Eur J Cancer Clin Oncol, Vol. 22, No. 4, pp. 401–409, 1986.
Printed in Great Britain.

0277-5379/86$3.00+0.00

Adenovirus Replication as an *in Vitro* Probe for Drug Sensitivity in Human Tumors*

PETER G. PARSONS,†‡ KEVIN R. MAYNARD,† JOHN H. LITTLE§ AND G. RODERICK MCLEOD§

†*Queensland Institute of Medical Research, Herston, Queensland, Australia and* §*Princess Alexandra Hospital, Woolloongabba, Queensland, Australia.*

Abstract—*The feasibility of using adenovirus* 5 *as an* in vitro *probe for chemosensitivity in short-term cultures of human tumors was evaluated using human melanoma cell lines and primary cultures of melanoma biopsies. A convenient immunoperoxidase method was developed for quantitating viral replication* 2 *days after infection. Two different approaches were explored: the host cell reactivation assay (HCR) using drug-treated virus; and the viral capacity assay using drug-treated cells. The HCR assay detected sensitivity to* 5-(3-*methyl*-1-*triazeno*)*imidazole*-4-*carboxamide (MTIC) in Mer*$^-$ *(methyl excision repair deficient) cell lines as decreased ability of the cells to replicate MTIC-treated virus. This test should be applicable to DNA-damaging agents and repair-deficient tumors. Adenovirus replicated readily in nonproliferating primary cultures of melanoma biopsies; application of the HCR assays to this material identified one Mer*$^-$ *sample of* 11 *tested. Herpes viruses were not suitable for use in HCR because herpes simplex virus type* 1 *failed to distinguish Mer*$^-$ *from Mer*$^+$ *melanoma cells; and nonproductive infection of MTIC-sensitive lymphoid cells with Epstein–Barr virus yielded an MTIC-resistant cell line. The second assay (viral capacity) involved determination of the inhibition of replication of untreated virus in treated cells. This approach correctly predicted sensitivity to hydroxyurea and deoxyadenosine in melanoma cell lines when compared with clonogenic survival assay. Viral capacity was also inhibited by cytosine arabinoside, fluorouracil, vincristine, adriamycin,* 6-*mercaptopurine and ionising radiation, and may therefore be useful for detecting sensitivity to a wide range of antitumor agents.*

INTRODUCTION

IN RECENT years there has been an increasing interest in the use of *in vitro* assays utilising human tumor cells for the screening of anticancer drugs and for prediction of clinical response on an individual basis. Such tests include clonogenic survival assays of tumor cells treated and subsequently cultured *in vitro* [1–8] or *in vivo* [8–10] and assays based upon inhibition of DNA synthesis in short term cultures [7, 11, 12]. These approaches suffer from the disadvantages of the low plating efficiency of human tumor cells, the inability of primary cultures to continue DNA replication even for a brief period, and the variety of biochemically-undefined resistance mechanisms available to a particular agent. In addition, it has been difficult to identify the response of tumor as distinct from normal cells present in the culture, and to correctly predict chemosensitivity as distinct from the less useful parameter of resistance.

We propose a new approach using adenovirus 5 as a probe for chemosensitivity. Adenovirus has a double-stranded DNA genome of 2.3×10^7 daltons which is closely associated with host cell chromosomes during lytic infection [13] and relies heavily on cellular enzymes for replication [14–16]. The virus replicates in cells from most human tissues and can be quantitated on an individual cell basis 2 days after infection by immunofluorescent detection of viral antigen [17]. Of critical importance for the present purpose, the virus replicates readily in proliferating and nonproliferating cells [14–16]. It therefore seemed reasonable to apply to chemotherapeutic agents and primary cultures of tumor biopsies two different types of virus assay used previously to compare the toxicity of carcinogens in continuous cell lines: the host cell reactivation (HCR) and viral capacity assays.

HCR compares the ability of host cells, not treated with the agent, to repair and hence replicate drug-damaged viral DNA. The dose-survival

Accepted 27 September 1985.
*This work was supported by a grant from the Queensland Cancer Fund.
‡To whom correspondence and requests for reprints should be addressed. The abbreviations used are: HCR, host cell reactivation; MTIC, 5-(3-methyl-1-triazeno)imidazole-4-carboxamide; ID, infectious dose; HSV-1, herpes simplex virus type 1; EBV, Epstein–Barr virus.

response for viral replication closely parallels the differences in cell survival found for cell lines sensitive to u.v. [18], ionizing radiation [17], carcinogenic methylating agents [19, 20] and MTIC, the active metabolite of the antitumor methylating agent DTIC [21]. Human tumour cell lines of the Mer^- phenotype (methyl excision repair deficient) are highly sensitive to methylating agents with respect to cell survival and HCR [19–21]. For DNA-damaging agents, the HCR approach could therefore be expected to correctly predict tumor sensitivity resulting from a deficiency in DNA repair.

