

Effects of Febrile Temperature on Adenoviral Infection and Replication: Implications for Viral Therapy of Cancer

Stephen H. Thorne, Gabriel Brooks, Yeun-Ling Lee, Tina Au,
Lawrence F. Eng, and Tony Reid*

Veteran's Administration Palo Alto and Stanford University, Palo Alto, California

Received 6 February 2004/Accepted 26 May 2004

We previously conducted a phase I/II study using arterial infusions of ONYX-015 (*dl1520*), a replication-selective adenoviral vector, with E1b deleted, for patients with metastatic colorectal cancer. No dose-limiting toxicities occurred, but >90% of the patients experienced fever. The effects of temperature on the replication of *dl1520* in normal and transformed cells had not been studied. Therefore, replication and cell viability assays were performed with a panel of nontransformed and transformed cell lines cultured at 37 and 39.5°C and treated with adenovirus type 5 (Ad5) or *dl1520*. Ad5-mediated cytolytic effects were inhibited and production of infectious particles decreased by >1,000-fold in the nontransformed cells at 39.5°C. Seven of nine of the tumor cell lines retained significant cell-killing effects when treated with Ad5 at 39.5°C. When *dl1520* was used, no cytolytic effects were observed at 39.5°C in the nontransformed cell lines; however, cytolytic effects occurred in six of nine tumor cell lines at 39.5°C. Notably, a subset of the tumor cell lines demonstrated increased *dl1520*-mediated cytolytic effect and replication at 39.5°C. Suppression of Ad5 and *dl1520* replication at 39.5°C was not related to p53 status or HSP70 expression. Also, at 39.5°C, E1a expression was inhibited in nontransformed cells but was still abundant in the transformed cells, indicating that a novel early block in viral replication occurred in the nontransformed cells. Fever may therefore augment the therapeutic index of oncolytic viruses by inhibiting replication in normal cells while permitting or enhancing viral replication in some tumor cells.

Following the initial indications of antitumor activity and safety of ONYX-015 (*dl1520*) (10, 32), a host of different replicating oncolytic adenoviral strains are currently entering the clinic for use in the treatment of cancers. A variety of genetic alterations have been incorporated into these viruses in order to achieve tumor selectivity (reviewed in reference 14a). However, the conditions used to assay the tropism and effectiveness of these viruses in vitro are very different from those encountered among patients. As a result, these screens may not yield optimal therapeutic viruses. In addition, the routes of entry and doses administered are very different from those of a typical viral infection and so may produce unexpected responses among patients. Therefore, it is critical to carefully examine the experiences of patients during clinical trials with the first-generation vectors in order to design safe and effective second-generation viruses.

We have previously reported the results of a phase I/II study of hepatic arterial infusion of ONYX-015 (*dl1520*) (32, 33). This virus, which has the E1B-55K gene deleted, was used with the intention of allowing lytic infections to occur in tumor cells with mutations in the p53 pathway while preventing viral replication in normal cells that have wild-type p53 (4, 5, 32, 33). During the course of this study, 35 patients received a total of nearly 200 arterial infusions of ONYX-015 (*dl1520*). No defined dose-limiting toxicities were observed (34, 40). The most common toxicities reported were flu-like symptoms occurring

several hours after each infusion. Patients experienced fevers, chills, and myalgias for up to 9 days after the infusion, suggesting that viral infection and replication was occurring. The fevers started within 1 to 2 h of the infusion of ONYX-015 (*dl1520*) and ranged from 38 to 41°C. Greater than 90% of the patients experienced fevers despite the prophylactic use of antipyretics (32, 33). Analysis of the blood from these patients confirmed the presence of adenoviral genomes for 3 to 9 days after infusion of the virus, suggesting ongoing viral replication (33).

This clinical study highlighted the importance of the febrile response. Unlike controlled cell culture conditions, viral replication among patients occurred during periods when the patients were febrile. The impact of fever on the replication of *dl1520* has not been evaluated and may be critical to the effectiveness of strategies that depend upon tumor-selective viral replication. Anti-inflammatory agents, such as aspirin and Tylenol (acetaminophen), and steroids, such as prednisone, are used to control the fever and inflammatory reactions. However, the use of such agents may be counterproductive if the presence of a fever provides some level of protection to normal tissues or enhances viral cytotoxicity against tumor cells. Therefore, it is important to understand the impact of fever on viral replication in both nontransformed and transformed cells. Much of what we know of viral replication under conditions of elevated temperature has come from studies examining nonphysiological temperatures (42°C [107.6°F] or higher). These effects have usually been studied in either the HeLa or KB cell line, both of which have been cultured for decades and are highly transformed.

In this study, the impact of physiologically significant tem-

* Corresponding author. Mailing address: Department of Hematology/Oncology, Moores UCSD Cancer Center, 9500 Gilman Dr., MC 0064, La Jolla, CA 92093. Phone: (858) 657-8710. Fax: (858) 657-8699. E-mail: tonyreid@ucsd.edu.

temperatures (37 and 39.5°C [102.2°F]) on viral replication, cell viability, viral gene expression, and subcellular localization of viral proteins has been examined in transformed and nontransformed cell lines. It was found that febrile temperature markedly reduced viral replication and virus-mediated cell killing for both Ad5 and *dl1520* in nontransformed cells. However, *dl1520* was able to maintain some level of replication at this increased temperature in most of the tumor cell lines studied, and it even demonstrated increased replication in some tumor cell lines. This indicates that fever may provide an increased therapeutic index for *dl1520* in some tumor types. Further attempts have been made to identify the characteristics required for this increased therapeutic potential; this effect was shown to be independent of E1B-55K's action on p53 and so may be related to its role in nucleocytoplasmic transport, but it does not appear to be related to HSP70 levels. However, the loss of a novel early block in the viral replication cycle in transformed cells may be implicated.

MATERIALS AND METHODS

Clinical data. The effects on patient temperature following hepatic arterial infusion with ONYX-015 (*dl1520*) were produced in a phase I/II clinical trial described elsewhere (32, 33) but have not previously been published.

Cell lines, virus, and viral preparation. Most of the cell lines used in this work were obtained from the American Type Culture Collection (Manassas, Va.); these were HEK 293, BEAS-2B, MRC-5, MCF-7, A549, Calu-6, PANC-1, MIA PaCa-2, Hep3B, HepG2, and LNCaP. The cell line A2780 was a gift from David Kirn, Imperial College, London, United Kingdom, and the RKO and RKO p53.13 matched cell lines were a gift from Michael Kastan, John Hopkins University, and have been described previously (37). All cell lines were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS).

Wild-type adenovirus serotype 5 (GenBank accession number M73260) was obtained from the American Type Culture Collection. *dl309* contains deletions in the E3 genes 14.7, 14.5, and 10.4K in an Ad5 background and was a generous gift from Bill Wold (St. Louis University School of Medicine, St. Louis, Mo.) (20). *dl1520* contains a deletion in the E1B 55K gene in a *dl309* background and was a generous gift from Arnold Berk (University of California, Los Angeles) (4).

Large-scale preparations of all of the viruses were performed in cell factories (Nunc), using the A549 cell line and Dulbecco's modified Eagle's medium plus 10% FCS. Ten-layer cell factories were seeded with A549 cells, and when the cells were 80% confluent the medium was changed to Dulbecco's modified Eagle's medium plus 2% FCS and the cells were infected with virus. Cell factories were then incubated (37°C, 5% CO₂) until the monolayer began to dissociate due to the viral infection (after 4 to 5 days), and the cells were shaken loose and cells and medium was collected. The infected cells were then collected by centrifugation and lysed by rounds of freezing and thawing. Virus was purified from this lysate by CsCl banding (41a), and the CsCl was subsequently removed by dialysis (Slide-a-Lyzer; Pierce). Titers of viral preparations were determined both for infectious viral units by 50% tissue culture infective dose (TCID₅₀) assay and for viral particles by optical density at 260 nm (25a).

