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Assessment of Genetic Shielding for Adenovirus Vectors

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Abstract: Development of adenovirus (Ad) vectors in the clinical context has highlighted that vector efficacy may be limited by the host humoral response due to pre-existing titers of neutralizing antibodies against the vector itself in humans. Further, multiple dosing of Ad vectors based on serotype 5 would be limited. Current immune evasion strategies being investigated by other laboratories are only applicable to non-replicating vectors. Therefore we have proposed genetic shielding as an alternate that would be applicable to both non-replicating and conditionally replicating Ad vectors. Genetic shielding would encapsulate fusion of a self-protein to Ad minor capsid protein, pIX, as a means to cloak immunogenic capsid epitopes and prevent neutralization of Ad vectors through Ad specific antibodies. In the development of a suitably shielded Ad vector we choose several self-proteins that we attempted to fuse to pIX. We have also used an indirect method to conjugate albumin to the capsid through an albumin binding domain fused to pIX. Despite attaining novel pIX modified Ad vectors we found that none of the pIX attached molecules in this study prevented neutralizing antibodies from halting gene transfer.

INTRODUCTION

Within the human population there are high titers of preexisting neutralizing antibodies against adenovirus serotypes 2 and 5 [1, 2] due to the general exposure to Ads. Therefore it has been proposed that efficacy of Ad vectors in the clinic would be initially limited and effective repeat administration of Ad vectors will be hindered by strong neutralizing antibody response to the vector. Thus far skeletal muscle was one of the few tissues where repeat Ad vector administration was successfully demonstrated [3]. However, the success of this procedure was highly dependent on the initial dose of Ad used in the experiment and therefore, it is still expected that repeat dosing in human will be problematic. With respect to conditionally replicative Ad vectors (CRAd) data from clinical trials were suggestive of limited efficacy due to strong innate immune responses [4]. Therefore development of non-replicating Ads, or CRAds, capable of evading the humoral response would improve clinical utility of these vectors.

There are two major efforts in an attempt to circumvent this issue with Ad vectors; (1) development of Ad vectors from different serotypes and (2) biochemical coating of the Ad vector. With respect to utilizing different serotypes, Ad11 and Ad35, to which the human population has a lower prevalence of neutralizing antibodies, have been investigated as alternative vectors to those of Ad5 [1, 5-7]. However, other human serotypes such as Ad6 [8], Ad19a [9, 10] and Ad41 [11], and adenovirus serotypes from other species are also being considered [12-16] as means to overcome preexisting Ad5 immunity. It is also likely that the development of humoral and cellular immune responses against these alternate serotypes will eventually occur. Another issue to consider is the different intrinsic properties of the various novel Ad serotype vectors such as the use of different cellular receptors for different serotype vector groups [17]. In this regard, it has been shown recently, that Ad vectors based on serotype B (like Ad35 and Ad11) binding to their cognate receptor CD46 down regulate immune responses [18] which has yet to be fully determined as a desirable attribute.

Biochemical modification of the Ad vector potentially provides shielding from the immune response through molecules covering the capsid. One established methodology is the conjugation of functional PEG to free lysine groups on the adenoviral capsid that enable Ad vectors to avoid neutralizing antibodies, *in vitro* or *in vivo* or limit the innate response [19-25]. In addition slower clearance rates of Ad from the blood have been demonstrated [26, 27]. Despite major improvements this method has several potential limitations including the ablation of the Ad receptor (CAR) specificity resulting in low levels of infection [19, 20, 23, 25]. Furthermore the heterogeneity of composition will confound potency in batch-to-batch production. These issues alone represent significant problems with respect to scale up and regulatory approval. Finally this method is suitable to protect only non-replicating Ad vectors as in the context of CRAd physiology the shielding molecule is lost from the progeny vectors.

We have previously proposed a concept based upon genetic shielding as an alternate and potentially much simpler method to cloak Ad vectors [28]. The concept was to utilize a self-protein fused to an adenovirus capsid protein to pro-

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vide a shield masking the immunogenic epitopes of the Ad vector capsid. Importantly it would be broadly applicable to both non-replicating vectors and conditionally replicating vectors. To prove this concept, we determined that the minor capsid protein, pIX, would be a suitable anchorage position for the shielding molecule. This is due to the large body of work indicating that carboxy terminus of pIX can be fused to an array of molecules including polylysine [29], the biotin acceptor peptide (BAP) [30] and larger complex proteins including various fluorescent proteins, HSV thymidine kinase (TK) and TK fusions [31-36]. In these studies it was demonstrated that virus viability was not significantly affected and that the pIX-fused molecules retained functionality. Furthermore, pIX is a triplet present at 80 locales, thus allowing for a defined number of shielding molecules to be included into the capsid, and allowing potentially for significant coverage of the capsid. However, the shielding protein of choice would need to embody several important characteristics, namely that it is a self-protein and has sufficient size to cover immunodominant Ad capsid epitopes. In this regard albumin could be a good choice as it is a large monomoeric non-gylcosylate polypeptide with wide *in vivo* distribution, long half-life and lack of substantial immunogenicity [37]. It has been fused to human growth hormone-recombinant human serum albumin (rHSA) [38], recombinant granulocyte colony stimulating factor-rHSA [39], and serum albumin-CD4 genetic conjugate [40] to enhance circulating half-life and improve stability for therapeutic applications. In addition it has a safe record in clinical practice, as Albuferon™ (albumin-interferon alpha) [41] has completed phase II clinical trials [42].