Viral capacity is a term used by Goohill *et al.* [22] to describe the ability of u.v.-treated cell lines to replicate untreated herpes simplex virus. The differences in viral capacity found comparing u.v.-sensitive and normal cells were similar to the differences in clonogenic cell survival. Since inhibition of DNA synthesis is a major effect of most chemotherapeutic agents, and drugs such as hydroxyurea, cytosine arabinoside and fluorouracil inhibit adenovirus replication [14–16], this assay is potentially much broader in scope than HCR because with adenovirus it forces a test, irrespective of the proliferative state of the cells and the cytotoxic mechanism, of the ability of treated cells to replicate DNA. A third and quite limited approach not addressed by this study involves combination of both assays to detect enhancement of HCR (induction of DNA repair) in cells pretreated with the agent. Studied extensively in bacteria, the relevance of this effect to repair of genotoxic damage in mammalian cells is not yet clear [23].

Using a rapid immunoperoxidase technique for quantitating adenovirus replication, we have explored the use of the HCR and viral capacity assays in human tumor cells using 10 agents chosen from the major classes of antitumor drugs. Herpes simplex virus type 1 (HSV–1) has been used to study HCR after u.v. irradiation [24] but requires cells to be proliferating. The virus was included in the present study because of the possibility of replicating in a small proportion of cells in a tumor biopsy. The aim of this work was to determine whether a viral probe could distinguish sensitive from resistant cell lines as clearly as clonogenic survival assays, and to determine whether adenovirus could replicate in primary cultures of human melanoma cells; correlation of the *in vitro* tests with clinical response has not yet been determined.

MATERIALS AND METHODS

Cell culture

The origins of the human melanoma MM96, MM253c1a and MM253–3D cell lines have been described [21, 25, 26]; the MM96L line is a late passage subline of MM96. The Burkitt lymphoma BJAB and Epstein–Barr virus (EBV)–infected B95–8/BJAB cell lines [27] were provided by Dr. D. J. Moss of this institute. Biopsies of histologically-confirmed malignant melanoma (lymph node metastases) were received from the Princess Alexandra Hospital. The samples were minced and cultured in Roswell Park Memorial Institute Tissue Culture Medium 1640 (Commonwealth Serum Laboratories, Melbourne, Australia) containing 10% inactivated fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 3 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. After 1 or 2 days adherent cells were detached (0.2 mg/ml trypsin in PBS) and reseeded for infection with adenovirus (see below). Some cultures were maintained separately for the purpose of establishing a continuous cell line; overgrowth by fibroblasts was prevented by the addition of cholera toxin (2 nM) or by culture in calcium-depleted medium [25]. All cell lines were periodically assayed for mycoplasma [21].

Drug treatments for cell survival assays were carried out using duplicate cultures, seeded in Linbro plates (5×10^3 cells per 16-mm diameter well) 24 hr previously. After 6–8 days cells were labelled with [^{3}H/methyl]-thymidine (2 μCi/ml, 40 Ci/mmol; Radiochemical Centre, Amersham, Bucks, U.K.) for 3 hr and harvested (trypsin) onto glass fibre discs prior to liquid scintillation counting. Survival curves for each drug were obtained using five dose levels, the highest dose usually giving approx. 1% survival. For lymphoid cell lines, the plates were incubated at an angle of 30° to enhance cell growth, and detachment with trypsin was not required for harvesting.

Because some of the dose–response curves exhibited a shoulder, cell and virus survivals were compared on the basis of D_0 (dose required to reduce survival by 0.37 on the linear part of the survival curve) and D_{37} (dose required to reduce survival to $1/e$, or 37%). The D_{37} value was therefore influenced by the shoulder as well as by the slope of the survival curve and was equal to the D_0 in the absence of a shoulder. Values could not be established for the HCR assay in the most resistant cells because of the solubility limit of MTIC in aqueous solution.

Replication of adenovirus

A stock of partially-purified virus was prepared by infecting 10^8 HeLa cells with 5×10^5 infectious doses (ID) of virus in 50 ml medium. The cells were harvested after 3 days, frozen and thawed five times in 20 ml medium and extracted with Arklone (2×10 ml) at 4°C. The aqueous supernatant (7×10^6 ID/ml) was stored at −70°C. Virus replication

was determined using a modification of the immunofluorescent method of Rainbow and Howes [17]. Infected cultures (5×10^3 cells per well in microtitre plates) were washed with phosphate-buffered saline, fixed with methanol for 1 min and incubated for 30 min at 37°C with a 1/30 dilution of adenovirus 5 neutralising rabbit antiserum (Microbiological Associates, Walkersville, MD) or a 1/15 dilution of human plasma in phosphate-buffered saline (20 μl). After washing with buffer (3×0.2 ml), the cultures were incubated for 30 min at 37°C with a 1/100 dilution of peroxidase-conjugated protein A (Kirkegaard and Perry Laboratories, Gaithersburg, MD) in phosphate-buffered saline (20 μl), and washed with 0.02 M Tris buffer, pH 7.4 (3×0.2 ml). Cells containing replicating virus were identified microscopically by brown nuclear staining developed after 5 min treatment at ambient temperature with a mixture of *O*-dianisidine (1 mM) and hydrogen peroxide (2.4 mM) in Tris buffer. After washing with water to terminate the reaction, the plates could be stored at ambient temperature for months without loss of staining. The background was typically <3 stained cells per well. Wells containing >400 stained cells were not scored. The two successive dilutions at the end point were scored giving a total of four replicates. One ID was defined as the minimum amount (maximum dilution) of virus required to produce one infected cell under the above conditions.

