns,* means (n = 3); *bars,* SD. *B,* cytotoxicity. After 72 hours of serum starvation, 2×10^4 IGROV1 cells were transfected with *dl*922-947, 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.

animals was only 19 days, treatment with Ad LM-X prolonged median survival to 26.5 days (P < 0.001). At a 10-fold lower concentration than that of the control virus, *dl*922-947 significantly prolonged median survival to 49.5 days (comparison with Ad LM-X, P < 0.001). At the higher dose of *dl*922-947, the survival advantage was such that median survival was not reached (P < 0.001), with 18 mice still alive at day 125.

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 vehicle had no effect on survival (Fig. 4*B, left*). In the lower dose *dl*922-947 cohort, median survival was 55 days in icodextrin and 43 days in PBS (P = 0.15), with two mice surviving long term in the icodextrin cohort compared with none in the PBS group (Fig. 4*B, right*). At the higher dose of *dl*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 = 0.12).

In separate experiments, using the same xenograft model and treatment schedule, 10 mice per group were treated with Ad LM-X, Ad5 WT, or *dl*922-947 (all 5×10^9 ppi) delivered in 20% icodextrin (Fig. 4*C*) and *dl*922-947, *dl*309 (both 5×10^9 ppi), or vehicle alone (Fig. 4*D*). In these experiments, *dl*922-947 was at least as effective at prolonging survival above control as both Ad5 WT and *dl*309.

Some *dl*922-947-treated mice were tumor-free at the time of sacrifice. Postmortem examination revealed that all mice treated with either vehicle alone had significant ascites and i.p. disease at the time of sacrifice (median tumor mass PBS, 3.53 g; range, 1.93-5.00 g; median tumor mass icodextrin, 3.22 g; range, 1.93-4.72 g; P = not significant). Regardless of vehicle, all mice treated with Ad LM-X also had visible i.p. tumor at time of sacrifice, although less than with vehicle alone (median, 1.56 g; range, 1.03-2.70 g; P < 0.001 compared with PBS). The lowest tumor masses were seen in those mice treated with *dl*922-947, especially at the higher dose (median tumor mass, 0.73 g; range, 0.00-2.53 g; P < 0.001 compared with Ad LM-X; Fig. 4*E*). Ten mice treated with high-dose *dl*922-947 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 *dl*922-947 with Ad5 WT, a similar reduction in tumor mass at sacrifice was again seen in the *dl*922-947-treated mice (median mass *dl*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 *dl*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. 5*A, 1*). By contrast, the Ad5 WT livers were grossly necrotic and showed diffuse eosinophilic degeneration (Fig. 5*A, 2*). The livers of *dl*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. 5*A, 3*). By contrast, in *dl*922-947-treated mice that died with significant tumor burdens, liver architecture was grossly normal (Fig. 5*A, 4*). The Ad5 WT– and *dl*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 *dl*922-947 within tumor cells contributed to hepatotoxicity. Therefore, tumor-bearing or non–tumor-bearing mice were treated with *dl*922-947 (5×10^9 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. 5*B, 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. 5*B, 3* and *4*). By day 39, some improvement was seen (Fig. 5*B, 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. 5*C, 1*). By day 32, a few E1A-positive cells were still detected, but this did not correlate with areas or extent of necrosis (Fig. 5*C, 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. 6*A*). 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. 6*A*). *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. 6*B*). In contrast, infection with *dl*922-947 (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 ($P < 0.05$).

When mice bearing i.p. IGROV-LUC xenografts were treated i.p. with vehicle alone or Ad LM-X (5×10^9 particles daily for 5 days), a steady increase in bioluminescence was observed over time