Cell viability assay. Losses of cell viability following viral infection were assayed by MTS assay according to standard methods (42). Briefly, cells were plated at a density of 10⁴ cells per well in 96-well plates (RPMI with 2% FCS). This constitutes a confluency of 50 to 70%, depending on the cell type. Once the cells adhered to the plate (after approximately 6 h), virus was added to give a range of multiplicities of infection (MOIs) of between 0.001 and 10 PFU/cell. An additional 25% volume of RPMI plus 2% FCS was added after 5 days to ensure sufficient nutrients. Cell viability was assayed once complete cell death was observed at the highest MOI (typically at 5 to 9 days postinfection). Viability was then assayed according to the manufacturer's instructions, by staining with MTS (Promega) and reading the absorbance at 490 nm. The percentage of viable cells in each well relative to an uninfected control (100% viable) was then calculated and plotted against the MOI. A nonlinear regression curve fit was applied using PRISM (Graphpad) software, and the MOI at which 50% loss of viability occurred (50% effective concentration [EC₅₀]) was calculated. This was taken to be the viral dose at which 50% cell killing was produced.

Burst assay. Cell lines to be tested were grown to 80 to 90% confluency before infection with different strains of adenovirus at an MOI of 1.0 infectious unit/cell.

Infection times of 90 min in medium with 2% FCS were used, before replacement with fresh medium with 2% FCS. At 72 h postinfection, cells were scraped into the medium, this mixture was collected, and the cells were lysed by three rounds of freezing in liquid nitrogen and thawing at 37°C. Cellular fragments were removed by gentle centrifugation, and the numbers of infectious viral units released into the supernatant was assayed.

Infectious unit assay was performed by the standard TCID₅₀ assay (31a). Briefly, 96-well plates were seeded with 10³ HEK 293 cells in 200 μl of medium and allowed to attach overnight. Twenty microliters of the viral suspension to be tested, or a dilution of this, was inoculated into each of the top 12 wells of the plate before serial dilutions (transfers of 22 μl) were performed down successive rows. Each well was then scored as positive if there was any viral cytopathic effect (CPE) evident after 10 days of incubation. The produces a TCID₅₀ titer for the virus, which is expressed as infectious units per milliliter.

Western blotting. Cell lines were grown to 90% confluency in six-well plates before a 90-min infection with virus at an MOI of 1.0 PFU/cell was performed. At various time points postinfection, the medium was removed and the cells were collected. Protein extraction was performed by standard methods, using 1% sodium dodecyl sulfate (SDS) in phosphate-buffered saline (pH 7.2), and protein concentrations were measured by the method of Lowry et al. (23a). Appropriate amounts of protein were run on SDS-10% polyacrylamide gels and transferred onto Hybond nitrocellulose membranes (Amersham Life Science Inc.) by transblotting. A polyclonal 13 S-5 anti-E1A antibody (Santa Cruz Biotechnology Inc., Santa Cruz, Calif.) and a polyclonal antihexon antibody (Accurate Chemicals & Scientific Corp., Westbury, N.Y.) were used to detect viral proteins, and these were visualized with the Vectastain avidin-biotin complex (ABC) kit (Vector Laboratories Inc., Burlingame, Calif.) according to the manufacturer's instructions. Alternatively, uninfected cells were grown at the indicated temperatures for 48 h before protein extraction and assay. Membrane blots were treated with anti-p53 or anti-HSP70 antibodies (Santa Cruz Biotechnology Inc.) and visualized as before.

Immunohistochemical staining. Cells were grown to 60% confluency in cell chamber slides (LabTek) before virus (Ad5) was added at 0.1 PFU/cell. At different times postinfection, the medium was removed and the cells were fixed with 0.3 M NaCl in 70% ethanol. The slides were then rinsed in phosphate-buffered saline before exposure to polyclonal anti-E1A antibody (Santa Cruz Biotechnology Inc.) or polyclonal antihexon antibody (Accurate Chemicals & Scientific Corp.). Slides were developed with the Vectastain ABC kit (Vector Laboratories Inc.) according to the manufacturer's instructions.

RESULTS

Induction of fevers by *dl1520* during a phase II clinical trial.

The average baseline temperature prior to the first infusion of ONYX-015 (*dl1520*) for 11 patients treated in the phase II study was 97.4 ± 0.72°F ($n = 11$; range, 96.5 to 98.8°F). The average of temperatures taken 2 to 12 h after the infusion was 99.8 ± 2.04°F ($n = 48$; range, 96.7 to 104.7 35.9 to 40.4°C) (Fig. 1A). The temperatures taken 2 to 12 h after the infusion were significantly higher than the temperatures at baseline ($P < 0.001$). An average of four to five temperatures were taken after the viral infusion, starting 1 to 2 h postinfusion, and most patients used acetaminophen before the infusion and every 4 to 6 h as needed after the infusion. Despite this prophylactic use of acetaminophen, nearly all of the patients developed significant fevers. The fever curve for a single patient, comparing the first and fourth viral treatments is shown in (Fig. 1B). The patient's temperature increased to 102.5°F (39.2°C) within 4 h of each infusion and reached a maximum of 104.4°F (40.2°C) at 24 h after the first infusion, when the patient's maximum temperature exceeded 100°F (37.8°C) for 6 days. Following the fourth infusion the patient developed a systemic inflammatory response requiring treatment with prednisone and oxygen (33). Treatment with prednisone rapidly inhibited the inflammatory and completely abolished the febrile response (Fig. 1B). This treatment, while controlling the systemic inflammatory response, may have also affected the replication