HCR assay

Adenovirus (5×10^6 ID/ml) was treated with MTIC in medium for 90 min and stored at −70°C in 150-μl aliquots. Cells seeded 24 hr previously ($5–10 \times 10^3$ per well in microtitre plates) were infected with duplicate 10-fold dilutions of virus in medium (0.1 ml) for 2 hr. The medium was then replaced and viral replication determined after 48 hr as described above. In one experiment, HCR was determined by plaque formation in cell monolayers cultured under agar as previously described [21].

Viral capacity

Cells seeded as for the HCR assay were infected with dilutions of untreated adenovirus for 1 hr. The medium was then replaced and, unless otherwise stated, appropriate doses of drugs were added. After 48 hr, viral replication was quantitated as described above.

Replication of HSV-1

A pool of HSV-1 (2×10^7 ID/ml) was obtained as supernatant medium from 5×10^7 MM253cla cells infected 2 days previously with plaque-purified HSV-1. For quantitation of viral replication, six replicates of cells seeded 24 hr previously (5×10^3 cells per well in microtitre plates) were infected with 10-fold dilutions of virus in medium (0.1 ml) for 2 hr and the medium was then replaced. After 5 days the wells were scored under the microscope for viral replication (lysis of the cell monolayer) and the ID calculated as the virus dilution required to lyse 50% of wells [28]. For the HCR assay, HSV-1 was treated with MTIC and viral replication determined as described above.

RESULTS

Immunoperoxidase assay for replication of adenovirus

The immunoperoxidase method developed for this work was more convenient than fluorescence detection of viral antigens because a large number of tests could be carried out rapidly in microtitre plates giving permanently-stained cultures which could be readily scored using a low power light microscope. In addition, the plates could be stored at 4°C for several weeks at the intermediate stages before staining.

Although an infection period of 48 hr was used routinely, use of periods ranging from 36 to 72 hr gave qualitatively similar results. Plasma from humans with a high adenovirus antibody titre gave the same results as strain-specific neutralising rabbit serum. Uninfected melanoma cells and fibroblasts were always negative, irrespective of the source of antibody. Varying the initial cell density (3000–20000 cells/well) did not affect the dose response curves obtained.

Virus-infected tumor cells (HeLa, melanoma) were stained over the whole of the cell, which in some cases became rounded with condensation of the cytoplasm. Fibroblasts, on the other hand, retained their spindle shape and staining was clearly limited to the nucleus. This enabled melanoma cells to be distinguished from fibroblasts, the latter being a minor contaminant (< 10% of cells) in 2–3-day-old primary cultures of melanoma biopsies and other tumors. A separate experiment showed that macrophages did not interfere with the test. Macrophages infected with virus did not remain attached in the microtitre wells during the fixing and washing procedures.

The possibility that expression of viral antigen in cells 2 days after infection represented complementation or abortive infection and not replication of infectious virus was excluded by a number of independent observations. First, the multiplicity of infection (number of ID per cell) in virus dilutions used for assay calculations was less than 1, thus excluding the possibility of complementation by defective virions. Secondly, no viral antigen could be detected immediately after infection, suggesting that a considerable degree of virus replication is

required before antigen becomes detectable. Thirdly, similar results were obtained when the plaque assay was used to compare the replication of MTIC-treated virus in sensitive and resistant cell lines (see Fig. 1B below). Finally, an independent though somewhat more qualitative estimate of viral replication was available in the present assay from the degree of cell lysis and detachment observed at virus concentrations 10–100 times higher than the immunologically-determined endpoint. In both the HCR and viral capacity assays this visual endpoint always changed to the same extent as that determined quantitatively by staining.

Detection of MTIC sensitivity in cell lines by adenovirus HCR

An autologous pair of Mer$^-$ and Mer$^+$ cell lines was used to evaluate the present approach. As found previously [21, 26] with the Mer$^-$ line MM253c1a was highly sensitive to MTIC compared with the Mer$^+$ derivative MM253–3D in a cell survival assay (Fig. 1A) and in an HCR assay where viral replication was determined by plaque formation after culture of a cell monolayer under agar for 14 days (Fig. 1B). A similar difference in HCR between the two cell lines was found when immunoperoxidase staining was used to quantitate viral replication 2 days after infection (Fig. 1B). The same differential HCR response was found comparing three other Mer$^-$ human tumor cell lines (MM384, MM138 and HeLa-S_3) with six allogeneic Mer$^+$ melanoma lines and three Mer$^+$ fibroblast strains (results not shown).

The Mer$^-$ phenotype and herpesviruses

HSV-1 replicated well in MM253c1a and MM253–3D cells. Treatment of virus with MTIC concentrations 5–10-fold less than those used for adenovirus inhibited replication but no difference in virus survival was found between the two cell lines (Fig. 2A).

The effect of EBV on MITC sensitivity in stably-infected cells was determined using an autologous pair of Burkitt lymphoma cell lines. The uninfected line BJAB was shown to be highly sensitive to MTIC (D_0 21 μM) compared with a large number of other lymphoid cell lines tested in this laboratory (D_0 100–200 μM) and was therefore designated Mer$^-$. The EBV-infected derivative B95–8/BJAB was found to be highly resistant to MTIC (D_0 190 μM; Fig. 2B).