To examine our concept of genetic shielding we decided to use two different strategies with human albumin (hALB), direct fusion to pIX for capsid incorporation into the capsid and indirect linkage through conjugation methods (Fig. **1**). In addition to using hALB directly fused to pIX we decided to utilize another self-protein, alpha-1-antitrypsin (A1A), in parallel for direct incorporation. Although it is not as large as hALB, A1A is of a comparable size to HSV-TK, which has been successfully fused to pIX in replicating Ad vectors [31]. A1A is a serine proteinase inhibtitor with a primary function to inhibit the enzyme neutrophil elastase. We were concerned using an active enzyme and therefore mutated A1A through site directed mutagenesis to obtain A1A (P1-P1') which retains normal folding but results in ablation of enzyme function [43]. In an alternate approach to attempt to prove the concept of shielding with molecules located at the pIX locale, a second strategy was used that would involve conjugating hALB to the capsid through linkage to a binding motif (Fig. **1**). Bacteria express surface proteins that interact with human extracellular proteins including several natural albumin binding proteins that are well characterized, such as motifs in Protein G and Protein PAB [44]. Pathogens use these proteins to avoid detection by the human immune system. The small ligand BAP has been successfully incorporated into pIX and been shown to conjugate to appropriately labeled ligands [30], validating our approach and therefore we decided to incorporate the albumin binding domain 3 from Streptococcal Protein G (ABD) [44, 45] into pIX to be utilized as a docking site for albumin.

MATERIALS AND METHODOLGY

Cell Line Culture

HEK 293 cells and A549 cells were purchased from ATCC (Manassas, VA). All cells were propagated in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. FBS was purchased from HyClone

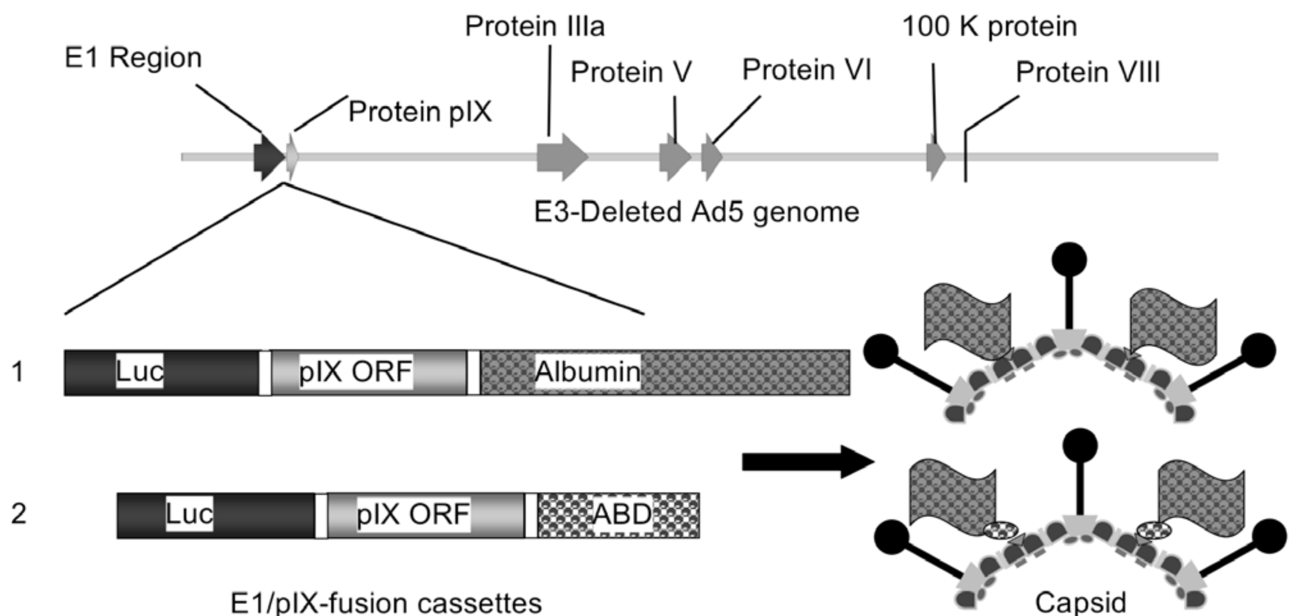

Fig. (1). Concept of direct and indirect incorporation of genetic shield proteins into Ad vector capsid. The E1/pIX fusion cassettes illustrate the concept of (**1**) direct incorporation of albumin through genetic fusion to the pIX molecule, and (**2**) indirect incorporation of albumin through conjugation to an albumin binding domain (ABD) genetically fused to the pIX molecule. The E1/pIX fusion cassettes are recombined into the Ad genome at the position shown. In the capsid configuration, the ABD domain is shown as a spotted circle and albumin is shown as a spotted flag.