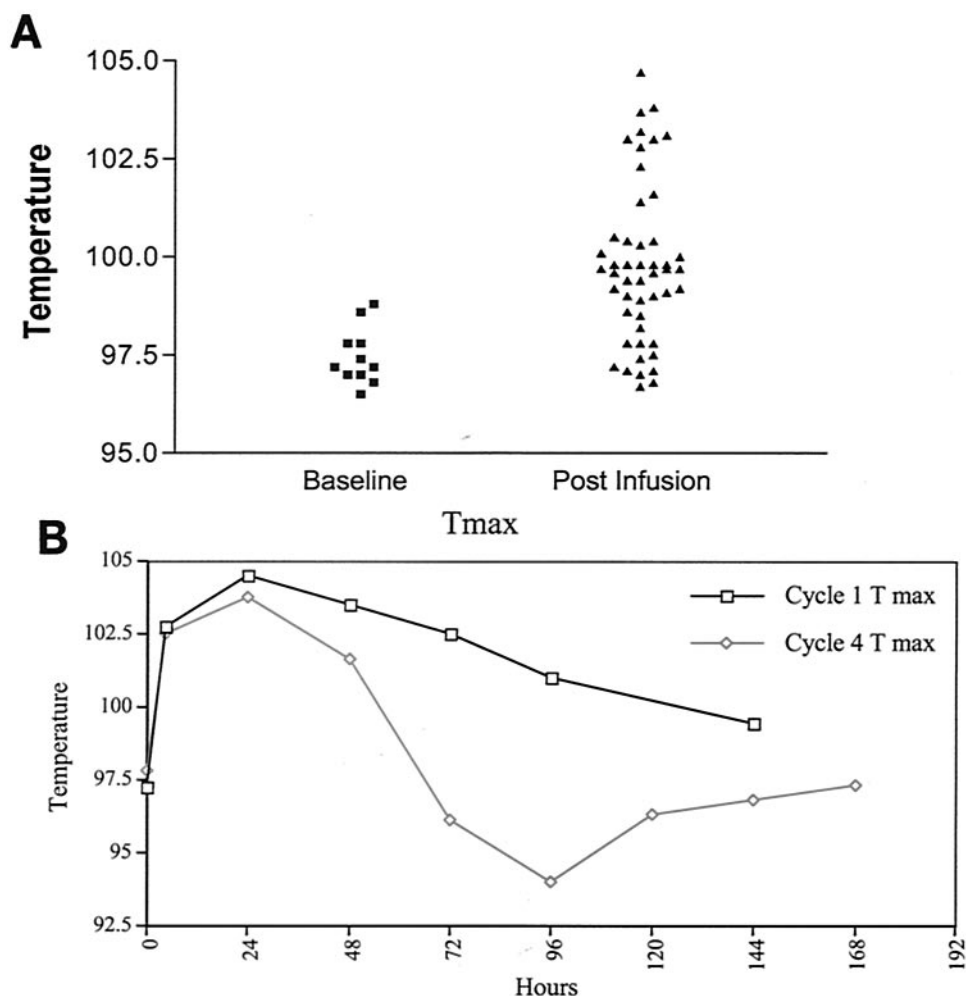


FIG. 1. (A) Induction of fevers following intra-arterial administration of ONYX-015 (*dl1520*) for 11 patients in the phase II clinical trial. (B) Fever produced following the first and fourth infusion of virus in a single patient. The patient received prednisone at 24 h following the fourth infusion.

of ONYX-015 in normal and/or tumor cells. It is therefore important to understand what effects these increases in patient temperature have on viral replication, selectivity, and antitumor activity. It is also vital that the treatments used to alleviate the symptoms do not interfere with the benefits provided by the viral therapy.

Comparison of the losses of cell viability induced by Ad5 and *dl1520* at 37°C. The survival of a panel of nontransformed and transformed cell lines infected with Ad5 and *dl1520* at 37°C is shown in Fig. 2. The percentage of surviving cells was determined 9 days after infection with serial dilutions of either wild-type Ad5 or *dl1520* relative to uninfected controls. The EC_{50} s (the number of viral particles per cell needed to achieve 50% loss of viability of the cell monolayer) are shown in Fig. 2. At 37°C, Ad5 produced significant cell death in all cell lines tested; however, the EC_{50} s varied considerably (range, <0.001 to 2.8). Meanwhile, *dl1520* was attenuated compared to Ad5 at 37°C in all of the cell lines tested (except HepG2). This is likely due to E1B 55K functions other than p53 suppression (see Discussion). No loss of cell viability was observed with *dl1520* at any of the doses tested (up to an MOI of 10 infectious

units/cell) in either of the two nontransformed cell lines. These cell lines, BEAS-2B (normal human bronchial epithelial cells) and MRC-5 (normal human lung fibroblasts) are immortalized, but nontransformed cell lines that are contact inhibited do not form colonies in soft agar and do not form tumors in nude mice. The lack of killing in these two cell lines is consistent with the hypothesis that *dl1520* cannot form productive infections in cells containing functional p53 and an intact p53 pathway. In addition, no loss of cell viability was observed with *dl1520* in three of the nine transformed cell lines tested (MCF-7, Calu-6, and MIA PaCa-2). However this does not correlate with p53 status, since MCF-7 has a functional p53 (19, 41), MIA PaCa-2 has a mutant p53 (9, 26), and Calu-6 is p53 null (1). The remaining six tumor cell lines tested all displayed some level of CPE after treatment with *dl1520* (EC_{50} range, <0.001 to 2.4). Perhaps surprisingly, the greatest level of cytolytic activity was found to occur in the p53-positive HepG2 cells (although it cannot be ruled out that some other point on the p53 pathway is defective in this cell line).

Comparison of the loss of cell viability produced by Ad5 and *dl1520* at 39.5°C. Initially studies were run that verified that the

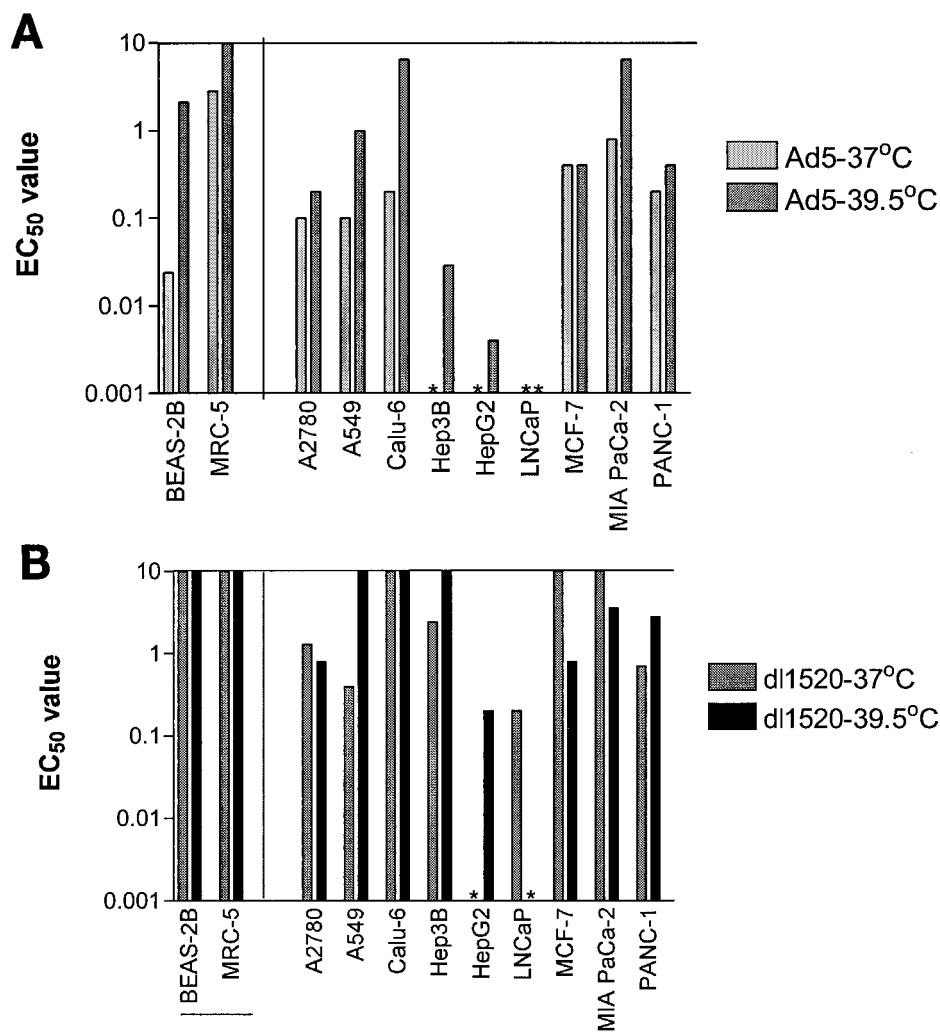


FIG. 2. EC_{50} s (i.e., number of viral units per cell required to produce a 50% loss of viability relative to an uninfected control). Different cell lines were incubated at 37 or 39.5°C following treatment with increasing doses of Ad5 or *dl1520*. Cell survival was measured at 6 days postinfection by MTS assay, and standard curves were used to identify the value at which 50% loss of cell viability was being produced. (The assay used was unable to detect values of >10 or <0.001 .) BEAS-2B and MRC-5 are nontransformed cell lines.

cell lines used were not killed or did not undergo significant growth inhibition at 39.5°C (data not shown). Ad5 was found to show reduced cytotoxicity in the two normal cell lines at 39.5°C compared to 37°C (as measured by loss of cell viability with the MTS assay). No cytotoxic effects were seen in the MRC-5 cells when they were incubated at 39.5°C, and the cytotoxicity of Ad5 was inhibited more than 85-fold in BEAS-2B cells. Reductions in cell viability mediated by Ad5 were also diminished at 39.5°C in the tumor cell lines (with the exception of LNCaP, which displays complete cell death at both temperatures, and MCF-7, which shows no change between the two temperatures). However, among the tumor cells, only A549, Calu-6, and HepG2 demonstrated greater-than-10-fold inhibition of Ad5 mediated cell killing at 39.5°C. None of the cell lines tested demonstrated any increase in cell death at 39.5 compared to 37°C.