HCR of MTIC-treated adenovirus in primary cultures of melanoma biopsies

Of 22 biopsies received, five yielded insufficient adherent cells for analysis ($< 10^5$), one contained heavily melanised cells which gave a high background in the peroxidase staining assay and two gave insufficient viral replication to be quantitated. Cultures were > 95% melanoma cells based on morphology and whole-cell peroxidase staining characteristic of tumor cells. The replication of control and MTIC-treated virus in the remaining 14 biopsies is described in Table 1 and compared with Mer$^-$ and Mer$^+$ cell lines. Biopsies were designated Mer$^+$ if the D_0 for HCR was ≥ 10 mM and Mer$^-$ if the D_0 was ≤ 8 mM MTIC.

The results showed that untreated cells were able to replicate adenovirus as effectively and usually better than permanent cell lines such as MM253c1a. Only one biopsy (MM446) had a D_0 in the HCR assay as low as the Mer$^-$ cell lines. In some cases permanent cell lines were established from these biopsies and it was therefore possible to compare the biopsy HCR with the HCR and clonogenic cell survival of the derived cell lines.

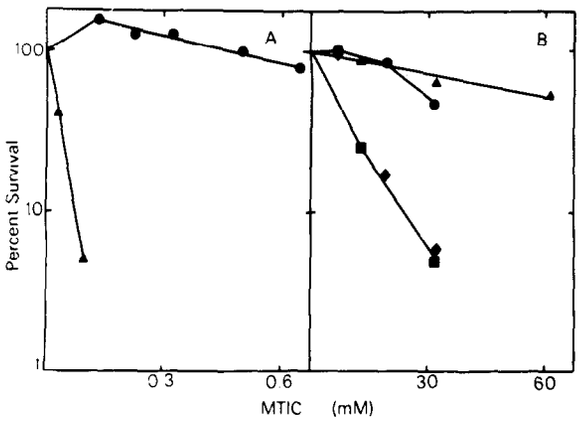

Fig. 1. Comparison of cell survival and adenovirus HCR in sensitive (Mer$^-$) MM253c1a and resistant (Mer$^+$) MM253–3D cell lines. A, survival of MTIC-treated cells. ▲, MM253c1a; ●, MM253–3D, B, Replication of MTIC-treated adenovirus in cell lines. Plaque assay: ■, MM253c1a; ▲, MM253–3D. Immunoperoxidase assay: ◆, MM253c1a; ●, MM253–3D. Points are means of duplicates.

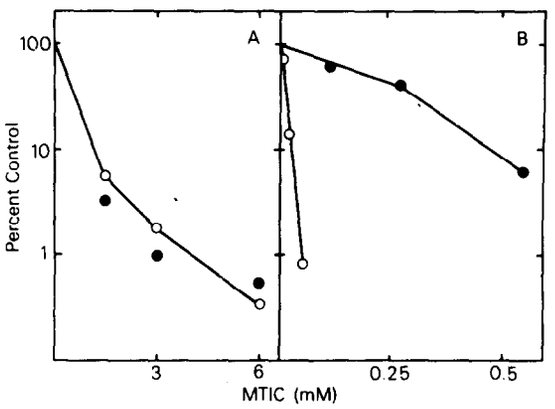

Fig. 2. Interaction of herpesviruses with the Mer$^-$ phenotype. A, HSV-1 replication in Mer$^-$ and Mer$^+$ cells treated with MTIC. ○ MM253c1a; ●, MM253–3D. Infectivity in control cells was 1–2 × 10^7 ID/ml. B, Cell survival in autologous lymphoid cell lines with and without the EBV genome. ○ BJAB(EBV-negative); ● B95–8/BJAB (EBV-positive). Points are means of duplicates.

Table 1. Replication of adenovirus in melanoma cells and HCR of MTIC-treated virus

Sample	Control virus titre (ID/ml)	Replication of MTIC-treated virus D_0(mM)	D_{37}(mM)
Primary cultures of melanoma biopsies			
MM423	8.6×10^6	19	22
MM426	3.5×10^8	12	19
MM432	3×10^6	NA*	>38
MM434	1×10^7	16	22
MM435†	1.3×10^8	NA	>38
MM440	2.2×10^7	13	13
MM444	5×10^5	12	12
MM446	2.3×10^5	7	7
MM447	2.8×10^5	21	21
MM448	3.7×10^5	NA	>38
MM451	4.3×10^5	22	33
MM452	1.8×10^6	15	15
MM454	2.6×10^5	13	32
Mer⁻ cell lines			
MM253c1a	6.6×10^5	6.6 ± 2.5‡	8.7 ± 4.6
HeLa-S_3	6.4×10^8	6.4	6.4
Mer⁺ cell lines			
MM96L	9×10^7	15 ± 5	32 ± 3.4
MM253-3D	4.8×10^5	24	39
MM418	3.7×10^5	40	59

*Not applicable; virus replication not decreased sufficiently to determine D_0.
†Same patient as MM426, biopsied 6 months later.
‡Mean and S.D. for three experiments.

