JPP Journal of Pharmacy and Pharmacology

JPP 2004, 56: 1371–1378

Received May 20, 2004
Accepted July 28, 2004
DOI 10.1211/0022357044643
ISSN 0022-3573

Microsphere–liposome complexes protect adenoviral vectors from neutralising antibody without losses in transfection efficiency, in-vitro

Jason C. Steel, Heather M. A. Cavanagh, Mark A. Burton and Wouter H. J. Kalle

Abstract

Adenoviral vectors have been commonly used in gene therapy protocols but the success of their use is often limited by the induction of host immunity to the vector. Following exposure to the adenoviral vector, adenoviral-specific neutralising antibodies are produced, which limits further administration. This study examines the effectiveness of a novel combination of microspheres and liposomes for the shielding of adenovirus from neutralising antibodies in an in-vitro setting. We show that liposomes are effective in the protection of adenovirus from neutralising antibody and that the conjugation of these complexes to microspheres augments the level of protection. This study further reveals that previously neutralised adenovirus may still be transported into the cell via liposome–cell interactions and is still capable of expressing its genes, making this vector an effective tool for circumvention of the humoral immune response. We also looked at possible side effects of using the complexes, namely increases in cytotoxicity and reductions in transfection efficiency. Our results showed that varying the liposome:adenovirus ratio can reduce the cytotoxicity of the vector as well as increase the transfection efficiency. In addition, in cell lines that are adenoviral competent, transfection efficiencies on par with uncomplexed adenoviral vectors were achievable with the combination vector.

Introduction

Recombinant adenoviruses are attractive gene vectors for gene therapy. They are capable of infecting a large range of cell types in all stages of cell division and have shown a high efficiency in gene expression studies (St George 2003). Their relative inability to insert into the host genome limits the risk of insertional mutagenesis but as a drawback also limits their therapeutic life-time (Prince 1998). Most adenovirus-based vectors have been rendered incapable of initiating viral replication, which, although limiting their toxicity, causes the non-replicating virus to be lost during cellular division making multiple treatment necessary for an effective therapy. The ability to re-dose with adenoviral vectors is limited by the host's humoral immunity, which develops quickly following initial exposure to the viral coat proteins (Mack et al 1997). Further exposure to the virus results in the large-scale production of neutralising antibodies, which effectively inactivates the adenoviral vector thereby limiting the therapeutic value of the treatment (Benihoud et al 1999). Furthermore, the majority of the human population have previously been exposed to adenovirus and therefore already possess a humoral immunity to the virus (Verma & Somia 1997).

For recombinant adenovirus to be an effective vector for gene therapy the immune system needs to be circumvented. A number of strategies have been put forward to achieve this, including the use of broad immunosuppressants, varying adenoviral serotypes and using cytoablative agents (Engelhardt et al 1994; Vilquin et al 1995; Mack et al 1997; Wilson et al 1998; O'Riordan et al 1999). Many of these methods, however, have limitations to their use in the clinical setting, including questions of safety to the patient.

School of Biomedical Science, Charles Sturt University, P.O. Box 588, Wagga Wagga 2678, Australia

Jason C. Steel, Heather M. A. Cavanagh, Mark A. Burton, Wouter H. J. Kalle

Correspondence: J. C. Steel, School of Biomedical Science, Charles Sturt University, P.O. Box 588, Wagga Wagga 2678, Australia.
E-mail: jsteel@csu.edu.au

This study evaluates the use of immuno-shielding to prevent adenoviral neutralisation by adenovirus-specific antibody, thus providing a method to circumvent the immune response. Shielding is provided by the complexing of the adenovirus to liposomes to form what has been titled AL complexes. Further, the AL complexes have been bound to sustained-release ion-exchange microspheres to form MAL complexes. In this study both the MAL and AL complexes are tested for their ability to shield the adenoviral component from neutralising antibody. The mechanism of protection is examined, as well as whether the liposome component of the AL complexes may provide a non-receptor-mediated avenue for cellular entry of neutralised adenovirus.

In addition, this study evaluates possible side effects of the complexes, namely, whether the formation of the AL complexes and the MAL complexes induces increased toxicity and how the addition of the complexes affects adenoviral transgene expression.

Materials and Methods

Microspheres

The microspheres used for the formation of the combination vector were purchased commercially (Aminex 50W-X4; Bio-Rad, Australia). They are a 4% cross-linked cation-exchange resin formulated from divinalbenzene with particle diameters of $32.5 \pm 2.5\,\mu$m. They contain a sulfonic acid functional group and are non-porous. Before use the microspheres were washed with 5 changes of nanopure sterile water before being pelleted and dried at 37°C overnight to remove water.