In comparison, *dl1520* did not cause any measurable loss of cell viability in the normal cell lines, BEAS-2B and MRC-5, at

either 37 or 39.5°C but did cause cell death at 37°C in six of the nine tumor cell lines tests. Of these, cytotoxicity was diminished at 39.5°C in four of the tumor cell lines (A549, PANC-1, Hep3B, and HepG2) (Fig. 2). Interestingly however, four other of the cell lines (MCF-7, LNCaP, A2780, and MIA PaCa-2) demonstrated increased cytotoxic effects with *dl1520* when the cells were cultured at 39.5 compared to 37°C. The increase in cell death at 39.5°C was most noticeable in MCF-7 and LNCaP cells; MCF-7 cells required $<10\%$ and LNCaP cells required $<0.5\%$ of the input virus to cause 50% cell viability at 39.5 compared to 37°C.

To further illustrate the relative cytotoxic effects of Ad5 and *dl1520*, time course experiments were performed with these viruses. These results also demonstrate that the time-dependent increase in virus-mediated cell death with Ad5 in BEAS-2B cells at 37°C is attenuated when the cells are grown at 39.5°C (Fig. 3A). In contrast, no virus-mediated cell death

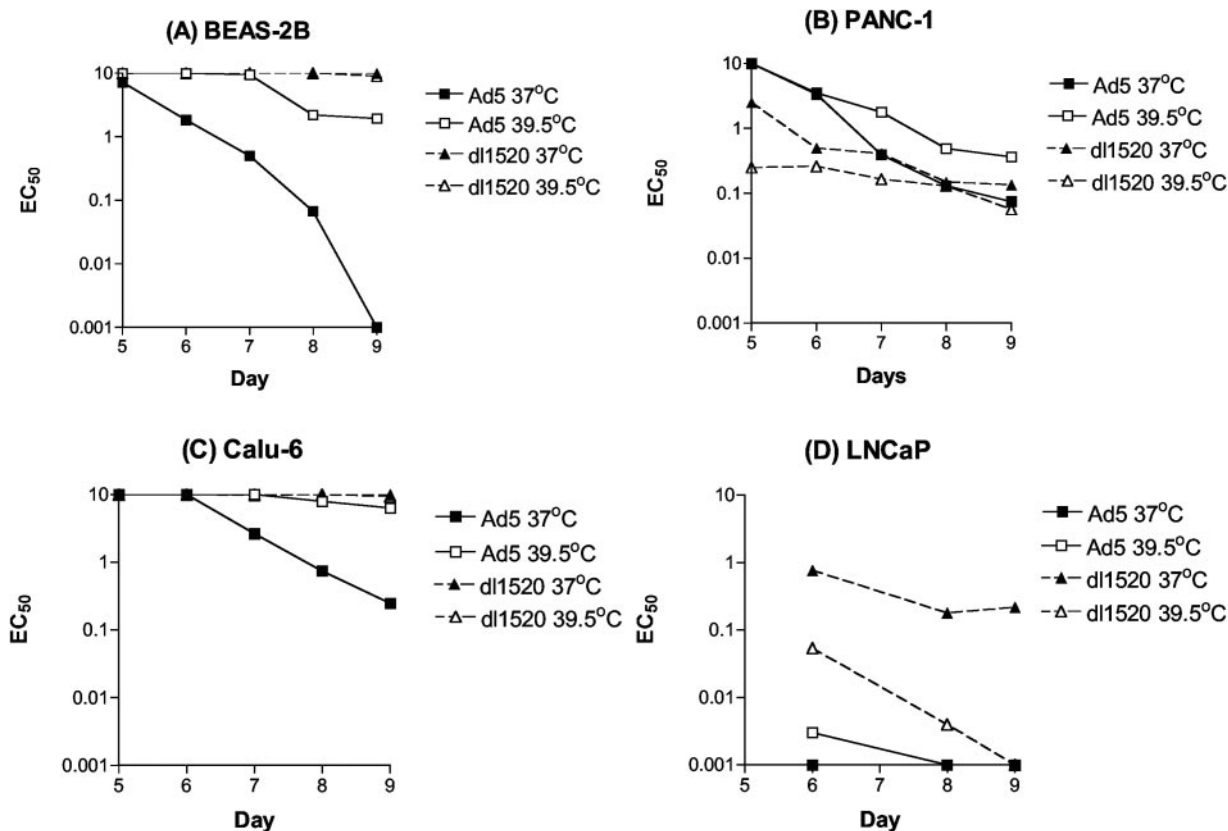


FIG. 3. Experiments were repeated as described for Fig. 2. MTS assays were performed at different times postinfection to create a time course of EC₅₀ values.

was observed in BEAS-2B cells at either 37 or 39.5°C with *dl1520*, again demonstrating the attenuation of *dl1520* compared to Ad5 in nontransformed cells.

Time courses for the cytotoxic activities of Ad5 and *dl1520* in transformed PANC-1, Calu-6, and LNCaP cells are also shown. Ad5-infected cells demonstrate a time-dependent loss of viability in PANC-1 cells, with modest attenuation of these effects at 39.5°C. *dl1520* also demonstrates a time-dependent cytolytic response in PANC-1 cells that is equivalent to that with Ad5 at 37°C while slightly enhanced (compared to that with Ad5) at 39.5°C (Fig. 3B). In contrast, the cytotoxic effects of Ad5 are potentially inhibited at 39.5°C in Calu-6, cells while *dl1520* does not cause any CPE at either temperature in this cell line (Fig. 3C).

Finally, a time course of cell survival in LNCaP cells is shown in Fig. 3D. These cells are particularly sensitive to adenoviral infection, and Ad5 demonstrates significant cell killing, even at the lowest MOIs and earliest time points for both temperatures. *dl1520* is slightly attenuated compared to Ad5 but demonstrates marked cytotoxic effects in this tumor cell line, especially compared to nontransformed cells such as the BEAS-2B and MRC-5 cells. Interestingly, the cytotoxic effect of *dl1520* is markedly enhanced at 39.5 compared to 37°C.

These results clearly indicate that, unlike in nontransformed cell lines, *dl1520* shows increased cell killing at 39.5°C in at least a subset of the tumor cell lines tested. This effect is not seen with the wild-type Ad5, where the cell killing is reduced at

39.5°C in all of the cell lines for which results were obtained. Therefore, the therapeutic index for *dl1520* can be greater at febrile temperatures in some, but not all, tumor cell lines.