(Logan, UT), and media and supplements were from Mediatech (Herndon, VA). All cells were propagated at 37°C in a 5% CO_2 atmosphere.

Capsid Modified Adenoviral Vectors

To generate the proposed shielded Ad vectors in this study, we used pIX-modified vectors which were based on the pSILucIXFlag-NheI shuttle vector [29] (or linker variants, 45Å and 75Å based on those described by Vellinga and coworkers [46]). To fuse proposed shield proteins to the pIX capsid protein, the shield proteins were cloned into the NheI sites of the shuttle vectors as follows. The cDNA of human alpha 1 antitrypsin (A1A) and human preproalbumin are commercially available in the plasmids pCMV6-XL6 and pCMV4-XL6 respectively (Origene, Rockville, MD). To generate a PCR fragment of A1A with NheI ends the following primers (IDT, Coralville, IA) were used sense 5'-tgacaagctagccgaggatccccagggagat-3' and anti-sense 5'-gcgcatggctagcttatttttgggtgggat-3' and to generate the mature form of hALB with NheI ends the following primers were used sense 5'- gacaagctagccgatgcacacaa-3' and anti-sense 5'-ggatggctagcttataagcctaag-3' (NheI sites underlined). PCR fragments once purified were NheI digested and cloned into the shuttle vectors. Site directed mutagenesis of A1A to generate Met358Pro and Ser359Pro (A1A(P1-P1')) took place in over two rounds using the following primers (altered nucleotides in bold), Step 1: sense 5'-ggccatacccatgtctatccc**a**cc**a**gaggtcaagttcaacaaac-3' and anti-sense 5'- gtttgttgaacttgacctc**t**gg**t**gggatagacatgggtatggcc-3' and Step 2: sense 5'-gttttagaggccatacc**acc**g**c**ctatcccaccagaggtcaag-3' and anti-sense 5'-cttgacctctggtgggatag**g**c**ggt**ggtatggcctctaaaaac-3'. The two steps were performed using the QuikChange Site Directed Mutagenesis kit following the manufacturer's protocol (Stratagene, La Jolla, CA). As a control for pIX modification and viral rescue we also made an AdLuc vector containing a mutated TK gene (sr39TK) as this is a known successful pIX modification [31]. For sr39TK the NheI fragment was out of pShuttle.wt.E1-pIX-flag-TK [31]. The albumin binding domain 3 (ABD) from streptococcal Protein G (46 amino acid) [44, 45] (Genbank X06173) was generated using two commercially synthesized single-stranded oligonucleotides (IDT), one starting at the 5' end, the other starting at the 3' end, with an overlapping central 15 nucleotide region. The oligonucleotides were annealed and extended, using Pfu Hifidelity TAQ (Stratagene), to form a double stranded (ds) cDNA of ABD with NheI restriction sites at both the 5' and 3' ends. The ds cDNA was digested with NheI to allow for subcloning into the pSILuc shuttle vectors.

Once the shuttle vectors were confirmed, they were linearized with PmeI (NEB, Ipswich, MA) and recombined into the pAdEasy backbone [47]. Two control vectors, containing wild type pIX were used for this work, AdLuc1 and AdCMVLuc (kindly provided by Dr Igor Dmitriev, UAB), both containing the wild type pIX, but with slightly differing backbone. AdLuc1 was used only in ELISA experiments. For gene transfer experiments the control vector AdCMVLuc made from the identical parental shuttle plasmid (pSI) was used to provide a better comparison. Viral genomes and rescued viruses were termed AdLucIXLinker-ligand.

Rescue and propagation of viruses was carried out on HEK 293 cells (success/failure summarized in Table **1A**), and viruses were purified from infected cells by three freeze-thaw cycles followed by two successive bandings on cesium chloride (CsCl, Sigma, St. Louis, MO) gradients. Particle units (pu) of viruses were determined with standard OD260 methods. Infectious virus titer was determined by focus forming unit (ffu) assay. Monolayers of HEK 293 cells were infected with serial dilutions of the vector. Following a suitable incubation period, the infected cells were probed with an adenovirus antibody and than with a secondary antibody conjugated with Fluorescein-Isothiocyanate (FITC). Fluorescent foci are viewed under a UV microscope and enumerated. Specific viral concentrations and titers are listed in Table **1B**.

Analysis of pIX-Modified Protein Capsid Incorporation by Western Blot

Virus (5 x 10^9 pu per sample) was denatured by boiling in Laemmli loading buffer (Bio-Rad, Hercules, CA). The viral capsid proteins were separated by a 4-20% gradient polyacrylamide gel (Bio-Rad) and the electrophorectically resolved viral capsomers were transferred to polyvinylidenedifluoride membrane (Bio-Rad) and probed with anti-FLAG monoclonal antibody (Sigma) as all pIX fusions contain a FLAG motif as previously described [29]. The blots were developed with the WesternBreeze Immunodetection system (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Additionally during propagation cell lysates from cells infected with vectors were also tested. In this case 10 μl of 293 cell lysates from the rounds of propagation were instead denatured by boiling in Laemmli loading buffer and analyzed on western blot using the anti-FLAG antibody.