Adenoviral vectors

For the neutralisation and toxicity studies, a replication competent adenovirus serotype 5 (donated by K. Hunt, VIDRL, Australia) was used. The adenovirus was propagated in HeLa cells. At maximum cytopathic effect, the cells were harvested and pelleted. The adenovirus was extracted from the HeLa cells by three consecutive freeze/thaw cycles and purified by two sucrose gradient ultracentrifugation steps followed by dialyses (Croyle et al 1998). Adenovirus was then diluted to a titre of 2.7×10^{12} particles/mL. Human anti-adenoviral serum was used in the neutralisation study (donated by K. Hunt, VIDRL, Australia).

For the expression study an E1, E3-deleted adenovirus type 5 expressing LacZ (Qbiogene, Carlsbad, USA) was used. The adenoviral vectors were propagated in HEK293 cells and harvested and purified as above.

Cell culture

HeLa cells were propagated in Basal media Eagle (BME; GIBCO BRL) + 10% fetal bovine serum (FBS; GIBCO BRL) + penicillin/streptomycin antibiotics and maintained at 37°C, 5% CO_2 in a humidified incubator. Cells were grown in 6-well plates.

HEK293 (human embryonic kidney + E1) cells were purchased from Qbiogene for propagation of the LacZ expressing adenoviral vector. HEK293 cells were maintained in Dulbecco's modified Eagles's medium (DMEM; GIBCO, NSW), supplemented with 10% foetal calf serum (FCS; GIBCO, NSW), 100 U mL^{-1} penicillin/streptomycin (Life technologies, NSW).

In addition to the HeLa cell line, CSU-SA1 (a rat-derived salivary adenocarinoma) cell line was also used for the in-vitro expression studies. For this study, cells were grown on 6-well plates and maintained in BME + 10% FCS + penicillin/streptomycin antibiotics at 37°C, 5% CO_2 in a humidified incubator.

Preparation of liposomes

Small unilamellar vesicles (SUV) were prepared by the injection of an ethanoic solution of lipids into an aqueous solution as previously described (Dass et al 2002). Briefly, eight milligrams of dimethyldioctadecylammonium bromide (DDAB; Sigma) and four milligrams of dioleoyl-L-phosphatidylethanolamine (DOPE; Sigma) were dissolved in 1.0 mL absolute ethanol to give mass ratios of 2:1 of DDAB:DOPE. Ethanoic lipid mix (50 μL) was rapidly injected (over 0.5 s) into vortexing water. This method resulted in spontaneous rearrangement of the lipids into SUVs. The liposome mix was filtered sequentially through 3.0-, 0.6- and 0.2-μm polycarbonate filters to size liposomes to a maximum diameter of 0.2 μm (Dass et al 2002).

Production of AL complexes

Complexes of adenovirus (2.73×10^{12} particles/mL) and DDAB-DOPE (540 μg mL^{-1}) liposomes were made by gently mixing and incubating at 25°C for 60 min (Steel et al 2004). Adenovirus (50 μL) was complexed to 500, 250 and 100 μL of liposomes, forming volume ratios of liposome:adenovirus (lip:adv) of 10:1, 5:1 and 2:1.

The adenoviral–liposome (AL) complexes were separated from unbound liposomes or adenovirus by ultracentrifuging (25 000 *g* for 16 h at 4°C) on a continuous sucrose density gradient (Steel et al 2004). The complexing of liposomes to adenovirus caused a shift in the density of the AL complex compared with uncomplexed liposomes or adenovirus. The shift in density was used to separate the bound AL complexes from unbound adenovirus and liposomes. A sample of AL complexes were removed and assayed for adenoviral particle content and liposomal content to confirm the complex's ratios.

Production of MAL complexes

MAL complexes were formulated as described by Steel et al (2004). Briefly, 1 mg of microspheres was added to the AL complexes (formulated as above) in 1 mL of phosphate-buffered saline (PBS) and incubated at 25°C with gentle mixing for 120 min. The MAL complexes were washed with 5 changes of nanopure sterile water to remove any unbound complexes.

Electron microscopy

MAL complexes and microspheres were examined under a scanning electron microscope (SEM). MAL complexes and microspheres were freeze dried and then mounted onto aluminium stubs for sputter coating. Sputter coating was performed with an Emitech K550 sputter coater using a gold/palladium target. Microspheres were viewed with a Hitachi S-4000 scanning electron microscope with a field emission electron source.

Cytotoxicity of complexes in-vitro

Complexes of replication incompetent adenoviral vectors and liposomes (AL complexes) with or without microspheres were made as above with 50 μL of adenovirus (2.73×10^{12} particles/mL) and 500, 250, 100 or 50 μL of liposome (540 $\mu g\,mL^{-1}$). The MAL complexes, AL complexes, liposome alone and adenovirus alone were tested for their cytotoxicity in HeLa cells (in-vitro). The vectors were added to the 6-well tissue culture plates containing a 75% confluent monolayer of HeLa cells. After 24 h the level of cytotoxicity induced by each of the complexes was determined by the trypan blue exclusion assay (Fujita et al 2000). Cells were detached from the wells and sample of the cell suspension removed. The samples were diluted 1:1 (v/v) with 0.4% trypan blue and the cells retaining the dye (dead cells) and those which did not (live cells), were counted with a hemocytometer. Cytotoxicity was calculated as the percentage of cells retaining dye (dead cells).