The four biopsies studied in this way (Table 2) showed Mer$^+$ values in each assay.

Detection of sensitivity to antimetabolites by viral capacity

The MM96L and MM253cla cell lines were highly sensitive to killing by deoxyadenosine and hydroxyurea respectively (Figs. 3A and 3B). Viral capacity was determined from the dose response of viral replication (virus survival) in drug-treated cultures. The results (Figs. 3C and 3D) paralleled those of cell survival in that viral replication was strongly inhibited in the drug-sensitive cell lines.

Quantitative comparisons made using D_0 and D_{37} values for cell and virus survival (Table 3) showed that both assays gave a similar differential between sensitive and resistant cells for deoxyadenosine and hydroxyurea. Approximately twice as much deoxyadenosine was required to inhibit viral capacity compared with cell survival, and 10-fold more hydroxyurea.

Effect of various agents on viral capacity

The ability of 10 agents to affect viral capacity was compared in the MM96L cell line (Table 4). Two agents which are considered to exert toxicity primarily by damaging DNA and which have little immediate effect on DNA synthesis (MTIC and melphalan) had no effect on viral capacity even at doses supratoxic to cells and when viral infection was delayed for 24 hr to allow the agents to have maximum effect. MTIC inhibited the viral capacity of MM253c1a cells at the highest doses but this was probably a secondary effect due to loss of cells from the cultures. Antimetabolites (deoxyadenosine, 5-fluorouracil, cytosine arabinoside) on the

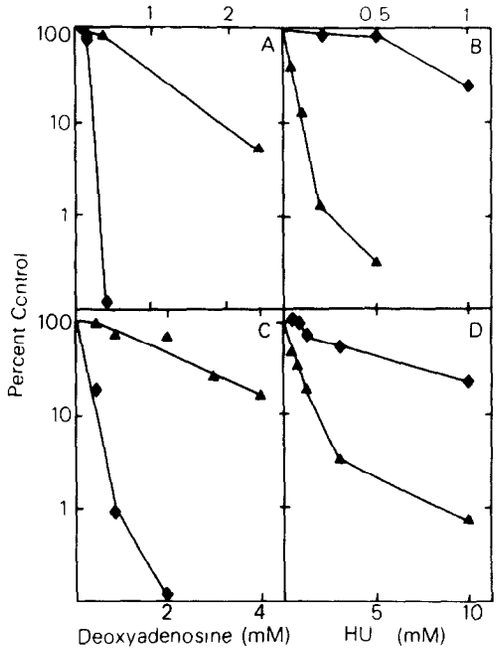

Fig. 3. Comparison of the cell survival and adenovirus capacity assays for detecting sensitivity to deoxyadenosine and hydroxyurea. A, Cell survival of MM96L (◆) and MM253c1a (▲) cells treated with deoxyadenosine. B, Survival of MM96L (◆) and MM253c1a (▲) cells treated with hydroxyurea. C, Viral capacity of MM96L (◆) and MM253c1a (▲) cells treated with deoxyadenosine. D, Viral capacity of MM96L (◆) and MM253c1a cells (▲) treated with hydroxyurea. Points are means of duplicates.

Table 2. HCR of MTIC-treated adenovirus in melanoma biopsies compared with HCR and cell survival in the derived cell lines

Biopsy No.	HCR in biopsy			Derived cell line					
	Virus titre (ID/ml)	D_0(mM)	D_{37}(mM)	Passage No.	HCR			Cell survival	
					Virus titre	D_0 (mM)	D_{37} (mM)	D_0 (μM)	D_{37} (μM)
MM418	1.7×10^7	NA*	>38	40	1.34×10^7	NA	>38	192	280
MM426	3.5×10^8	12	19	12	1.43×10^9	9 ± 2.6†	20 ± 4.4	60	140
MM435	1.3×10^8	NA	>38	10	1.77×10^8	NA	>38	320	320
MM444	5×10^5	12	<12	5	4.9×10^5	NA	29	80	86
Mer⁻ cell line									
MM253c1a				100	6.6×10^7	6.6 ± 2.5‡	8.7 ± 4.6	26 ± 4	48 ± 6

*Not applicable; survival not decreased sufficiently to determine D_0.
†Mean and S.D. for two experiments.
‡Mean and S.D. for three experiments.

Table 3. Comparison of the cell survival and viral capacity assays for detection of drug sensitivity

Cell line	Deoxyadenosine		Hydroxyurea	
	D_0 (mM)	D_{37} (mM)	D_0 (mM)	D_{37} (mM)
Cell survival				
MM96L	0.08 ± 0.01*	0.09 ± 0.02	0.31 ± 0.07	0.73 ± 0.08
MM253c1a	0.65 ± 0.03	0.98 ± 0.03	0.07 ± 0.02	0.16 ± 0.06
Ratio†	0.12	0.09	4.42	12
Viral capacity				
MM96L	0.22 ± 0.02	0.22 ± 0.02	2.5	3.81 ± 1.94
MM253c1a	1.51 ± 0.31	1.94 ± 0.54	0.65 ± 0.15	0.8 ± 0.2
Ratio	0.15	0.12	3.85	5

*Mean and S.D. (two to four experiments)
†Ratio = MM96L/MM253c1a

other hand, inhibited viral capacity generally at a concentration similar to that required to inhibit cell proliferation. This was also the case for gamma radiation, adriamycin and vincristine. As with hydroxyurea, a 10-fold higher level of 6-thioguanine was required to inhibit viral capacity compared with cell survival.