Assessment of ABD Adenovirus Vector Binding to Albumin

Human, murine, bovine or canine albumin (all obtained from Sigma) prepared at 5 μg/ml were adsorbed to 96-well Nunc Immuno-plates plates (Fisher Scientific, Pittsburgh, PA) overnight in 100 μl binding buffer (50 mM carbonate buffer (Ph 8.6)) at 4°C. An additional plate was treated with binding buffer alone (data not shown, as identical to bovine). The following day, wells were blocked with 0.5% casein prepared in 1xTBS/0.05% Tween and then viruses were added at the above pu/well (highest 30x10^9 pu/well) diluted in blocking buffer. In between all steps wells were washed with 1xTBS/0.05% Tween. Wells were probed for bound adenovirus particles with anti-Ad2 polyclonal antibody (NIAID) for 1hr at RT and then with the secondary HRP antibody being goat anti-rabbit immunoglobulin antibody (Dako, Carpinteria, CA) for 1 hour at RT. The color was developed with the Sigma FAST o-phenylenediamine dihydrochloride tablet kit (Sigma) as recommended by the manufacturer and then fixed with 1N HCL. The color intensity was measured at 450 nm with an EL800 plate reader (Bio-Tek Instruments, Winooski, VT). It should be noted that the presence of the various albumins in the ELISA plates were probed in separate wells with specific anti-albumin antibodies (data not shown, antibodies from Bethyl Laboratories, Montgomery, TX) and it was confirmed that the albumins absorbed to the plates.

Table 1A. Analysis of pIX-Modified Vector Rescue, Propagation and Incorporation of pIX-Protein Fusions into the Ad Vector Capsid

pIX Configuration	pIXFlag		pIX45A		pIX75A	
Protein	Rescue & Propagation	WB	Rescue & Propagation	WB	Rescue & Propagation	WB
Control Vectors						
Stop Codon	Yes	Yes	Yes	Yes	Yes	Yes
Direct Concept Vectors						
A1A	Yes	Yes	Yes	Yes	ND	ND
A1A(P1-P1')	Yes	Yes	Yes	Yes	ND	ND
sr39TK	Yes	Yes	ND	ND	ND	ND
hALB	Yes/No	ND*	ND	ND	Yes/No	ND
Indirect Concept Vectors						
ABD	Yes	Yes	Yes	Yes	Yes	Yes

WB Western Blot detection of pIX-fusion in capsid of CsCl purified vectors.
ND Not Done.
hALB Yes//No - Virus could be rescued but was not successfully propagated.
ND* Not Done on CsCl purified vector.

Table 1B. Viral Titers and pu/ffu Ratio Attained for Successfully Propagated pIX-Modified Vectors

	Virus Name	Particle Concentration (pu/ml)	Titer (ffu/ml)	Ratio (pu/ffu)
1	AdCMVLuc	1.2×10^{12}	2.0×10^{10}	60
2	AdLuc.IXFlag-stop	1.7×10^{12}	4.4×10^{10}	39
3	AdLuc.IXFlag-ABD	1.5×10^{12}	4.0×10^{10}	38
4	AdLuc.IX45A-stop	1.7×10^{12}	4.0×10^{10}	43
5	AdLuc.IX45A-ABD	1.2×10^{12}	2.6×10^{10}	46
6	AdLuc.IX75A-stop	1.2×10^{12}	7.1×10^{10}	17
7	AdLuc.IX75A-ABD	3.2×10^{11}	4.7×10^{9}	68
8	AdLuc.IXFlag-sr39TK	1.2×10^{12}	2.7×10^{10}	44
9	AdLuc.IXFlag-A1A	5.5×10^{11}	4.7×10^{9}	117
10	AdLuc.IXFlag-A1A(P1-P1')	1.4×10^{12}	7.3×10^{9}	192
11	AdLuc.IX45A-A1A	4.6×10^{11}	2.1×10^{9}	219
12	AdLuc.IX45A-A1A(P1-P1')	1.8×10^{12}	1.4×10^{10}	129

Adenoviral Infection of A549 Cells to Assess pIX-Protein Shielding Properties

A549 cells were seeded into 96 well plates at 1×10^4 cells/well 24 hours before infection with adenovirus vectors at an MOI of 100 pu/cell. An MOI of 100 pu/cell is a standard quantity to use with A549 cells as demonstrated in numerous studies. In studies with A1A or sr39TK containing vectors (and appropriate controls), vectors were incubated for 30 minutes at 37°C with a 1:2500 dilution of anti-Ad5 antibody (Abcam, Cambridge, MA) or were incubated for 30 minutes at 37°C with 1:2500 dilution of polyclonal hexon antibody (GeneTex, Inc., San Antonio, TX) pre-infection. Cells were then incubated with virus for 2 hours at 37°C, washed with PBS and refed with normal media. After 24 hours cells were lyzed with Luciferase Reporter Lysis Buffer (Promega, Madison, WI) and assayed for luciferase activity, measured as relative light units (RLU) according to the manufacturer protocol (Promega Luciferase Kit). The 1:2500 dilution of anti-Ad antibody and anti-hexon antibody was determined by mixing AdCMVLuc with a serial dilution of the antibodies prior to infection of cells, and determining a dilution that resulted in a 2 log reduction in luciferase activity when compared to AdCMVLuc with no antibody (data not shown). Viruses were also assessed after pre-mixing with naïve or immunized mouse serum at 1, 10 25, 50 and 90% serum concentration. Infection of cells and luciferase activity was assessed as previously described. Naïve serum was obtained from C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) that were not exposed to wt Ad5 vector while