Neutralisation assay

MAL and AL complexes at ratios of 2:1, 5:1 and 10:1 and an adenovirus–microsphere complex were tested for their ability to protect the adenovirus from neutralising antibodies. Following complex formation each of the treatments was incubated in 2-fold serial dilutions (1:2 to 1:2048) of complement-inactivated serum containing adenoviral neutralising antibodies for 45 min at 37°C. Each treatment was added to HeLa cells at 75% confluence and incubated at 37°C, 5% CO_2. Cells were examined 24–72 h post treatment for signs of adenoviral-induced cytopathic effect (CPE). The neutralisation point was calculated as the lowest dilution of sera at which adenoviral infection was clearly visible. Each assay was performed in triplicate, a minimum of 5 times.

Infectivity of neutralised adenovirus

Adenovirus was incubated in anti-adenoviral sera (diluted 1:64) for 45 min at 37°C. Half of the neutralised adenovirus (40 μL) was added to 80 μL of DDAB-DOPE liposomes and allowed to complex at 25°C for 1 h. The complexed virus and the remaining uncomplexed adenovirus were individually added to separate flasks containing 7 mL BME and HeLa cells (75% confluent). Cells were examined for signs of adenoviral induced CPE for up to 96 h post treatment.

β-galactosidase expression

HeLa and CSU-SA1 cell lines were grown in 6-well plates until 75% confluence level was reached at which point the media was removed and the cell monolayer washed with PBS. MAL complexes (at 2:1, 5:1 and 10:1 volume ratios), AL complexes (at 2:1, 5:1 and 10:1 volume ratios) or 50 μL adenovirus (2.7×10^{12} particles/mL) were made up and diluted in 5 mL of complete media before being added to the cell lines. Cells were washed and harvested 48 h later, for assaying of β-galactosidase expression levels.

β-galactosidase levels were assayed using the β-galactosidase reporter gene activity detection kit (Sigma) according to standard protocols. Briefly, cells were lysed with lysis buffer (Sigma) at room temperature for 15 min. The lysate was collected following microcentrifuge (5 min) and made up to 150 μL with lysis buffer. The lysate was added to 150 μL of assay buffer and incubated for 30 min. At that point stop buffer was added (500 μL) and the optical absorbance measured at 420 nm.

The samples were then standardised for protein levels using the Micro BCA protein assay reagent kit (Pierce). Measurements of β-galactosidase (rU) were expressed as OD units of β-galactosidase/OD units of protein. Each treatment was repeated in 12 wells.

Statistical evaluation

Statistical evaluation of the differences between each of the treatments was determined using the one-way analysis of variance test followed by the Student–Newman–Keuls multiple comparison procedure. Statistical analysis was performed at 95% confidence level ($P = 0.05$). This was consistent for all experiments requiring statistical evaluation.

Results

Immuno-shielding

The AL and MAL complexes were tested for their ability to shield adenovirus from neutralising antibody in-vitro. Several modified serum neutralisation tests were performed on the MAL and AL complexes and compared with that of uncomplexed adenoviral vectors. The results showed the uncomplexed adenoviral vectors are neutralised at a serum dilution of 1 in 128 (Figure 1). When examining both the MAL and the AL complexes at ratios of 5:1 and 10:1 (lip:adv) it was found that the adenoviral component could not be neutralised at the lowest dilution. The AL complex at a ratio of 2:1 (lip:adv) could, however, be neutralised at a 1 in 2 dilution. MAL complexes at the same ratio were unable to be neutralised, indicating increased immuno-shielding for complexes containing microspheres. When comparing the immuno-protection of microspheres alone (without the liposome component) we found that the level of protection was significantly lower than the AL complexes or the MAL complexes