Effect of temperature on viral replication. During infection with adenovirus, host protein synthesis is shut down and viral replication results in the formation of thousands of viral particles that form arrays in the nucleus. Ultimately, cellular lysis releases these particles, allowing ongoing infection. We therefore evaluated the effect of temperature on the ability of the virus to form infectious progeny. Cells were infected with either Ad5 or *dl1520* and incubated at 37 or 39.5°C for 3 days. The cells and the medium were then collected, and the cells were disrupted to release any intracellular virus. The number of infectious units produced per cell was determined by TCID₅₀ assay on HEK 293 cells.

The numbers of infectious particles produced by Ad5 and *dl1520* in BEAS-2B cells at 37 and 39.5°C is shown in Fig. 4A. *dl1520* is attenuated by over 2 log units compared to Ad5 at 37°C, and at 39.5°C, there was a large reduction in the amount of infectious virus produced with both *dl1520* and Ad5. Similar results were obtained with another nontransformed cell line, MRC-5 cells (data not shown). These results correlate closely with what was demonstrated by the viability assay. In contrast to the case for BEAS-2B and MRC-5 cells, significant Ad5 and *dl1520* replication was seen at 39.5°C in the transformed PANC-1 cell line (Fig. 4B). Interestingly, although the number of infectious virions produced in *dl1520*-infected cells at 39.5°C

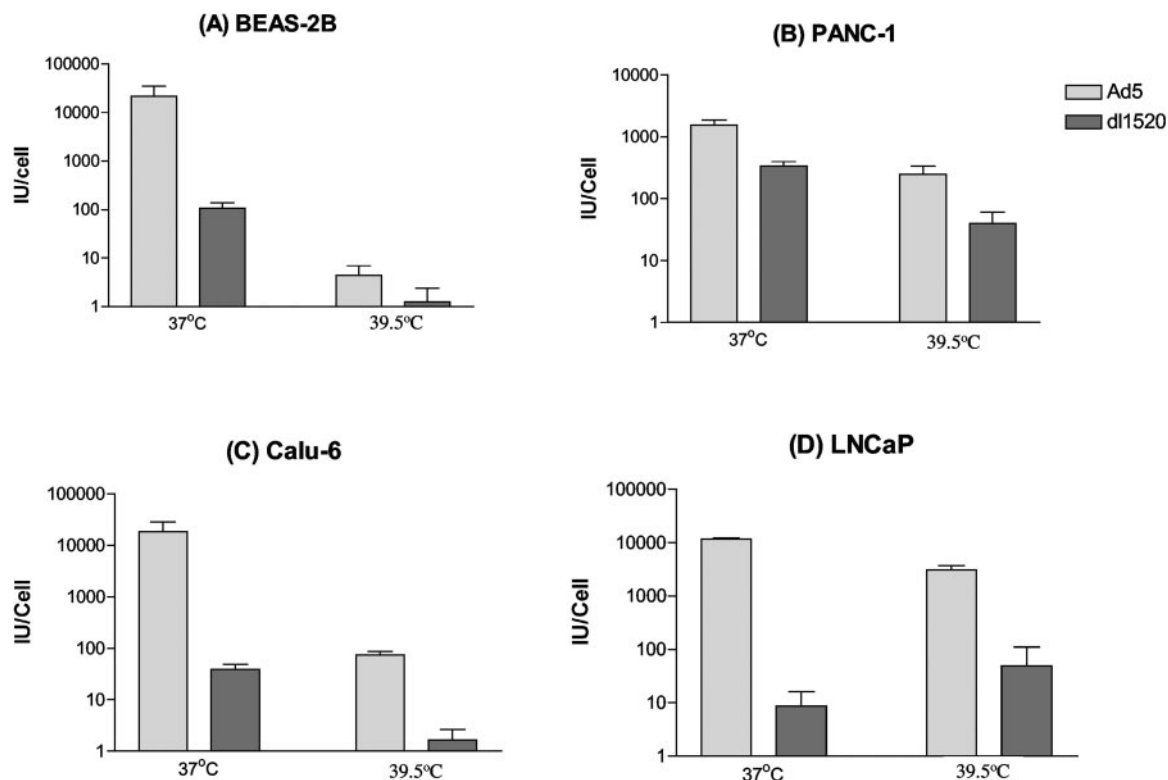


FIG. 4. Replication assay for Ad5 and *dl1520* incubated at 37 or 39.5°C postinfection. Infection was at an MOI of 1.0 PFU/cell, and cells and medium were collected at 3 days postinfection. Cells were then disrupted by cycles of freezing and thawing, and the numbers of infectious units (IU) were assayed by TCID₅₀. Results are calculated as viral units produced per cell at the time of infection. Error bars indicate standard deviations.

is almost 2 log units less than that produced in cells infected with Ad5 at 37°C, the cytolytic effects under these two conditions are approximately equal (Fig. 3B). These results suggest that although *dl1520* produces less infectious virus per cell, it is still able to produce equivalent cytotoxicity in this cell line. The cytolytic activity is controlled by factors in addition to the total number of infectious particles produced.

dl1520 is also attenuated compared to Ad5 in Calu-6 cells, while very little replication occurs in these cells with either virus at 39.5°C (Fig. 4C); these results are consistent with those for the cytotoxic effects (Fig. 3C). Finally, burst assay was also performed in the LNCaP cell line (Fig. 4D). As with the cell viability results, we saw very large levels of Ad5 replication at both temperatures, and *dl1520* produced more particles per cell when incubated at 39.5°C than when incubated at 37°C. However, as with the Panc-1 cell line, it is notable that *dl1520* is capable of producing high levels of cell killing, despite a relatively low production of viral particles. This effect is seen at both 37 and 39.5°C and may reflect different pathways of cell killing incorporated by Ad5 and *dl1520*.

Examination of a possible role of cellular p53 or HSP70 in the increased replication of *dl1520* at 39.5°C. *dl1520* has a deletion of the coding region for E1B 55K. One function of E1B 55K is to bind and inactivate p53, permitting entrance of the cell into S phase, where viral replication can occur. The E1B 55K deletion was therefore thought to confer selective replication of *dl1520* to cells with defects in the p53 pathway,

although results reported here and by other groups (11, 13) indicate a more complex cellular interaction of the E1B 55K deletion mutant. However, p53 is also induced in response to cellular stresses such as heat shock (28, 29). Therefore, it is possible that increased p53 induction is causing cell cycle arrest or apoptosis and so preventing viral replication at 39.5°C. This effect would be expected to be more marked for *dl1520* but would not be expected to occur in tumor cell lines that do not have a functional p53 pathway. However, p53 was not found to

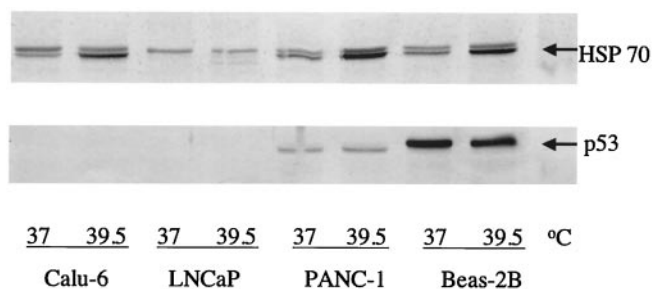


FIG. 5. Western blot analysis of p53 and HSP70 protein (lower band) expression following incubation at 37 or 39.5°C. Cells were grown at the indicated temperatures for 48 h and then disrupted, and protein was collected and assayed. The same amounts of protein were run on SDS-polyacrylamide gels before transfer to nitrocellulose membranes and Western blotting with antibodies to p53 and HSP70 and visualization by the ABC system.