immunized serum was obtained from C57BL/6 mice 14 days following tail vein injection 10^9 pu/mouse of wt Ad5 vector (HAdV-5, ATCC, formerly known as Adwt300 [48].

Two sets of experiment were carried out with the ABD containing vectors. In the first experiment, AdLucIX45A-ABD and control vector, AdLucIX45A-Stop, prior to antibody incubation, were pre-mixed with varying quantities of hALB (Sigma) for 30 minutes at 37°C. All viruses, including AdCMVLuc were then incubated without or with a 1:2500 dilution of anti-Ad5 antibody (Abcam) for 30 minutes at 37°C. Cells were then incubated with virus for 2 hours at 37°C, washed with PBS and refed with normal media. A further 24 hours later, cells were analyzed for luciferase activity using standard methods. In the second experiment pre-incubation the vectors were incubated for 30 minutes at 37°C in serum free media, with 90% naïve murine serum or 90% serum from mice immunized with Ad5. Naïve serum was obtained as above, but this time the immunized serum was obtained from C57BL/6 mice 28 days following tail vein injection 10^9 pu/mouse of wt Ad5 vector. Following this initial incubation, the pre-mixes were then either incubated without antibody or with a 1:2500 dilution of anti-Ad5 antibody (Abcam) for a further 30 minutes at 37°C. Cells were then incubated with virus for 2 hours at 37°C, washed with PBS and refed with normal media. A further 24 hours later, cells were analyzed for luciferase activity using standard methods. All gene transfer experiments were carried out once but with triplicates within the actual experiment. The experiments are represented at relative light units (RLU) for luciferase activity. The data was also normalized but the patterns per results remained identical (data not shown).

RESULTS

Concept 1: Assessment of Shielding Capacity of pIX Modified Ad Vectors Containing Shield Protein Directly Fused to pIX

The preferred method for genetic shielding would be to directly incorporate hALB into Ad5 vectors through genetic fusion with pIX (Fig. **1**) We also used A1A and A1A (P1-P1') along with sr39TK to test as shielding molecules in this concept. The molecules A1A and the enzymatic deficient A1A(P1-P1') were included as alternate self molecules while sr39TK was included because it is a proven pIX modification [31]. All the Ad vectors were generated using the pAdEasy system [47] and pIX_modified Ad vectors containing sr39TK and A1A molecules were rescued and propagated on 293 cells (as indicated in Table **1A**). Successful capsid incorporation of modified pIX proteins was indicated through western blot analysis (sr39TK is shown in Fig. (**2**), panel **A**). Following a similar propagation strategy to that used with control Ad vector, AdCMVLuc, these vectors could be propagated to titers between 5x 10^{11} pu/ml and $2x10^{12}$ pu/ml. While the pu/ffu ratio of pIX-sr39TK was at 44 those of the A1A related vectors were higher falling between 117 and 219 (Table **1B**). The current batch of AdCMVLuc had a pu/ffu ratio of 60. However difficulties arose with generating viruses with hALB fused directly to pIX.

Initially only the AdLucIXFlag-hALB genome was prepared as proteins larger than hALB, TK-luciferase [36] and TK-eGFP[35], have been successfully fused onto the shortest pIX-flag variant resulting in capsid incorporation. Plaques that arose on transfected 293 cells indicated that virus was