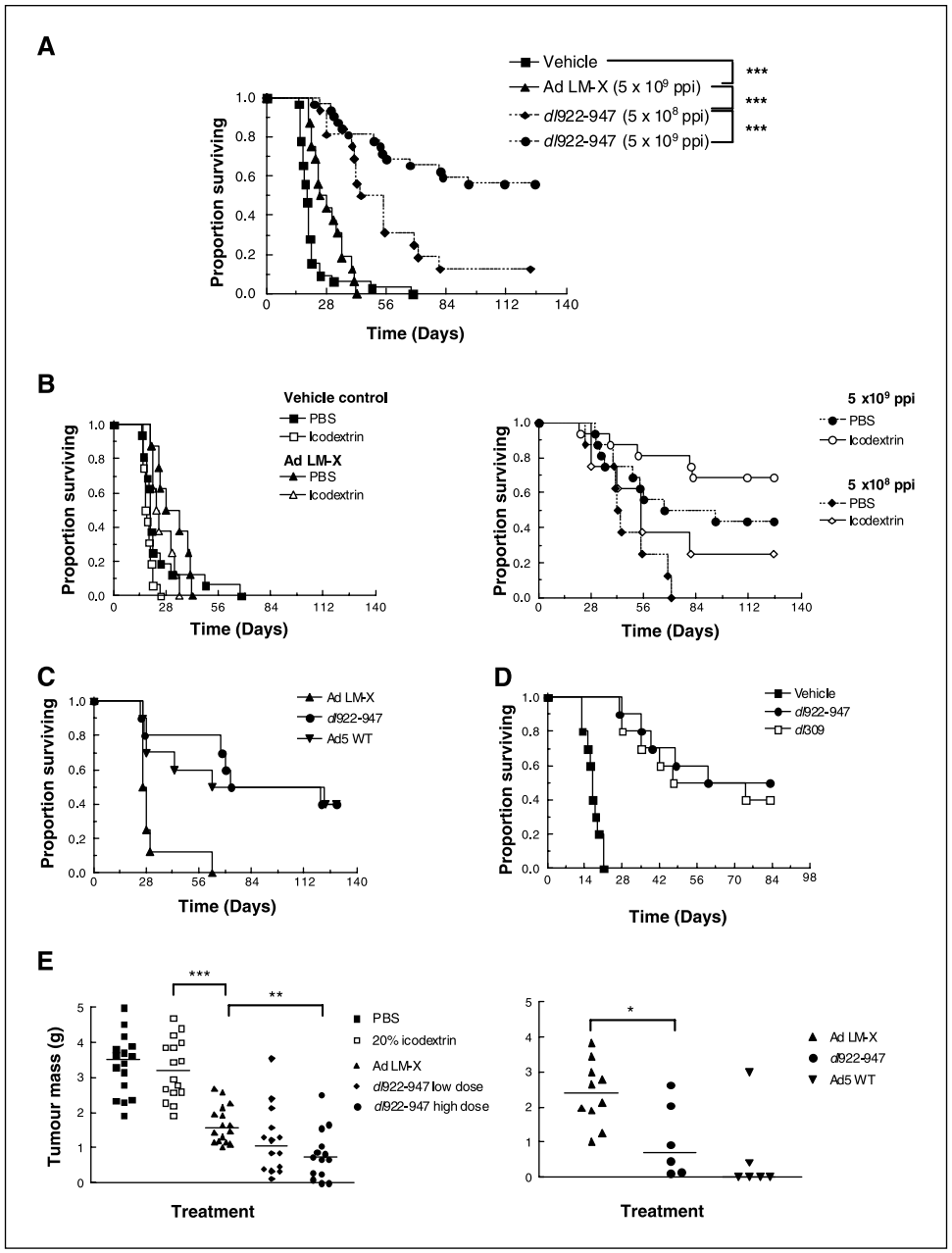


Figure 4. Effect of *dl*922-947 in IGROV1 ovarian carcinoma xenografts. *A,* comparison of two doses of *dl*922-947. Female nude mice were inoculated i.p. with 3×10^6 IGROV1 cells on day 1. On days 4 to 8 inclusive, mice received i.p. injections of Ad LM-X (5×10^9 ppi), *dl*922-947 (either 5×10^8 or 5×10^9 ppi), or an equal volume of vehicle (either PBS or 20% icodextrin; $n = 8$-16 per group). Animals were monitored for accumulation of ascites and for general well being. Two identical experiments were done sequentially, and Kaplan-Meier survival curves are presented from a joint analysis. ***, $P < 0.001$ (two tailed *t* test). *B,* delivery of *dl*922-947 in icodextrin may improve survival. Survival of the animals presented in (*A*) was analyzed according to the vehicle in which virus was delivered: vehicle alone (PBS or icodextrin) and Ad LM-X cohorts (*left*) and *dl*922-947 cohorts (*right*). *C* and *D,* comparison of *dl*922-947 with Ad5 WT or *dl*309 *in vivo*. Female nude mice were inoculated i.p. with 3×10^6 IGROV1 cells on day 1. On days 4 to 8 inclusive, mice received i.p. injections as follows: (*C*) *dl*922-947, Ad5 WT, or Ad LM-X (all 5×10^9 ppi in 20% icodextrin); (*D*) 20% icodextrin, *dl*922-947, or *dl*309 (5×10^9 ppi in 20% icodextrin). Animals were again monitored for accumulation of ascites and for general well being. *E,* tumor mass at time of sacrifice. At the time of sacrifice, postmortems were done on all mice presented in (*A*) to (*C*), and all visible i.p. tumors were dissected out and weighed. Each symbol represents the mass of tumor in each mouse; horizontal line represents the mean. *Left,* results from experiments presented in (*A*) and (*B*); *right,* results from experiment presented in (*C*). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (two tailed *t* test).