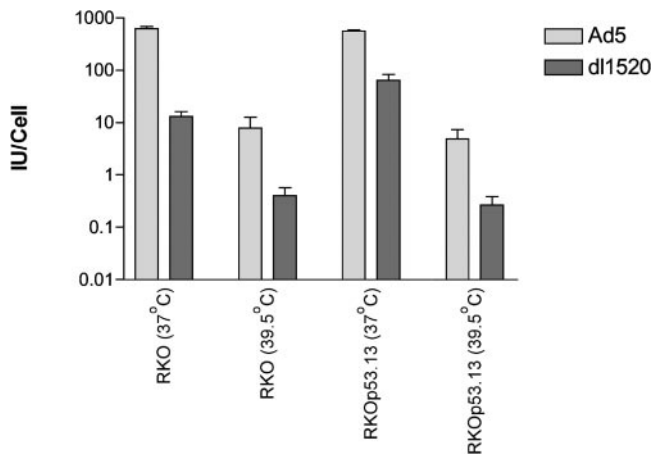


FIG. 6. Effect of p53 status on replication of Ad5 and *dl1520* at 37 and 39.5°C. Matched cell lines with normal (RKO) or dominant negative (RKOp53.13) p53 were used to compare burst assays as described for Fig. 4.

be induced in Panc-1 and Beas-2b cells at 39.5°C (Fig. 5). In addition, Calu-6 and LNCaP cells lack detectable levels of p53 but still suppress the replication of Ad5.

Tumor cell lines have numerous mutations in addition to possible abnormalities in p53, and so correlations between the expression of p53 and the permissiveness of viral replication at 37 and 39.5°C becomes difficult when comparing different cell lines. For this reason, the matched cell lines RKO and RKO p53.13 were tested. The RKO colon carcinoma cell line has been shown to express a wild-type p53 (3). However, the p53 alleles have been mutated in the RKO p53.13 cell line, and the p53 is nonfunctional (37). Results of replication assays at different temperatures following Ad5 or *dl1520* infection in these cell lines are shown in Fig. 6. As before, 39.5°C inhibited viral replication following Ad5 infection; however, no significant effects due to p53 status were seen at either temperature with Ad5. This provides further evidence that the inhibition of replication of wild-type Ad5 at 39.5°C is not due to increased induction of p53. The replication of *dl1520* is also inhibited at 39.5°C in both the parental and p53 mutant cells. At 37°C the replication of *dl1520* was increased in the p53-defective cells compared to the parental cells. However, this difference was lost at 39.5°C, and the replication of *dl1520* was inhibited at 39.5°C in both cells with wild-type p53 and cells with p53 deleted. Therefore, temperature had little effect on p53 levels in the cell lines tested, and the observed temperature effects were not related to the cell's p53 status (Fig. 5 and 6).

Alternatively, induction of HSP70 has been found to facilitate the replication of adenovirus, and so HSP70 induction at higher temperatures may act to improve viral replication. We therefore evaluated the expression of HSP70 in cells treated at 37.0 and 39.5°C. Cell lysates were made at the times indicated and analyzed by Western blotting (Fig. 5). The results demonstrated that HSP70 is induced at 39.5°C in Calu-6, PANC-1, and Beas-2b cells. No detectable HSP70 was found at baseline in LNCaP cells, and HSP-70 was not induced at 39.5°C (the upper band is a noninducible cross-reacting protein commonly observed with this antibody and can act as an internal control for protein levels). Therefore, HSP70 can be induced at the

temperatures where viral replication is inhibited. However, the hypothesis that induction of HSP70 may complement any defects in *dl1520* (such as in viral mRNA transport) is not supported by these results. While it appears that a cellular factor may complement a *dl1520* defect due to the E1B deletion, these results do not support HSP70 or p53 being the critical cellular factor.

Effect of temperature on expression of viral proteins. Further investigations were launched in order to try to pinpoint the block in the Ad5 or *dl1520* viral life cycle encountered at 39.5°C and to examine any differences between the effects of temperature in nontransformed and transformed cells with the different viruses. Western blotting (Fig. 7) and immunohistochemistry (Fig. 8) were used to measure the levels of expression of the early E1A protein and the late capsid protein hexon. A polyclonal antibody was used against hexon so that both the trimeric and monomeric version of the protein would be detected and protein expression rather than folding would be evaluated. *dl309* was also used to identify whether any differential effects seen with *dl1520* are due to the loss of E1B-55K or the E3B region.

It is noticeable that the E1A protein in *dl1520* appears to be slightly smaller than that of Ad5, and it is thought that this is due to preferential expression of the 12S splice variant of the gene product over the 13S form (Fig. 7). This raises interesting questions regarding the interactions of the E1A and E1B regions of the adenoviral genome.

It is also apparent that in the nontransformed cells (Beas-2B), expression of both E1A and hexon is reduced at 39.5°C, indicating that there is a very early block in viral replication. As the infection step was performed at 37°C in all experiments, the viral infection must be compromised at a stage after cell entry but before E1A expression. This result is in distinction with observations by other groups, who found no reduction in viral protein synthesis at increased temperatures but did demonstrate a late defect in virion assembly (44).

In the transformed cell lines, elevated temperature has little effect on E1A or hexon protein levels. In the PANC-1 cell line this may reflect the limited reduction in viral CPE and replication at elevated temperatures relative to that in the nontransformed cells. However, for the Calu-6 cell line it is noticeable that E1A and hexon appear to be unaffected at 39.5°C despite a 2-log-unit reduction in the levels of progeny virus produced following infection with Ad5 (Fig. 4 and 7). This indicates that a late block in the viral life cycle occurs in transformed cells, and this agrees with the previously reported data (which was obtained with transformed HeLa and KB cells). These results were further verified by immunohistochemical staining for E1A protein in Ad5-infected cells (Fig. 8), where it can clearly be seen that the inhibition of viral replication occurs prior to E1A production in the nontransformed Beas-2B cells, while E1A levels appear to be relatively unaffected by elevated temperature in the transformed PANC-1 cell line. Together these results imply that a previously unreported early inhibition of viral replication may be the predominant mechanism of inhibition of viral replication by fever in nontransformed cells and that this inhibition is lost in all of the transformed cell lines examined. It is also of note that *dl1520* actually displays increased levels of hexon expression at 39.5°C in some cell lines, such as LNCaP. This increase

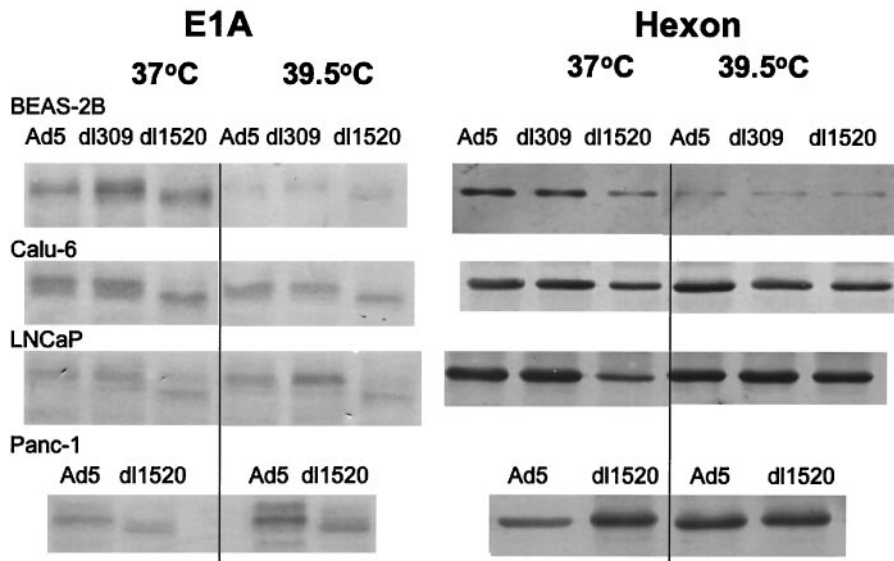


FIG. 7. Western blot analysis of E1A and hexon protein expression following infection with Ad5 or *dl1520* and incubation at 37 or 39.5°C. Cells were infected at an MOI of 1.0 PFU/cell, and cells were collected at 3 days postinfection. Cells were disrupted, and protein was collected and assayed. The same amounts of protein were run on SDS-polyacrylamide gels before transfer to nitrocellulose membranes and Western blotting with antibodies to E1A or hexon and visualization with the ABC system.