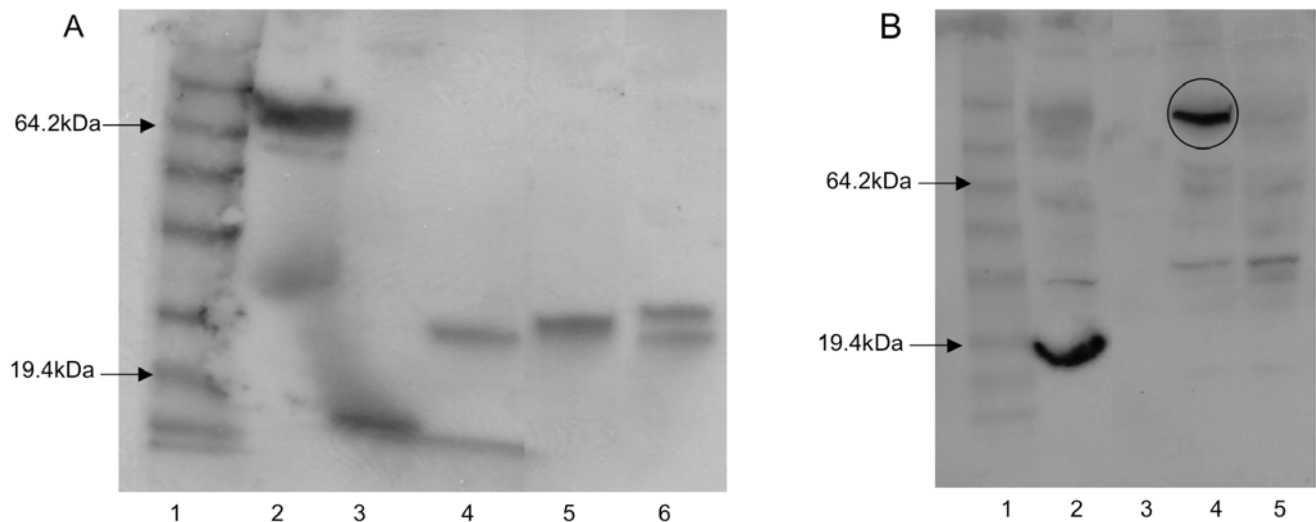

Fig. (2). Analysis of capsid incorporation of pIX-molecule fusions (panel **A**) and production of pIX-molecule fusions in cell lysates (panel **B**). CsCl purified virions of pIX-modified Ad vectors were analyzed for pIX-molecule capsid incorporation through western blot analysis (panel **A**). Ad vectors were loaded at 5 x 10^9 pu/lane after boiling and samples probed with anti-FLAG antibody. Lane order is as follows: (1) Marker, (2) AdLucIX-sr39TK (57kDa), (3) AdLucIXFlag-Stop (16kDa), (4) AdLucIXFlag-ABD (21kDa), (5) AdLucIX45A-ABD (25kDa) and (6) AdLucIX75A-ABD (28kDa). Cell lysates from 293 cells infected with AdLucIXFlag-hALB were analyzed for production of the pIX fusion during rescue and two rounds of propagation of vector (panel **B**). Lysate from cells infected with AdLucIXFlag-ABD was used as control for anti-FLAG antibody. Lane order is as follows: (1) Marker, (2) AdLucIXFlag-ABD, (3) AdLucIXFlag-hALB at rescue (35mm dish), (4) AdLucIXFlag-hALB at round one propagation and (5) AdLucIXFlag-hALB at round two of propagation. The presence of pIX-hALB (83kDa) in the cell lysate is circled and only seen at round one propagation.

rescued. However propagation of the virus to CsCl purification stage was unsuccessful due to ever increasing length of time, outside of the normal 2-3 days, to see cytopathic effect (CPE) on the infected cells. The gene fusion could be detected by PCR analysis of the cell lysates from infected cells (data not shown) but the protein was only detected in the second round of amplification (where CPE occurred at 4 days) by western blot (Fig. **2**, panel **B**). This also suggested that the fusion protein probably is not aggregating due to a lack of a specific band at higher molecular weight. A propagation strategy of harvesting cells every four days after virus infection was also attempted but no CPE was seen and resulted in empty capsids during CsCl purification. Therefore these hALB vectors were not pursued in tests of gene transfer experiments.

To determine if the capsid incorporated pIXFlag-A1A and sr39TK fusions could provide a shielding effect *in vitro* gene transfer analysis was used as the starting point. Briefly viruses were pre-mixed with neutralizing antibodies, or just in serum free media, and then luciferase activity was used to determine the level of infection of cells with vectors. Adenovirus infection properties of several pIX-modified vectors, including control vectors (AdLucIXFlag-stop and AdLucIX45A-stop), in the absence and presence of anti-Ad5 (1:2500 dilution) (Fig. **3**, panel **A**), were analyzed to determine if any of the modifications provided a shielding effect. The results demonstrated that the modified pIX region did not affect general infection properties of the vectors when compared to AdCMVLuc. However the results also demonstrated that none of the proteins fused to pIX provided a shielding effect, as the antibody effectively reduced luciferase activity for all vectors tested. We also tested serum from naïve and immunized C57BL/6 mice (serum taken 14 days after immunization with a wild type vector) and com-