DISCUSSION

This new approach to predicting chemosensitivity in human tumors relied upon a combination of four factors: the ability of adenovirus to replicate in nonproliferating cells, detection of sensitivity to DNA-damaging agents by HCR, the possibility of detecting sensitivity to other agents by inhibition of viral capacity, and development of a convenient assay for viral replication. The fact that adenovirus replicates readily in nonproliferating cells, already known from previous studies [14–16], was amply confirmed in this work using melanoma biopsies, few of which produced continuous cell lines. The problem occasionally experienced of melanin obscuring the brown staining of the peroxidase reaction could be overcome if required by using fluorescent labelling [17] or alternative peroxidase substrates. Such procedures may also be useful if identification of tumor cells by staining with monoclonal antibody is carried out. The technical

Table 4. Comparison of the effect of antitumor agents on survival and viral capacity in MM96L cells

	Cell survival		Viral capacity	
Agent (μM)	D_0	D_{37}	D_0	D_{37}
Deoxyadenosine	60	180	220	220
Hydroxyurea	310	730	2500	3800
5-Fluorouracil	38	38	23	23
Cytosine arabinoside	0.42	0.42	0.51	0.51
Adriamycin	2.6	3.3	0.5	1.47
6-Thioguanine	2.2	11	22	55
Vincristine	0.006	0.012	0.005	0.015
MTIC*	160	490	> 1000	> 1000
Melphalan*	1.9	1.9	> 33	> 33
Gamma radiation (rads)*	140	480	500	550

*Cells treated 24 hr before virus infection. Other agents added immediately after infection.

advantages of the assay are that no specialised equipment, culture media or techniques are required, few cells are needed (10^5 per drug), results are obtained within 3 days, and a virus of negligible pathogenicity is used.

Consistent with previous studies of MTIC [21] and carcinogenic methylating agents [19], the modified HCR assay clearly distinguished Mer^- from Mer^+ cell lines. The present study also showed agreement in HCR assays between four Mer^+ biopsies and their derived cell lines. It was of interest that one biopsy (MM446) gave a Mer^- HCR response, suggesting that the Mer^- phenotype occurs *in vivo*. This is consistent with reports that 20% of human tumor biopsies had decreased levels of methyltransferase [29], and that only 20% of human tumor cell lines are Mer^- [19, 20]. In this laboratory, three of 20 human melanoma cell lines have the Mer^- phenotype (unpublished results). It should be noted in this context that HCR is a more general test for deficiency in methylation repair than lack of methyl transferase activity; however, the HCR assay would not detect the rare Mer^+Rem^- phenotype which shows intermediate sensitivity to methylating agents [20]. A much larger number of patients will be required in order to determine the frequency of Mer^- tumours *in vivo* and to adequately compare the tumour response to DTIC therapy. The applicability of the HCR assay to other DNA damaging drugs will depend on whether sensitivity arises from defective repair. The first step in crosslinking DNA by bis(chloroethyl)nitrosourea involves O^6-guanine alkylation which is repaired poorly in Mer^- cells [30] and therefore should decrease the HCR response. It is also likely that sensitivity of human cells to other DNA crosslinking agents such as melphalan [31] and *cis*-platinum [32] may under certain conditions be associated with deficient repair.

HSV–1 was tested as a possible alternative to adenovirus for HCR assays. The similar virus inactivation response to MTIC obtained in Mer^- and Mer^+ cell lines, however, indicated that this virus, having a genome size five times that of adenovirus, was able to effect repair in Mer^- cells either by induction of a cellular repair mechanism or by providing a virally-coded repair system such as a methyl transferase. This was also consistent with the conversion on the basis of cell survival of Mer^- to Mer^+ phenotype found after infection of lymphoid cells with the herpes virus EBV. Expression of the EBV genome, however, does not invariably ensure resistance to methylation because some EBV-transformed lymphoblastoid lines have the Mer^- phenotype [33].

The viral capacity assay using adenovirus correctly predicted sensitivity to two drugs (hydroxyurea and deoxyadenosine) for which pairs of sensitive and resistant cell lines were available. Viral capacity was inhibited by six other agents including three antimetabolites (5-fluorouracil, cytosine arabinoside and 6-thioguanine) Since viral capacity depends largely on the ability of the treated cell to replicate DNA, this approach should therefore detect sensitivity resulting from alterations in such diverse properties as transport, enzyme affinity and gene amplification. It also overcomes the problem of DNA synthesis measurement in nonclonogenic assays being affected by changing pool sizes when using ^{3}H-thymidine.