in replication is likely to be in part due to a relief of its natural attenuation relative to Ad5 under these conditions. Therefore, it appears that at least two independent mechanisms contribute to the increased replication of *dl1520* in tumor cells at 39.5°C. First, the cellular mechanism responsible for the early

inhibition of viral replication 39.5°C in normal cells is lost in tumor cells, permitting greater viral replication in tumor cells. Second, the attenuation of *dl1520* due to deletion of the E1b-55K protein is complemented at 39.5°C in some tumor cells, permitting greater viral replication in tumor cells.

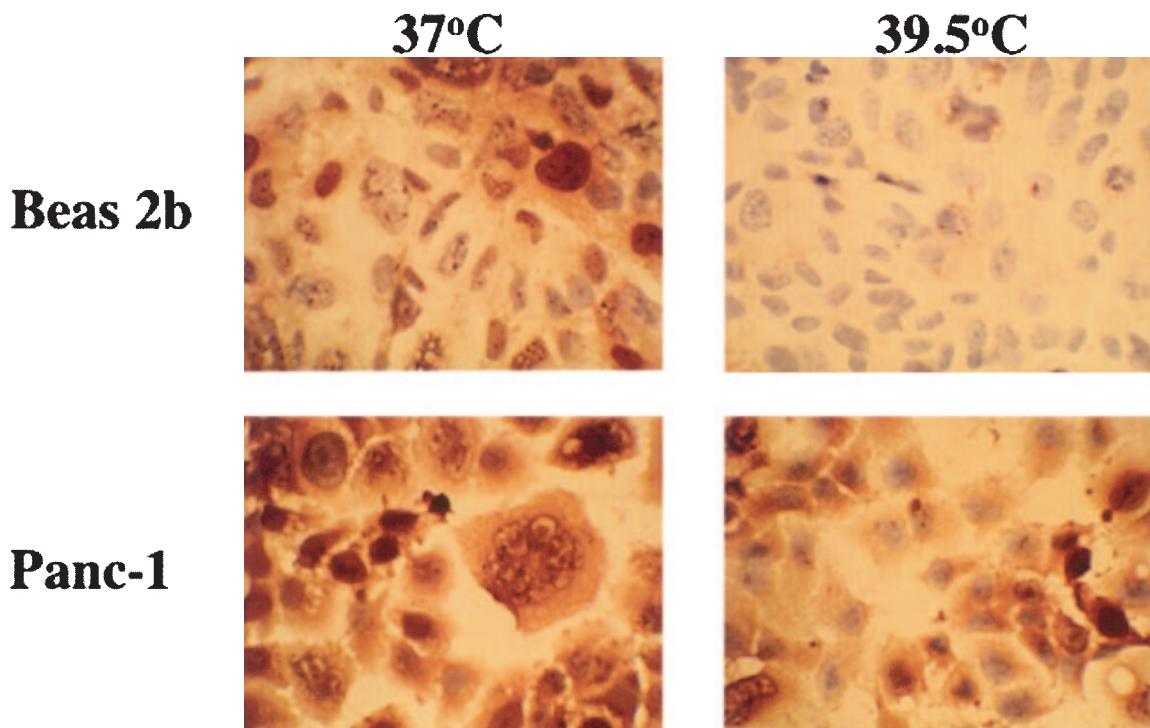


FIG. 8. Immunohistochemistry of E1A and hexon expression following incubation of Ad5-infected cells at 37 or 39.5°C. Cells were infected at an MOI of 1.0 PFU/cell, and cells were fixed at 3 days postinfection. Antibodies against E1A or hexon proteins were then used, and visualization was with the ABC system.

DISCUSSION

This study was initiated following the observation that fevers were commonly induced during intra-arterial *d11520* (ONYX-015) treatment of patients with colon carcinoma metastatic to the liver (Fig. 1). No previous work had compared the effects of febrile temperatures on the ability of oncolytic viruses to replicate in nontransformed and transformed cells. This is of particular relevance because the fevers were commonly treated with anti-inflammatory agents, such as aspirin and acetaminophen, and steroids, such as prednisone, yet we do not know if the effects of these agents will enhance or reduce the benefits of the viral treatment. It has been known for over 30 years that under extreme conditions of heat shock, adenovirus replication is inhibited at a late stage in the life cycle. Warocquier et al. (44) demonstrated that although the production of infectious adenovirus is reduced by over 99% when cells are grown at 42°C, replication of viral DNA and the transcription of RNA proceed normally. It was further shown that viral protein synthesis was not inhibited at this elevated temperature (30) but that there was a large reduction in the levels of capsid formation seen at 42 compared to 37°C. Only 3% of the monomeric hexon polypeptides assembled to form the trimeric capsomere, and 9% of the penton base and fiber polypeptide assembled to form the penton capsomere. This was not due to incorrect protein folding, as subsequently reducing the temperature to 37°C allowed assembly of the hexon trimer without the need for new protein synthesis (23). Additional studies indicated that transfer of cytoplasmic viral polypeptides to the nucleus postsynthesis was not impaired and, if anything, was increased at 42°C (43).

We therefore investigated the effects of the physiologically significant temperature of 39.5°C on viral replication and CPE in both normal and transformed cells (Fig. 2 to 4). This was done for the clinical virus *d11520* (ONYX-015) and for its parental wild type, Ad5. In the nontransformed cell lines (Beas-2B and MRC-5), both Ad5 and *d11520* were attenuated at 39.5°C. This attenuation correlated with a reduction in E1A expression, implicating a block at an early stage of the viral life cycle. As the initial infection took place at 37°C in all experiments, the block is likely to be after entry of the virus into the cell but before early gene expression (perhaps during escape from the endosome, during movement along microtubules, or during uncoating and entry to the nucleus). This early inhibition of viral replication, which occurs at a physiologically relevant temperature, has not been previously described and is likely to be the predominant mechanism of inhibition of viral replication by a fever in the nontransformed cells. In transformed cells, E1A and hexon expression appeared to be unaffected by elevated temperature, and so this represents a generalized means by which adenovirus may be targeted to tumor cells at febrile temperatures.

However, Ad5 and *d11520* still showed some reduction in replication at 39.5°C in most of the tumor cell lines. Expression of E1A and hexon was still observed in cells in which viral CPE and replication are reduced (such as CALU-6 cells), implying that although the early inhibition of viral replication is lost, a secondary block late in the viral life cycle is responsible for the approximately 10-fold reduction in viral titers in tumor cells cultured at 39.5°C. It is likely that this relates to the previously

described inability to assemble the viral capsid (44) and that the 3- to 4-log-unit reduction in viral titers in nontransformed cells results from the combination of both early and late blocks.