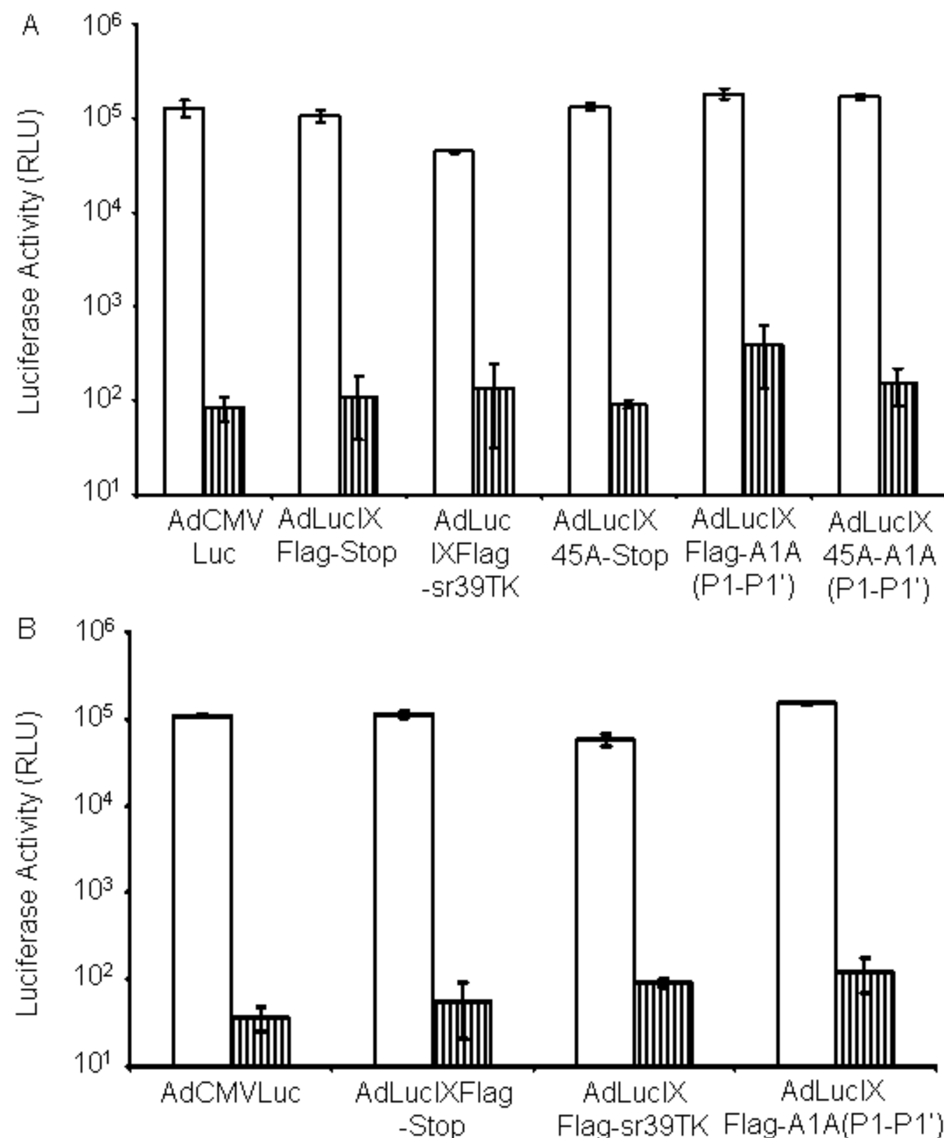

Fig. (3). Effect of neutralizing antibody on gene transfer of Ad5 vectors with direct incorporation of shielding proteins was assessed 24 hours following infection of A549 cells. Luciferase activity expressed as relative light units (RLU) is compared between vectors that were incubated with either a 1:2500 dilution of anti-Ad5 antibody (panel **A**) or a 1:2500 dilution of polyclonal hexon antibody (panel **B**) (striped columns) and vectors without antibody pre-incubation (white columns, both panels). All of the measurements are shown as mean RLU (n=3, +/- standard deviation).

pared 1%, 10%, 25%, 50% and 90% serum first on AdCMVLuc, and found that there was 0.5 to 1 log reduction in luciferase activity at 50% and 90% serum respectively (data not shown). This result may also suggest that there is less anti-Ad5 antibody present in immunized mouse serum than in the polyclonal antibody preparation but apart from this crude analysis, neutralizing titer of the serum was not compared to the polyclonal antibody preparation. When we compared AdCMVLuc, AdLucIXFlag-sr39TK and AdLucIX45-A1A at the 50% serum concentration, a similar reduction in luciferase activity was seen with all three viruses indicating that no shielding effect was seen (data not shown).

To assess whether any of the proteins provided some shielding of immunogenic epitopes a polyclonal hexon antibody was used. Hexon proteins form the largest surface on the capsid with the most dominant epitopes and consequently shielding against hexon neutralizing antibodies would be very important [49]. While penton and fiber also have immunogenic epitopes, we used the logic that inability of the pIX attached molecules to shield against hexon, probably would not allow shielding against penton and fiber neutralizing antibodies. This is due to the spatial arrangement of proteins in the capsid, especially fiber which extends away from the capsid surface and therefore decided to only test a hexon antibody. The adenovirus infection properties of AdLucIXFlag-Stop, AdLucIXFlag-sr39TK and AdLucIXFlag-A1A (P1-P1') pre-mixed, with or without anti-hexon antibody (used at the 1:2500 dilution), were then analyzed (Fig. **3**, panel **B**). The results clearly demonstrated that the polyclonal hexon antibody was able to neutralize adenovirus infection regardless of which protein was fused to pIX.