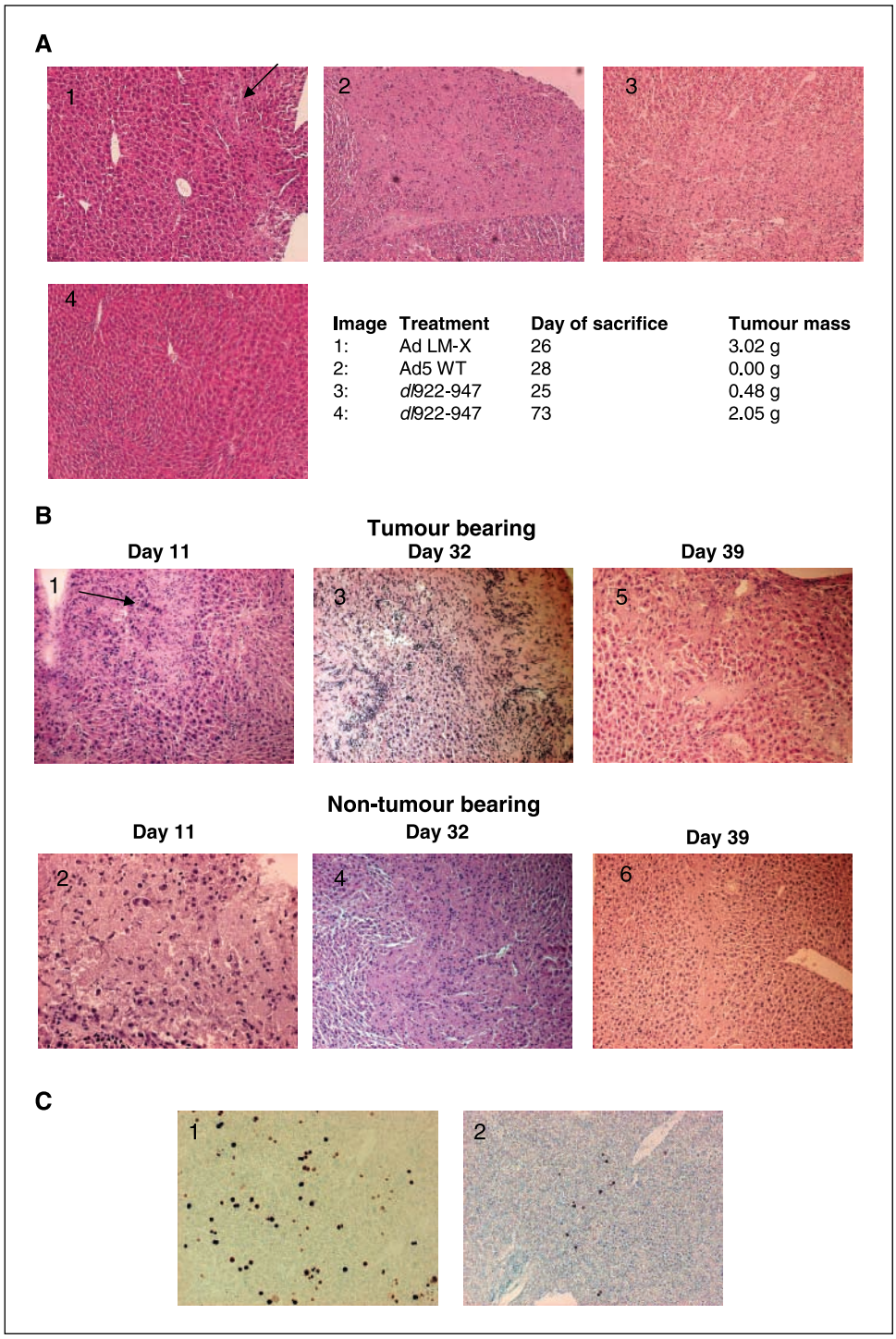


Figure 5. Histopathology. *A,* some *dl*922-947- and Ad5 WT–treated mice have liver necrosis. The mice sacrificed in the experiment represented in Fig. 4*C* underwent postmortem examination. Four-micrometer liver sections were stained with H&E. Representative images; magnification, ×100 in all images. Treatment, day of sacrifice, and the mass of dissected tumor from each animal. Samples are as follows: *1,* Ad LM-X treatment, liver with some necrosis (*arrow*); *2,* Ad 5 WT treatment, liver showing gross necrosis; *3,* *dl*922-947 treatment, liver showing necrosis in a mouse with minimal visible tumour at the time of sacrifice; *4,* *dl*922-947 treatment, normal liver architecture, in a mouse with significant tumor burden at sacrifice. *B,* sequential liver histopathology following *dl*922-947 treatment in tumor-bearing and non–tumor-bearing animals. Female nude mice were inoculated i.p. with 3×10^6 (tumor bearing) or 3×10^1 (non–tumor bearing) IGROV1 cells on day 1. On days 4 to 8 inclusive, mice received i.p. injections of *dl*922-947 (5×10^9 ppi in 20% icodextrin). Mice were sacrificed on days 11, 18, 25, 32, and 39 in groups of six, and full postmortem examinations were done. Four-micrometer liver sections were stained with H&E. Representative images; magnification, ×200 in all images, except (*3*) and (*4*), ×100. Images are as follows: *1-2,* focal areas of necrosis and eosinophilic degeneration (*arrow*) seen on day 11 in both tumor bearing (*1*) and non–tumor bearing (*2*); *3-6,* extensive necrosis evident in both groups of animals on day 32 (*3,* tumor bearing; *4,* non–tumor