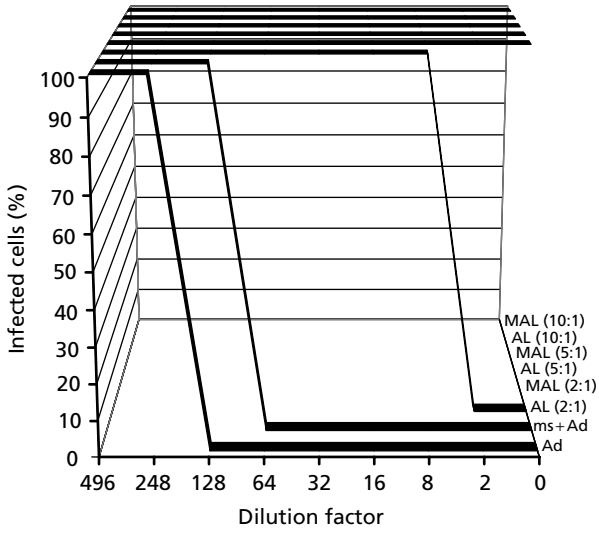

Figure 1 Percentage of infected cells for each neutralising antibody dilution factor. Adenovirus (Ad), microsphere/adenovirus (ms + Ad), MAL and AL complexes were tested for their ability to be neutralised in sera containing neutralising antibody. Each bar represents the mean of 5 separate experiments repeated in triplicate.

with a neutralisation point at 1:64. To eliminate the possibility that the MAL complexes themselves were binding the antibody and thus limiting the amount of antibody available for viral neutralisation, an experiment was performed in which the media was removed from flasks treated with MAL complexes and then tested to confirm it retained its neutralising capability (results not shown).

When we tested the ability of liposomes to carry adenovirus that had been pre-neutralised with antibody, we found that the AL complexes were able to induce adenoviral-mediated CPE in each of the cell flasks tested, indicating viral infection. In the flasks that contained the pre-neutralised adenovirus without liposomes, no viral CPE was evident, indicating there was no viral replication or no viral infection.

Cytotoxicity

The cytotoxicity of MAL complexes and their constituents was examined in a HeLa cell line (Table 1). Cytotoxicity was measured using the trypan blue exclusion assay. MAL complexes containing 500 μL of liposomes showed significant levels of cytotoxicity when compared with the controls with losses of cell viability up to 36%. When comparing this level of cytotoxicity with that of the AL complexes containing 500 μL of liposomes, or the liposomes alone, we found that the MAL complexes induced significantly higher levels of cell viability ($P < 0.01$) with AL complexes inducing 56% loss of viability and the liposomes alone inducing 62% loss of viability.

MAL complexes and their constituents at ratios of 5:1, 2:1, 1:1 and 1:2 (lip:adv) did not induce significant levels of cytotoxicity when compared with the controls, with no statistical differences between any of these ratios (Table 1).

Expression study

This in-vitro study aimed to examine the β-galactosidase expression levels in HeLa and CSU-SA1 cell lines following treatment with varying combinations of MAL complexes, AL complexes and adenoviral vectors (Figure 2).

When examining expression rates for the different ratio MAL complexes in either HeLa or CSU-SA1 cells, it was found that there was no significant difference in the expression levels of cells treated with either 2:1 or 5:1 (lip:adv) ratios ($P > 0.05$). The 10:1 ratio, however, induced significantly lower levels of β-galactosidase expression when compared with the other ratios ($P < 0.05$). The expression rates induced by AL complexes showed significant differences between 2:1, 5:1 and the 10:1 (lip:adv) when examining the CSU-SA1 cell line ($P < 0.05$). However, the HeLa cells showed no difference in expression levels between AL complexes at 2:1 and 5:1 (lip:adv) ratios but showed significant difference between these and the 10:1 (lip:adv) ratio.

On comparing the MAL complexes with the AL complexes it was found that at 10:1 (lip:adv) ratios, the MAL complexes induced significantly more expression than that

Table 1 The toxicity of MAL, AL complexes and liposomes in HeLa cell line

Liposome content ($540\,\mu g\,mL^{-1}$)	Cytotoxicity (percentage)		
	Liposome alone	Liposome + adenovirus (50 μL) (AL complexes)	Liposome + adenovirus (50 μL) + microsphere (1 mg) (MAL complexes)
500 μL (10:1)	62.04 ± 8.03	56.12 ± 4.82	36.79 ± 3.99
250 μL (5:1)	15.16 ± 4.04	15.99 ± 8.99	16.05 ± 5.02
100 μL (2:1)	10.99 ± 3.71	10.58 ± 5.10	7.67 ± 4.13
50 μL (1:1)	9.38 ± 3.11	14.10 ± 6.22	10.30 ± 3.92
25 μL (1:2)	13.01 ± 2.97	13.45 ± 3.73	7.08 ± 4.13
0 μL (control)	11.07 ± 5.62	13.37 ± 7.18	9.87 ± 2.29

Cytotoxicity was measured by the trypan blue exclusion assay and the results presented as the percentage of non-viable cells in each well ± s.d of the mean. Each treatment was repeated 6 times.