The finding that moderately cytotoxic doses of vincristine and adriamycin inhibit viral capacity may help clarify the mechanism of toxicity of these agents to human cells. Vincristine, for example, would appear to exert its lethal effects on cells before mitosis [34] but its site of action apart from the mitotic spindle is not known. The viral capacity of adriamycin-treated cells was inhibited even more than cell survival at the same dose. Further comparisons of virus and cell survival after adriamycin treatment may help to determine whether the primary site of drug toxicity is epigenetic, for example at the cell membrane as proposed by Tritton and Yee [35] or at the level of DNA damage.

Known DNA-damaging agents (MTIC and melphalan) had little effect on viral capacity, presumably because DNA synthesis is inhibited eventually as a result of template damage rather than enzyme damage. The large HSV–1 genome may prove to be more sensitive to these agents than that of adenovirus in the viral capacity assay. Using antimetabolites such as hydroxyurea, however, HSV–1 replication was not reduced in sensitive

cells [36]. The ability of ionising radiation to affect viral capacity was unexpected in view of the extended period of DNA synthesis which occurs in the MM96 line following ^{3}H-thymidine suicide [37]. It should be noted that since viral DNA synthesis is maximal 8–16 hr after infection [15], the optimal period between virus infection and drug treatment may need to be determined for each agent. This may account for high doses of 6-thioguanine, a slowly-acting agent [38, 39], being required to inhibit viral capacity compared with cell survival.

The present study demonstrated the feasibility of using adenovirus as a probe for *in vitro* sensitivity of melanomas to a wide variety of agents. The broad host range of adenovirus and ability to replicate in non-proliferating cells, not only melanomas but other tumors (unpublished results), many enable these methods to be applied generally to human neoplasms. Viral probes may also help elucidate the mechanism of action of certain antitumor agents. In this respect, HCR of adenovirus has already proven useful in studies of the allogeneic [19, 20] and autologous Mer^{-} phenotype [21] in cell lines.

REFERENCES

1. Kirkwood JM, Marsh JC. *In vivo* drug sensitivity assay of clonogenic human melanoma cells and correlation with treatment outcome. *Cancer Res* 1983, **43**, 3434–3440.
2. Rupniak HT, Marks P, Uratkins S, Bourne G, Slevin M, Bancroft-Livingstone GH, Simmons CA, Hill BT. Drug sensitivities of human tumour cells obtained directly from biopsy material. In: Periti P, Grassi GG, eds. *Current Chemotherapy and Immunotherapy*. Vol. 2, Washington D. C., American Society for Microbiology, 1982, 1274–1276.
3. Salmon SE, Hamburger AW, Soehlen B, Durie B, Alberts D, Moon T. Quantitation of differential sensitivity of human-tumor stem cells to anticancer drugs. *N Engl J Med* 1978, **298**, 1321–1328.
4. Shrivastav S, Bonar RA, Stone KR, Paulson DF. An *in vitro* assay procedure to test chemotherapeutic drugs on cells from human solid tumors. *Cancer Res* 1980, **40**, 4438–4442.
5. Tanigawa N, Kern DH, Hikasa Y, Morton DL. Rapid assay for evaluating the chemosensitivity of human tumors in soft agar culture. *Cancer Res* 1982, **42**, 2159–2164.
6. Tveit KM, Fodstad O, Lotsberg J, Vaage S, Pihl A. Colony growth and chemosensitivity *in vitro* of human melanoma biopsies. Relationship to clinical parameters. *Int J Cancer* 1982, **29**, 533–538.
7. Weisenthal LM, Marsden JA, Dill PL, Macaluso CK. A novel dye exclusion method for testing *in vitro* chemosensitivity of human tumors. *Cancer Res* 1983, **43**, 749–757.
8. Zirvi KA, Masui H, Giulliani FC, Kaplan NO. Correlation of drug sensitivity on human colon adenocarcinoma cells grown in soft agar and in athymic mice. *Int J Cancer* 1983, **32**, 45–51.
9. Berenbaun MC, Sheard CE, Reittie JR, Bundick RV. The growth of human tumors in immunosuppressed mice and their response to chemotherapy. *Br J Cancer* 1974, **30**, 13–32.
10. Smith EE, Courtenay VD, Gordon MY. A colony-forming assay for human tumor xenografts using agar diffusion chambers. *Br J Cancer* 1976, **34**, 476–483.
11. Dendy PD. Some problems in the use of short-term cultures of human tumours for *in vitro* screening of cytotoxic drugs. In: Hellmann K, Connors TA, eds. *Chemotherapy*. New York, Plenum, 1975, **7**, 341–380.
12. Wolberg W. The effect of 5-fluorouracil on DNA thymine synthesis in human tumors. *Cancer Res* 1969, **29**, 2137–2144.
13. McDougall JK. Adenoviruses—interaction with the host cell genome. *Progr Med Virol* 1975, **21**, 118–132.
14. Flint SJ, Broker TR. Lytic infection by adenoviruses. In: Tooze J. *Molecular Biology of Tumor Viruses*, Part 2. Cold Springs Harbor, Cold Spring Harbor Laboratory, 1980, 443–546.
15. Green M, Parsons JT, Pina M, Fujinga K, Caffier H, Landgraf-Leurs M. Transcription of adenovirus genes in productively infected and in transformed cells. *Cold Spring Harbor Symp Quant Biol* 1970. **34**, 808–818.
16. Philipson L, Linberg U. Reproduction of adenoviruses. *Compr Virol* 1974, **3**, 143–227.
17. Rainbow AJ, Howes M. Decreased repair of gamma-irradiated adenovirus in *Xeroderma pigmentosum* fibroblasts. *Int J Radiat Biol* 1979, **36**, 621–629.
18. Day RS. Cellular reactivation of ultraviolet-irradiated human adenovirus 2 in normal and xeroderma pigmentosum fibroblasts. *Photochem Photobiol* 1974, **19**, 9–13.
19. Day RS, Ziolkowski CHJ. Scudiero DA, Meyer SA, Mattern MR. Human tumour cell strains defective in the repair of alkylation damage. *Carcinogenesis* 1980, **1**, 21–32.
20. Scudiero DA, Meyer SA, Clatterbuck BE, Mattern MR, Ziolkowski CHJ. Day III RS. Relationship of DNA repair phenotypes of human fibroblasts and tumor strains to killing by *N*-methyl-*N*^{1}-nitro-*N*-nitrosoguanidine. *Cancer Res* 1984, **44**, 961–969.
21. Hayward IP, Parsons PG. Comparison of virus reactivation, DNA base damage, and cell cycle effects in autologous human melanoma cells resistant to methylating agents. *Cancer Res* 1984, **44**, 55–58.