bearing), with partial recovery by day 39 (*5,* tumor bearing; *6,* non–tumor bearing). *C,* E1A immunohistochemistry. Four-micrometer sections from livers of tumour-bearing mice were stained for E1A expression on day 11 (*1*) and day 32 (*2*). Magnification, ×100.

(Fig. 6*C*), consistent with tumor progression. With i.p. *dl*922-947 (5×10^9 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, *dl*922-947 was shown to have greater activity than Ad5 WT and *dl*1520 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 *dl*922-947 in this setting. In human ovarian cancer cell lines, the *in vitro* efficacy of *dl*922-947 consistently exceeded that of both Ad5 WT and *dl*309. The inclusion of *dl*309 as a second control indicated that the E3B deletion in *dl*922-947 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 *dl*922-947 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 *dl*922-947 than with Ad5 WT and replication of *dl*922-947 in IGROV1 cells also exceeded that of Ad5 WT and *dl*309. *dl*922-947 also produced greater cytopathic effect than *dl*309, Ad5 WT, and *dl*1520 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 *dl*922-947 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 *dl*922-947 induces less cytotoxicity than *dl*309 in ovarian surface epithelial cells with an intact Rb pathway (IOSE-C21) but far greater toxicity than *dl*309 in those immortalized with pRb inactivation (IOSE-80), we confirm that cellular Rb pathway status is an important determinant of *dl*922-947 activity. However, it remains unclear quite why *dl*922-947 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