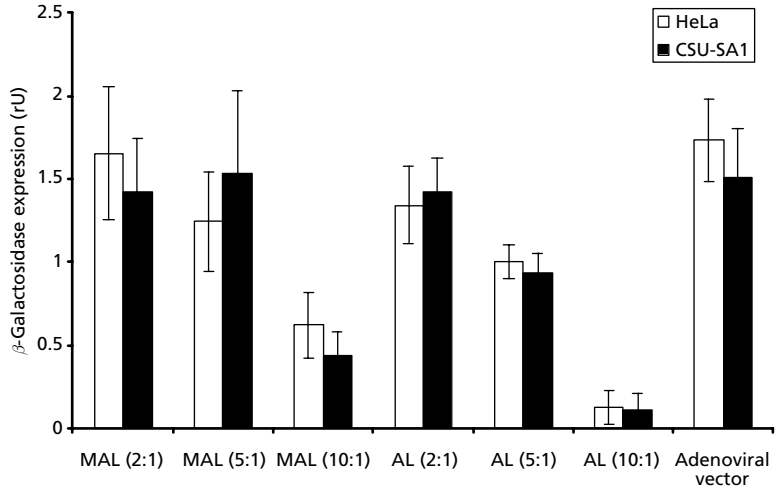

Figure 2 β-galactosidase expression levels in HeLa and CSU-SA1 cell lines following treatment with varying volume ratios (2:1, 5:1 and 10:1 (liposome:adenovirus)) of AL complexes alone and conjugated to microspheres as well as unconjugated adenovirus. Bars represent the mean of 12 wells (6-well plates) for each treatment $\pm$ s.d.

of the AL complex at the same ratio ($P < 0.05$). For the 2:1 and 5:1 (lip:adv) ratios there was no significant difference between expression levels ($P > 0.05$).

When comparing the adenoviral vector expression with that of the MAL complexes there was no significant difference for the 2:1 or the 5:1 (lip:adv) ratios ($P > 0.05$) whereas a significant difference was noted between the adenoviral vectors and MAL complexes at a ratio of 10:1 (lip:adv). The 2:1 (lip:adv) ratio AL complexes also exhibited no difference between the expression it induced and the adenoviral vector expression ($P > 0.05$). The 5:1 and 10:1 (lip:adv) AL ratios, however, induced significantly less β-galactosidase expression than the adenoviral vector ($P < 0.05$).

The level of β-galactosidase expression showed no significant difference when comparing the HeLa cell line and the CSU-SA1. This remained constant with all treatments tested.

Discussion

The success of using adenoviral vectors for gene therapy is often limited by the host's immune response to the virus (Mack et al 1997). While cell-mediated immunity can be reduced by the use of third generation adenoviral vectors containing little or no viral sequences, the induction of humoral immunity still remains a significant problem. The effectiveness of the re-administration of adenoviral vectors is limited by the large-scale production of adenoviral-specific neutralising antibodies by the host (Russell 2000). One method used to prevent the neutralisation of the adenoviral vectors is to bind cationic polymers or liposomes to the virus (Chillon et al 1998; O'Riordan et al 1999; Natsume et al 2000; Worgall et al 2000; Croyle et al 2002; Mizuno et al 2002). These substances then act to physically shield the adenoviral vector, preventing the binding of neutralising antibody. In this study we examined the use of liposomes, microspheres and combinations of liposomes and microspheres for their ability to shield the adenovirus from neutralising antibody while retaining high expression levels and not inducing significant cytotoxicity.

The first part of this study, looking at the ability of each of these combinations to provide immuno-shielding, found that liposomes, microspheres and a combination of liposomes and microspheres were able to shield the adenovirus from neutralising antibody. When comparing the level of immuno-shielding we found that the adenovirus complexed with liposomes (AL complexes) and the AL complexes bound to microspheres (MAL complexes) provided greater protection than the adenovirus complexed with microspheres. Adenovirus directly bound to the microspheres would only be afforded immuno-shielding while it is attached to the microsphere. As the adenovirus is released off the microsphere into the media it becomes exposed to neutralising antibody and as such would be vulnerable to neutralisation. Full protection would have been given only to those adenoviruses that were released from the microspheres directly onto the cells. However, with the AL complexes and the MAL complexes, the liposomes remain attached to the adenovirus mediating entry into the cell, thereby protecting the adenovirus right up until cellular internalisation.

When comparing the different adenovirus:liposome ratios we showed that each of the differing AL complex ratios was able to provide an effective level of protection from neutralising antibody; however, greater liposome quantity increased the level of shielding. Qiu et al (1998) showed that the complexing of adenovirus with liposome resulted in aggregations with lipid surfaces. This surface lipid is most likely responsible for the shielding effect of these complexes. Meunier-Durmort et al (1997) showed that at lower adenoviral-to-liposome ratios the penton

fibres were capable of penetrating the lipid layers. These penton fibres could then be neutralised by antibody, possibly limiting entry into cells. This effect was demonstrated in the 2:1 liposome: adenoviral ratio, which could be neutralised at high concentration of antibody. When looking at the 5:1 and 10:1 (lip:adv) ratios the results showed that the adenovirus within these complexes could not be neutralised even at the highest antibody concentration, indicating total covering of the aggregate with lipid.