As noted above, Ad5 replication was inhibited by 3 to 4 log units at 39.5°C in nontransformed cells but by only about 1 log unit in transformed cells (range), and so at febrile temperature, viral replication is favored in tumor cells by 1 to 2 log units compared to nontransformed cells, even with wild-type virus. The increase in therapeutic index was more dramatic with *d11520*. This virus is highly attenuated in nontransformed cells, and replication is undetectable in nontransformed cells cultured at 39.5°C. In contrast, replication of *d11520* was actually enhanced and CPE was increased in some of the tumor cell lines (LNCaP and MCF-7). Therefore, the selectivity and tumor specificity of *d11520* can be enhanced in tumor cells by three distinct mechanisms. First, the early cellular block in viral replication at 39.5°C prevents replication in nontransformed cells but is absent in all tumor cell lines tested. Second, the deletion of E1B results in attenuation in nontransformed cells. Third, the replication of *d11520* can be enhanced at 39.5°C in some tumor cell lines. The mechanism for this enhanced replication of *d11520* at increased temperatures in tumors is not known, but its elucidation will help to identify patients who might benefit most from this treatment.

There are several possible explanations for this effect. E1B-55K has multiple functions during adenovirus infection and replication, and the loss of these functions could be compensated for by the induction or loss of cellular proteins. In the early phase of infection, E1B-55K forms a stable complex with p53 (36) and inhibits p53-mediated transcriptional activation (45). In addition, E1B-55K and E4orf6 work together to transport p53 to the cytoplasm where it is degraded (38). The inactivation of p53 prevents apoptosis, allows cells to enter S phase, and so permits viral replication. As p53 levels are known to be increased at higher temperatures, it is possible that this could explain the increased attenuation in normal cells and the ability of *d11520* to replicate normally at 39.5°C in some tumor cell lines, where p53 function is lost. However, suppression of viral replication was observed in tumor cells that were devoid of p53. In addition, we found no induction of p53 protein at 39.5°C in the Panc-1 (mutant p53) and Beas-2b (wild-type p53) cell lines at the temperatures used in this study (Fig. 5). While previous studies have demonstrated increased levels of p53 with heat shock, this induction of p53 occurs only at very high temperatures. Therefore, p53-mediated effects are unlikely to account for the inhibition of viral replication at higher temperatures.

Another possible explanation for the enhanced replication of *d11520* at 39.5°C in tumor cells relates to nuclear-cytoplasmic transport functions of the E1B-55K protein. During the late phase of adenovirus infection, a protein complex containing E1B-55K and E4orf6 shuttles between the nucleus and the cytoplasm, mediating the transport of viral mRNA to the cytoplasm. This function occurs while the nucleocytoplasmic transport of most host cell mRNAs is inhibited (6, 31), and E1B-55K has also been implicated in the direct shutoff of host cell protein synthesis (2). The loss of these two late functions is thought to contribute to the general attenuation of *d11520* compared to Ad5. One possibility therefore is that the induction of cellular proteins in response to elevated temperature

partially compensates for these defects. For example, it was found that incubating cells at 39.5°C can compensate for E1B-55K mutations that affect host range in some cells (12, 14, 15). Harada and Berk (13) speculated that this effect might be due to the selective stimulation of late viral protein synthesis by heat shock proteins that substitute for the loss of E1B-55K.

For example, ribosome shunting is used by both HSP70 mRNA (35) and late Ad mRNA to allow preferential translation when the translation of host capped mRNA has been inhibited. It is possible that the induction of heat shock proteins, which function as chaperones, could substitute for the nucleocytoplasmic shuttling functions of E1B-55K. Indeed, HSP-70 protein has been found to colocalize with the Ad particles in the nucleus (18) and to associate with both hexon (27) and fiber (24) proteins. However, induction of HSP70 is unlikely to account for the enhanced replication of *dl1520*, as HSP70 was not induced in LNCaP cells (Fig. 5) (even though *dl1520* replication was improved at 39.5°C) but was induced in cells demonstrating suppression of viral replication.

Other possibilities may involve the cap binding complex eIF-4F, which stimulates the interaction of capped mRNAs with the 40S ribosomal subunit during translation initiation and is regulated by the phosphorylation of its eIF-4E subunit (21). Reduced phosphorylation of eIF-4E, with resultant inhibition of cellular translation, occurs during the heat shock response (22), and eIF-4E phosphorylation is also reduced late in adenovirus infection (17). This dephosphorylation step, mediated by the viral L4-100K protein (7), might be substituted for by the heat shock response at 39.5°C. Alternatively, E1B-55K targets the Y-box transcription factor YB-1 to the nuclei of infected cells (16). YB-1 then becomes associated with viral inclusion bodies, the sites of viral transcription and replication, and was shown to facilitate adenovirus E2 gene expression through the E2 late promoter. This role of E1B-55K could also be substituted for by hyperthermia, as a temperature of 39.5°C has been shown to cause translocation of YB-1 from the cytoplasm to the nucleus in several human colon carcinoma cell lines (39). Therefore, although the exact mechanism remains to be defined and does not involve p53 or HSP70, it seems likely that the heat shock response substitutes for E1B-55K functions at 39.5°C in some tumor cell lines, where the early block in viral replication is lost and where many heat shock proteins are often overexpressed.

It was also observed that although in most cases the results for *dl1520* and Ad5 viral cytotoxic effects and replication were closely correlated with E1A and hexon levels, there were exceptions. In the PANC-1 and LNCaP cell lines, *dl1520* was capable of inducing levels of CPE similar to those induced by Ad5 despite greatly reduced levels of replication. It is possible, therefore, that p53-mediated premature cell lysis during infection with *dl1520* in some of the cell lines results in rapid cell death prior to the formation of high levels of progeny virus. However, this would not account for the effects seen in PANC-1 and LNCaP cells, since these cells have no detectable p53 (LNCaP) or mutated p53 (PAMC-1) (Fig. 5).

Another notable difference between *dl1520* and Ad5 was that both appeared to produce E1A proteins of different sizes, with *dl1520* apparently producing the 12S splice variant (Fig. 7), although this is currently being investigated further. The E1A gene is known to produce at least six different polypep-

tides ranging in size from 38 to 51 kDa, with the most common being the 12S (48 kDa; 243R) and 13S (51 kDa; 289R) forms. Although some functional differences between these forms have been described, the roles of the different variants are unknown (8). Previous results have suggested that the 12S form is found predominantly in the cytoplasm and that the 13S form is found predominantly in the nucleus (25), and it was found in this study that staining of E1A in *dl1520* was predominantly cytoplasmic (data not shown). This result raises interesting questions regarding the interaction of E1B 55K and E1A.

This study was initially begun to examine the effects of febrile temperature on the tumor-targeting potential of the adenovirus *dl1520* (ONYX-015). It was found that although fever usually inhibited viral replication, physiologically significant temperatures could also increase the therapeutic index of viral replication in tumor over normal tissues. In addition, in some tumor cell lines, the E1B-55K deletion in *dl1520* could be at least partially compensated for by a cellular factor at the higher temperatures used, leading to increased viral replication at 39.5°C. Finally, it was also reported that the predominant factor inhibiting viral replication in nontransformed cells at febrile temperatures is likely to be at a previously undescribed early step in the viral life cycle.

ACKNOWLEDGMENTS

This work was funded by the Veteran's Administration.

We thank Ayn Kou and Ji Di Li for their help with many aspects of this work, David Kirn and Michael Kastan for the donation of cell lines used in this work, and Bill Wold and Arnold Berk for the donation of viral strains.

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