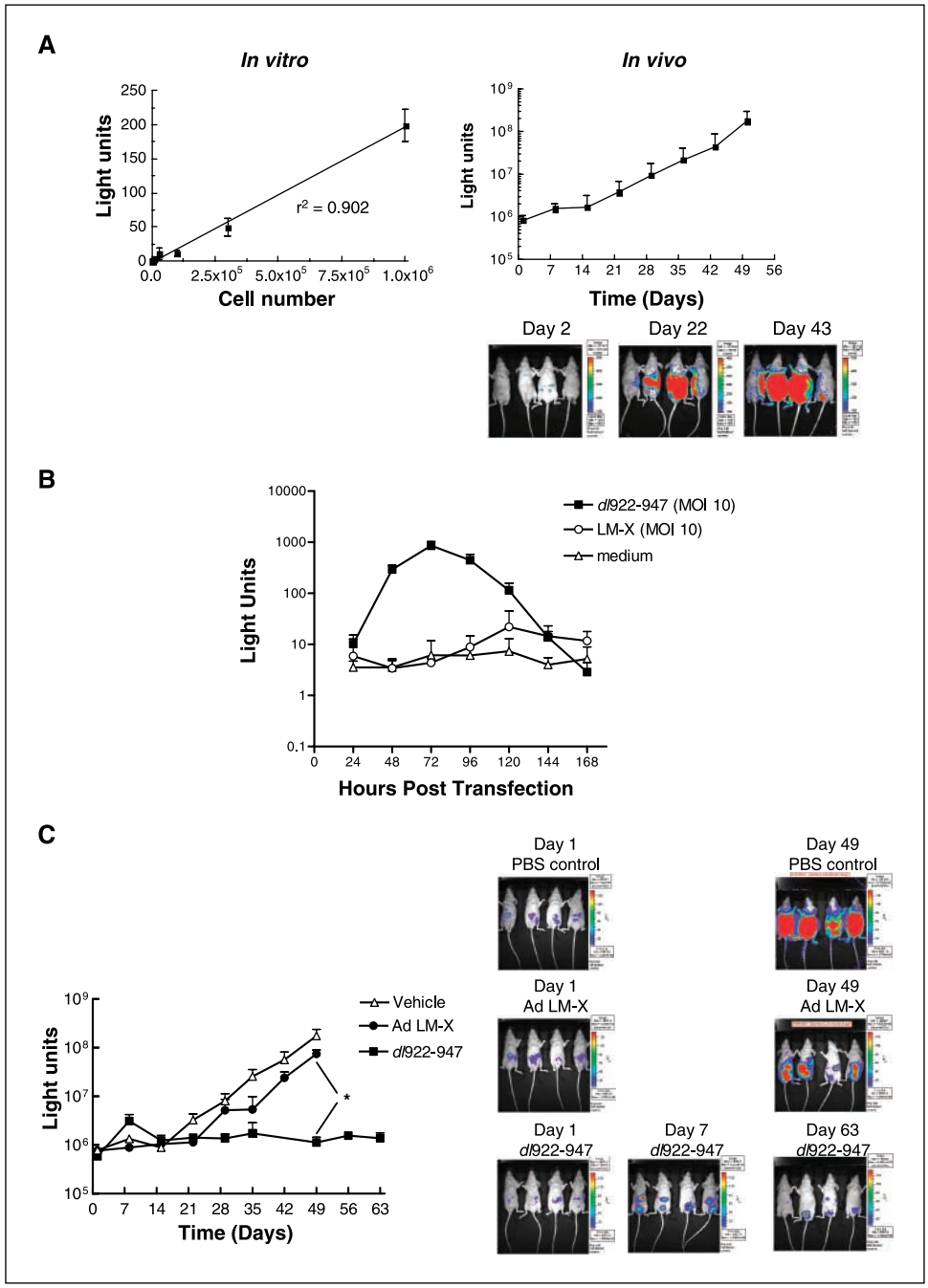


Figure 6. Bioluminescence monitoring. *A,* light emission from IGROV-LUC cells correlates with cell number *in vitro* and increases with time *in vivo*. Between 1×10^4 and 1×10^6 IGROV1-LUC cells were plated in six-well plates in groups of six. After overnight incubation, cells were lysed and *in vitro* firefly luciferase assays were done according to manufacturer's instructions. *Points,* means ($n = 6$); *bars,* SD. IGROV1-LUC cells (3×10^6) 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 (3×10^4) were either mock transfected or transfected with *dl*922-947 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 (3×10^6) were injected i.p. into nude female mice on day 1. On days 4 to 8, mice received daily i.p. injections of *dl*922-947, Ad LM-X (both 5×10^9 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 *dl*309-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 immortalized 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 arrested ovarian cancer cells in G_1 to investigate their response to viral infection. *dl*922-947 and Ad5 WT were equally capable of inducing S phase in G_1-arrested cells, implying that replicating viruses actively induce G_1-S transition in infected cells rather than simply taking advantage of cells already in the S phase. The cytolytic advantage of *dl*922-947 over Ad5 WT in cycling cells was, however, partially diminished in cells arrested in G_1, presumably due to loss of E1A-CR2 function.