It has been proposed that entry into the cell of the AL complexes may occur through lipid–lipid interaction with the membrane or via adenovirus- or liposome-induced endocytosis. Qiu et al (1998) reported that cellular entry of the adenovirus can occur via a pathway independent of the adenoviral fibre receptor and α_v-integrins, suggesting that entry of the complexes may occur in the absence of accessible penton fibres. In contrast, Meunier-Durmort et al (1997) suggested that the penton fibres were necessary for effective lipoadenofection and showed that the inactivation of these penton fibres may severely limit the efficiency of the combination vector. However, when we tested the ability of the AL complex to enter the cell, even when the adenovirus was already fully neutralised (adenovirus was incubated with neutralising antibody and then either complexed with liposomes, or not, before incubating on HeLa cells), we found a ratio as low as 2:1 was still able to infect cells. Conversely, uncomplexed neutralised adenovirus was unable to gain entry into the cells and thus initiate viral replication. This result presents an apparent disparity between entry of complexed pre-neutralised adenovirus and post-neutralised adenovirus at the 2:1 (lip:adv) ratio. The explanation may be found in the complex's ability to initiate lipid–lipid interactions with the cellular membrane. It is feasible that the pre-neutralised adenovirus when complexed with liposomes has a lipid layer surrounding the particle, and as such is able to gain entry into the cell via lipid–lipid interaction with the cell membrane. In the post-neutralised model, however, antibodies would attach to penton fibres penetrating the lipid surface of the complex possibly forming a physical barrier preventing lipid interactions.

The cellular entry of the neutralised adenovirus suggests that the AL complexes do not require binding to the coxsackievirus and adenovirus receptor (CAR) for internalisation. The neutralisation of the adenovirus prevents its binding to CAR and as such any cellular entry by the complex must be mediated by the liposomal component. This highlights the complex's ability to enter cells with a lack of CAR.

The benefits of using the MAL complexes were seen in their ability to augment the protection of the AL complexes from neutralising antibody, thus providing similar high protection levels with a lower quantity of liposome. This was shown with the 2:1 (lip:adv) ratio of AL complexes, which could be neutralised at an antibody dilution of 1 in 2 when not part of the MAL complexes but when conjugated to microspheres could not be neutralised at any antibody dilution. Looking at the SEM micrographs of the MAL complexes we can see that the liposomes aggregate to form a film over the majority of the microsphere and it might be this increased aggregation that provides the added protection to the adenovirus (Figure 3). In addition to the SEM micrographs, the direct association of the microspheres with the AL complexes to form MAL complexes has been shown in a previous study by Steel et al (2004). Using a density gradient and ultracentrifugation it was shown that the AL complexes directly associate with the microspheres to form complexes with an altered sedimentation density compared with either the AL complexes or the microspheres alone.

The drawback to using high liposome concentrations in the AL complexes can be seen when examining both the cytotoxicity of the complexes and the expression levels able to be induced by these complexes. While the 10:1 (lip:adv) ratio AL complexes were able to provide high levels of immuno-shielding, these complexes also induced significant cytotoxicity. This cytotoxicity may have also contributed to the significantly reduced expression levels of β-galactosidase seen in cells treated with the 10:1 (lip:adv) AL complexes. The significant difference in expression levels

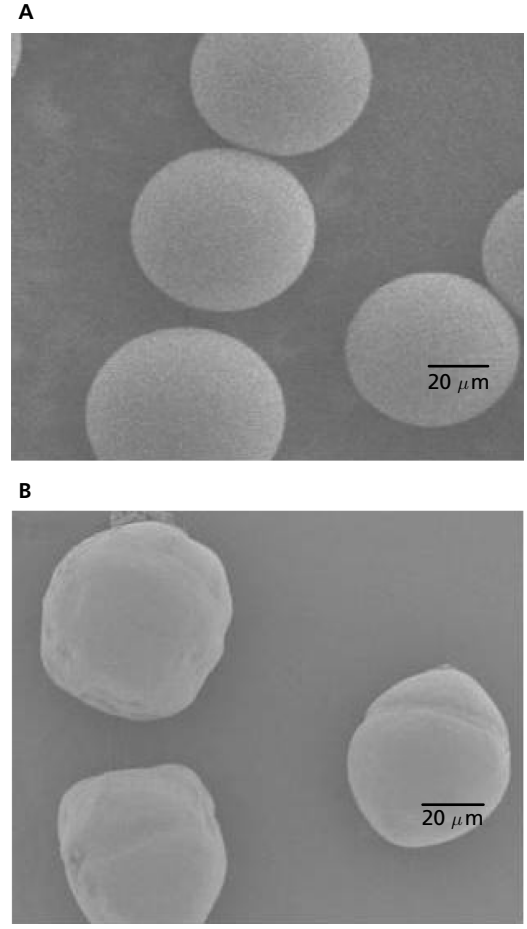

Figure 3 A. Scanning electron micrograph (SEM) of microsphere before AL complexing. B. SEM of MAL complex. The MAL complexes were freeze dried and then mounted onto aluminium stubs and sputter coated with a gold/palladium target.