22. Coohill TP, Moore SP, Drake S. The wavelength dependence of ultraviolet inactivation of host capacity in a mammalian cell–virus system. *Photochem Photobiol* 1977, **26**, 387–391.
23. Bockstahler LE. Induction and enhanced reactivation of mammalian viruses by light. *Progr Nucl Acid Res* 1981, **26**, 303–313.
24. Lytle CD, Aaronson SA, Harvey E. Host-cell reactivation in mammalian cells, II. Survival of herpes simplex virus and vaccinia virus in normal human and xeroderma pigmentosum cells. *Int J Radiat Biol* 1972, **22**, 159–165.
25. Parsons PG. Selective proliferation of human tumour cells in calcium-depleted medium. *Aust J Exp Biol Med Sci* 1978, **56**, 297–300.
26. Parsons PG, Smellie SG, Morrison LE, Hayward IP. Properties of human melanoma cells resistant to 5–(3′,3′–dimethyl–1–triazeno)imidazole–4–carboxamide and other methylating agents. *Cancer Res* 1982, **42**, 1454–1461.
27. Klein G, Sugden B, Leibold W, Menezes J. Infection of EBV-genome-negative and -positive human lymphoblastoid cell lines with biologically different preparations of EBV. *Intervirology* 1974, **3**, 232–244.
28. Reed LJ, Muench H. A simple method of estimating fifty per cent end points. *American J. Hygiene* 1938, **27**, 493–497.
29. Myrnes B, Norstrand K, Giercksky K, Sjunneskog C, Krokan H. A simplified assay for 0^6-methylguanine-DNA methyltransferase activity and its application to human neoplastic and non-neoplastic tissues. *Carcinogenesis* 1984, **5**, 1061–1064.
30. Robbins P, Harris AL, Goldsmith I, Lindahl T. Cross-linking of DNA induced by chloroethylnitrosourea is prevented by 0^6–methylguanine–DNA methyltransferase. *Nucl Acids Res* 1983, **11**, 7743–7758.
31. Parsons PG. Treatment-time dependence of melphalan resistance and DNA cross-linking in human melanoma cells. *Cancer Res* 1984, **44**, 2773–2778.
32. Fraval HNA, Roberts JJ. Excision repair of *cis*-diamminedichloroplatinum(II)–induced damage to DNA in Chinese hamster cells. *Cancer Res* 1979, **39**, 1793–1797.
33. Sklar R, Strauss B. Removal of 0^6-methylguanine from DNA of normal and xeroderma pigmentosum-derived lymphoblastoid cell lines. *Nature* 1981, **289**, 417–420.
34. Mujagic H, Chen S–S, Geist R, Occhipinti SJ, Conger BM, Smith CA, Schuette WH, Shackney SE. Effects of vincristine on cell survival, cell cycle progression and mitotic accumulation in asynchronously growing sarcoma 180 cells. *Cancer Res* 1983, **43**, 3591–3597.
35. Tritton JR, Yee G. The anticancer agent adriamycin can be actively toxic without entering cells. *Science* 1982, **217**, 248–250.
36. Leary K, Bratton J, Francke B. Replication of herpes simplex virus type 1 on hydroxyurea-resistant baby hamster kidney cells. *J Virol* 1983, **47**, 224–226.
37. Sheridan JW, Simmons RJ. Tritiated-thymidine-induced increased DNA content and irreversible differentiation in a human melanoma cell line. *Br J Exp Pathol* 1981, **62**, 289–296.
38. Nelson JA, Carpenter JW, Rose LM, Adamson DJ. Mechanisms of action of 6-thioguanine, 6-mercaptopurine, and 8-azaguanine. *Cancer Res* 1975, **35**, 2872–2878.
39. Tidd DM, Paterson ARP. A biochemical mechanism for the delayed cytotoxic reaction of 6-mercaptopurine. *Cancer Res* 1974, **34**, 738–746.