We have shown that the promising *in vitro* results seen with *dl*922-947 translate into a dramatic *in vivo* survival advantage, with many mice surviving long term following *dl*922-947 treatment. This is at least comparable with treatment with Ad5 WT or *dl*309, but the large advantage that existed between *dl*922-947 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 G_2-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 *dl*922-947 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 *dl*922-947 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 *dl*922-947 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 *dl*922-947 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 *dl*922-947. 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 *dl*1520 in ovarian cancer, but i.p. delivery of 10^7 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 *dl*922-947. 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 *dl*922-947 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 *dl*922-947 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 *dl*922-947-treated mice correlates with the powerful antitumor effect in mice treated with this virus.

In summary, the E1A-CR2 mutant *dl*922-947 has impressive activity in ovarian carcinoma. Although i.p. delivery in murine models was associated with some delayed hepatotoxicity, *dl*922-947 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.

Acknowledgments

Received 8/1/2005; revised 10/13/2005; accepted 11/10/2005.

Grant support: Cancer Research UK grant C9423/A4119 and Medical Research Council grant G84/6553.

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We thank Derek Davies for his assistance with the FACS analyses, Sandra Peak for the *in vivo* experiments, Krishna Caulee and Vipul Bhakta for histopathology, and Dr. Nelly Auersperg for access to the Canadian Ovarian Tissue Bank.