between cells treated with AL or MAL complexes at this ratio may also be explained in terms of cytotoxicity with the AL complexes inducing significantly more toxicity than the MAL complexes and as such there were less cells able to express the transfected gene. While the exact mechanism for cytotoxicity reduction is unknown it has been hypothesised that it may be due to the MAL complexes' ability to induce sustained release of the AL complexes from the microsphere component. The sustained release of the AL complexes would reduce the concentration of AL complexes in the media at any one time and as such limit the amount of AL complexes able to induce cytotoxic effects.

When looking at the β-galactosidase expression levels for the 2:1 and 5:1 (lip:adv) ratios of the MAL complexes and the 2:1 (lip:adv) ratio AL complexes, the results showed there to be no significant difference between these and adenoviral vectors in either cell type tested. This shows that, at these ratios of MAL and AL complexes, there is no loss to the high expression efficiency achievable by adenovirus. The complexes maintain expression levels comparable to adenoviral vectors in HeLa cells (which have been show to be readily infected by adenovirus (Kim et al (2001)) allowing their use in most, if not all, cell types where high levels of expression are required.

In addition to the MAL and AL complexes' ability to maintain high levels of expression in cell lines with high expression of CAR, the literature suggests that these complexes would provide significant expression efficiency advantages in those cell types devoid of CAR. A number of previous studies using cell lines expressing reduced or no levels of CAR have shown that AL complexes are able to induce significantly higher levels of expression when compared with uncomplexed adenoviral vectors (Arcasoy et al 1997; Fasbender et al 1997; Meunier-Durmort et al 1997; Byk et al 1998; Chillon et al 1998; Kaplan et al 1998; Qiu et al 1998; Toyoda et al 1998). This highlights another benefit to the use of this gene delivery system.

This study has shown that MAL complexes have the ability to provide adenovirus with significant immunoshielding while reducing the levels of cytotoxicity of AL complexes. This coupled with the MAL complexes' ability (at ratios of 2:1 and 5:1 (lip:adv)) to maintain high levels of transgene expression, comparable with the uncomplexed adenoviral vectors, makes MAL complexes an attractive vector for future gene therapy studies.

References

Arcasoy, S. M., Latoche, J. D., Gondor, M., Pitt, B. R., Pilewski, J. M. (1997) Polycations increase the efficiency of adenovirus-mediated gene transfer to epithelial and endothelial cells in vitro. *Gene Ther*. **4**: 32–38

Benihoud, K., Yeh, P., Perricaudet, M. (1999) Adenovirus vectors for gene delivery. *Curr. Opin. Biotechno.*, **10**: 440–447

Byk, T., Haddada, H., Vainchenker, W., Louache, F. (1998) Lipofectamine and related cationic lipids strongly improve adenoviral infection efficiency of primitive human hematopoietic cells. *Hum. Gene Ther*. **9**: 2493–2502

Chillon, M., Lee, J. H., Fasbender, A., Welsh, M. J. (1998) Adenovirus complexed with polyethylene glycol and cationic lipid is shielded from neutralizing antibodies in vitro. *Gene Ther*. **5**: 995–1002

Croyle, M., Anderson, D., Roessler, B., Amidon, G. (1998) Development of a highly efficient purification process for recombinant adenoviral vectors for oral gene delivery. *Pharm. Dev. Technol*. **3**: 365–372

Croyle, M., Chirmule, N., Zhang, Y., Wilson J. (2002) PEGylation of E1-deleted adenovirus vectors allows significant gene expression on readministration to liver. *Hum. Gene Ther*. **13**: 1887–1900

Dass, C. R., Walker, T. L., Burton, M. A. (2002) Liposomes containing cationic dimethyl dioctadecyl ammonium bromide: formulation, quality control, and lipofection efficiency. *Drug Deliv*. **9**: 11–18

Engelhardt, J., Ye, X., Doranz, B., Wilson, J. (1994) Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver. *Proc. Natl Acad. Sci. USA* **91**: 6196–6200

Fasbender, A., Zabner, J., Chillon, M., Moninger, T. O., Puga, A. P., Davidson, B. L., Welsh, M. J. (1997) Complexes of adenovirus with polycationic polymers and cationic lipids increase the efficiency of gene transfer in vitro and in vivo. *J. Biol. Chem*. **272**: 6479–6489

Fujita, R., Ishikawa, M., Takayanagi, M., Takayanagi, Y., Sasaki, K. (2000) Enhancement of doxorubicin activity in multidrug-resistant cells by mefloquine. *Methods Find. Exp. Clin. Pharmacol*. **22**: 281–284

Kaplan, J. M., Pennington, S. E., St George, J. A., Woodworth, L. A., Fasbender, A., Marshall, J., Cheng, S. H., Wadsworth, S. C., Gregory, R. J., Smith, A. E. (1998) Potentiation of gene transfer to the mouse lung by complexes of adenovirus vector and polycations improves therapeutic potential. *Hum. Gene Ther*. **9**: 1469–1479

Kim, J., Lee, S., Cho, Y., Kim, Y., Lee, J. (2001) Ectopic expression of the coxsackievirus and adenovirus receptor increases susceptibility to adenoviral infection in the human cervical cancer cell line, SiHa. *Biochem. Biophys. Res. Commun*. **288**: 240–244

Mack, C., Song, W., Carpenter, H., Wickham, T., Kovesdi, I., Harvey, B., Magovern, C., Isom, O., Rosengart, T., Falck-Pedersen, E., Hackett, N., Crystal, R., Mastrangeli, A. (1997) Circumvention of anti-adenovirus neutralizing immunity by administration of an adenoviral vector of an alternate serotype. *Hum. Gene Ther*. **8**: 99–109

Meunier-Durmort, C., Picart, R., Ragot, T., Perricaudet, M., Hainque, B., Forest, C. (1997) Mechanism of adenovirus improvement of cationic liposome-mediated gene transfer. *Biochim. Biophys. Acta* **1**: 8–16

Mizuno, M., Ryuke, Y., Yoshida J. (2002) Cationic liposomes conjugation to recombinant adenoviral vectors containing herpes simplex virus thymidine kinase gene followed by ganciclovir treatment reduces viral antigenicity and maintains antitumor activity in mouse experimental glioma models. *Cancer Gene Ther*. **9**: 825–829

Natsume, A., Mizuno, M., Ryuke, Y., Yoshida, J. (2000) Cationic liposome conjugation to recombinant adenoviral vector reduces viral antigenicity. *Jpn J. Cancer Res*. **91**: 363–367

O'Riordan, C. R., Lachapelle, A., Delgado, C., Parkes, V., Wadsworth, S. C., Smith, A. E., Francis, G. E. (1999) PEGylation of adenovirus with retention of infectivity and

protection from neutralizing antibody in vitro and in vivo. *Hum. Gene Ther.* **10**: 1349–1358

Prince, H. (1998) Gene transfer: a review of methods and applications. *Pathology* **30**: 335–347

Qiu, C., De Young, M. B., Finn, A., Dichek, D. A. (1998) Cationic liposomes enhance adenovirus entry via a pathway independent of the fiber receptor and alpha(v)-integrins. *Hum. Gene Ther.* **9**: 507–520

Russell, W. C. (2000) Update on adenovirus and its vectors. [Review]. *J. Gen. Virol.* **81**: 2573–2604

Steel, J. C., Cavanagh, H. M., Burton, M. A., Dingwall, D., Kalle, W. H. (2004) In-vitro evaluation of ion-exchange microspheres for the sustained release of liposomal-adenoviral conjugates. *J. Control. Release* **95**: 601–611

St George, J. A. (2003) Gene therapy progress and prospects: adenoviral vectors. [Review]. *Gene Ther.* **10**: 1135–1141

Toyoda, K., Ooboshi, H., Chu, Y., Fasbender, A., Davidson, B. L., Welsh, M. J., Heistad, D. D. (1998) Cationic polymer and lipids enhance adenovirus-mediated gene transfer to rabbit carotid artery. *Stroke* **29**: 2181–2188

Verma, I., Somia, N. (1997) Gene therapy — promises, problems and prospects. *Nature* **389**: 239–242

Vilquin, J., Guerette, B., Kinoshita, I., Roy, B., Goulet, M., Gravel, C., Roy, R., Tremblay, J. (1995) FK506 immunosuppression to control the immune reactions triggered by first-generation adenovirus-mediated gene transfer. *Hum. Gene Ther.* **6**: 1391–1401

Wilson, C., Embree, L., Schowalter, D., Albert, R., Aruffo, A., Hollenbaugh, D., Linsley, P., Kay, M. (1998) Transient inhibition of CD28 and CD40 ligand interactions prolongs adenovirus-mediated transgene expression in the lung and facilitates expression after secondary vector administration. *J. Virol.* **72**: 7542–7550

Worgall, S., Worgall, T. S., Kostarelos, K., Singh, R., Leopold, P. L., Hackett, N. R., Crystal, R. G. (2000) Free cholesterol enhances adenoviral vector gene transfer and expression in CAR-deficient cells. *Mol. Ther.* **1**: 39–48