References

1. Bray F, Loos AH, Tognazzo S, La Vecchia C. Ovarian cancer in Europe: cross-sectional trends in incidence and mortality in 28 countries, 1953–2000. Int J Cancer 2005;113:977–90.
2. Ozols RF, Bundy BN, Greer BE, et al. Phase III Trial of carboplatin and paclitaxel compared with cisplatin and paclitaxel in patients with optimally resected stage III ovarian cancer: a Gynecologic Oncology Group study. J Clin Oncol 2003;21:3194–200.
3. Alberts DS, Liu PY, Hannigan EV, et al. Intraperitoneal cisplatin plus intravenous cyclophosphamide versus intravenous cisplatin plus intravenous cyclophosphamide for stage III ovarian cancer. N Engl J Med 1996;335:1950–5.
4. Zeimet AG, Marth C. Why did p53 gene therapy fail in ovarian cancer? Lancet Oncol 2003;4:415–22.
5. Heise C, Sampson-Johannes A, Williams A, McCormick F, von Hoff D, Kirn D. ONYX-015, an E1B gene attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemotherapeutic agents. Nat Med 1997;3:639–44.
6. Sherr CJ, McCormick F. The RB and p53 pathways in cancer. Cancer Cell 2002;2:103–12.
7. Kirn D. Clinical research results with dl1520 (Onyx-015), a replication-selective adenovirus for the treatment of cancer: what have we learned? Gene Ther 2001;8:89–98.
8. Vasey PA, Shulman LN, Campos S, et al. Phase I trial of intraperitoneal injection of the E1B-55-kd-gene-deleted adenovirus ONYX-015 (dl1520) given on days 1 through 5 every 3 weeks in patients with recurrent/refractory epithelial ovarian cancer. J Clin Oncol 2002;20:1562–9.
9. O'Shea C, Johnson L, Bagus B, et al. Late viral RNA export, rather than p53 inactivation, determines ONYX-015 tumor selectivity. Cancer Cell 2004;6:611–23.
10. D'Andrilli G, Kumar C, Scambia G, Giordano A. Cell cycle genes in ovarian cancer: steps toward earlier diagnosis and novel therapies. Clin Cancer Res 2004;10:8132–41.
11. Heise C, Hermiston T, Johnson L, et al. An adenovirus E1A mutant that demonstrates potent and selective systemic anti-tumoral efficacy. Nat Med 2000;6:1134–9.
12. Fueyo J, Gomez-Manzano C, Alemany R, et al. A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect *in vivo*. Oncogene 2000;19:2–12.
13. Hosie K, Gilbert JA, Kerr D, Brown CB, Peers EM. Fluid dynamics in man of an intraperitoneal drug delivery solution: 4% icodextrin. Drug Deliv 2001;8:9–12.
14. Engler H, Machemer TR, Schluep T, et al. Development of a formulation that enhances gene expression and efficacy following intraperitoneal administration in rabbits and mice. Mol Ther 2003;7:558–64.
15. Li NF, Gayther SA, Balkwill F. A cellular model for studying human ovarian surface epithelium carcinogenesis. Proc Am Assoc Cancer Res 2005;46:LB–176.
16. Li NF, Wilbanks G, Balkwill F, Jacobs IJ, Dafou D, Gayther SA. A modified medium that significantly improves the growth of human normal ovarian surface epithelial (OSE) cells *in vitro*. Lab Invest 2004;84:923–31.
17. McNeish IA, Tenev T, Bell S, Marani M, Vassaux G, Lemoine N. Herpes simplex virus thymidine kinase/ganciclovir-induced cell death is enhanced by co-expression of caspase-3 in ovarian carcinoma cells. Cancer Gene Ther 2001;8:308–19.
18. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
19. Tubiana M, Malaise E. Growth rate and cells kinetics in human tumours: some prognostic and therapeutic implications. In: Symington T, Carter R, editors. Scientific foundations of oncology. London: Heinnemann; 1976. p. 126–36.
20. Bauerschmitz GJ, Lam JT, Kanerva A, et al. Treatment of ovarian cancer with a tropism modified oncolytic adenovirus. Cancer Res 2002;62:1266–70.
21. Kanerva A, Zinn KR, Chaudhuri TR, et al. Enhanced therapeutic efficacy for ovarian cancer with a serotype 3 receptor-targeted oncolytic adenovirus. Mol Ther 2003;8:449–58.
22. Weedon S, Green N, McNeish I, et al. Sensitisation of human carcinoma cells to the prodrug CB1954 by adenovirus vector-mediated expression of E coli nitroreductase. Int J Cancer 2000;86:848–54.
23. Steinwaerder DS, Carlson CA, Lieber A. DNA replication of first-generation adenovirus vectors in tumor cells. Hum Gene Ther 2000;11:1933–48.
24. O'Connor RJ, Hearing P. The E4–6/7 protein functionally compensates for the loss of E1A expression in adenovirus infection. J Virol 2000;74:5819–24.
25. Shayakhmetov DM, Li Z-Y, Ni S, Lieber A. Analysis of adenovirus sequestration in the liver, transduction of hepatic cells, and innate toxicity after injection of fiber-modified vectors. J Virol 2004;78:5368–81.
26. Shayakhmetov DM, Gaggar A, Ni S, Li Z-Y, Lieber A. Adenovirus binding to blood factors results in liver cell infection and hepatotoxicity. J Virol 2005;79:7478–91.
27. Engler H, Machemer T, Philopena J, et al. Acute hepatotoxicity of oncolytic adenoviruses in mouse models is associated with expression of wild-type E1a and induction of TNF-α. Virology 2004;328:52–61.
28. Heise C, Ganly I, Kim YT, Sampson-Johannes A, Brown R, Kirn D. Efficacy of a replication-selective adenovirus against ovarian carcinomatosis is dependent on tumor burden, viral replication and p53 status. Gene Ther 2000;7:1925–9.
29. Raki M, Kanerva A, Ristimaki A, et al. Combination of gemcitabine and Ad5/3-Delta24, a tropism modified conditionally replicating adenovirus, for the treatment of ovarian cancer. Gene Ther 2005;12:1298–05.
30. Engler H, Machemer T, Philopena J, et al. Acute hepatotoxicity of oncolytic adenoviruses in mouse models is associated with expression of wild-type E1a and induction of TNF-[α]. Virology 2004;328:52–61.
31. Kanerva A, Zinn KR, Peng KW, et al. Noninvasive dual modality *in vivo* monitoring of the persistence and potency of a tumor targeted conditionally replicating adenovirus. Gene Ther 2005;12:87–94.