Concept 2: Assessment of Indirect Shielding Capacity of pIX Modified Ad Vectors Containing a Binding Motif Conjugated to Shield Protein

As with the hALB, A1A, A1A(P1-P1') and sr39TK vectors for strategy one, we generated ABD vectors with pAdEasy as indicated in Table **1**. All three ABD vectors and their corresponding control vectors (pIX-linker-stop) could be rescued and propagated on 293 cells, to attaining titers above 1-2x10^{12} pu/ml under similar propagation strategies to AdCMVLuc. Only AdLucIX75A-ABD did not grow to a high titer (< 5x10^{11} pu/ml). However all these vectors had pu/ffu ratios within the 17 to 68 range (see Table **1B**). The pIX fusions were detected through western blot analysis (Fig. **2**, panel A). One interesting facet for AdLucIX75A-ABD was that two pIX bands were detected on western blot suggesting that this construct is not completely stable and that perhaps the ABD portion on some of the pIX-fusions is being degraded. This data corroborates another report that the 75Å linker is not necessarily the best linker for pIX attached protein constructs [50].

Prior to gene transfer experiments, the functionality of ABD in the pIX capsid incorporated context was tested through ELISA methodology (Fig. **4**). Albumins from various sources, human (Fig. **4,** panel **A**), murine (Fig. **4,** panel **B**), bovine (Fig. **4,** panel **C**) and canine (Fig. **4,** panel **D**) were adsorbed to ELISA plates and then the appropriate

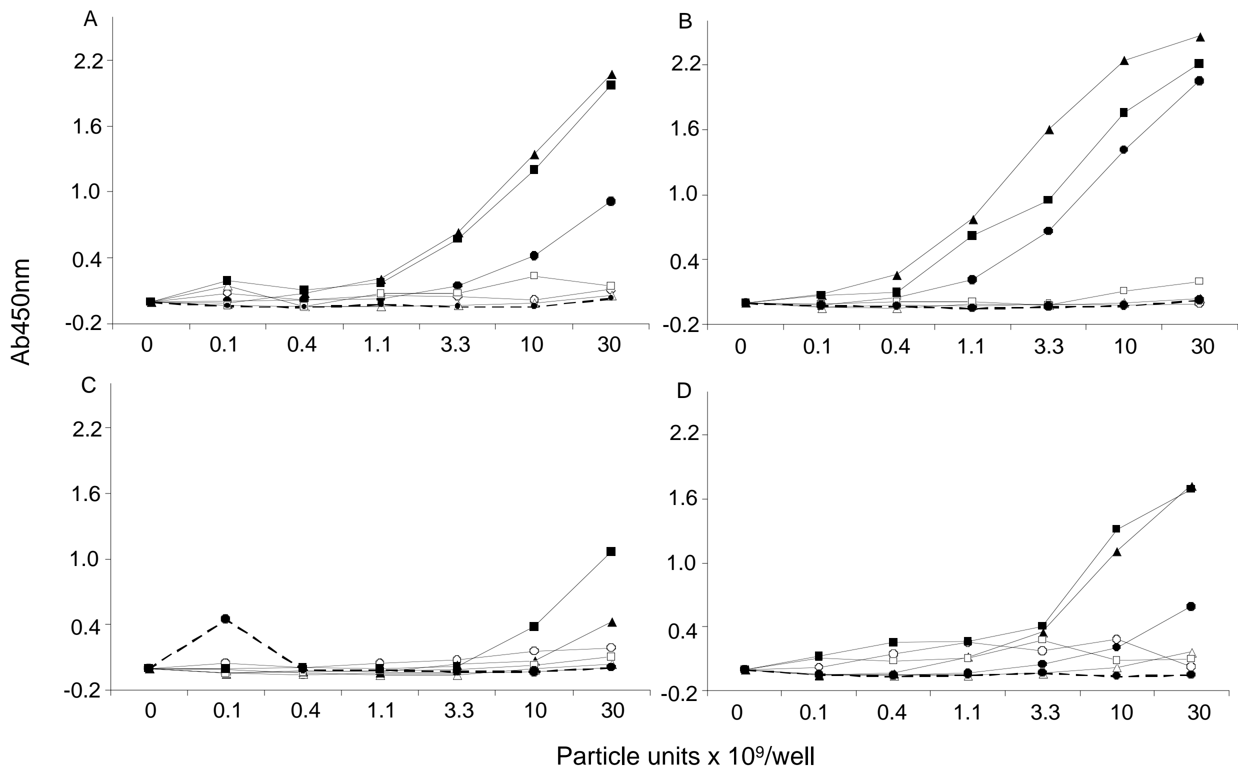

Fig. (4). Analysis of Ad5 capsid incorporated pIX-ABD interaction with human (panel **A**), murine (panel **B**), bovine (panel **C**) or canine (panel **D**) albumin through ELISA methodology with increased binding indicated by increase in absorbance reading. The viruses are represented as follows: AdLucIXFlag-ABD (solid circle), AdLucIXFlag-Stop (open circle), AdLucIX45A-ABD (solid triangle), AdLucIX45A-Stop (open triangle), AdLucIX75A-ABD (solid square), AdLucIX75A-Stop (open square), AdLuc1 (dashed line).

ABD and control viruses were added to the wells. The ABD viruses had most affinity with hALB (Fig. **4,** panel **A**) and murine albumin (mALB) (Fig. **4,** panel **B**) by virtue of the higher OD readings obtained when compared to control viruses at concentrations greater than 3.3 x 10^9 pu/well and 1.1 x 10^9 pu/well respectively. There was less affinity with canine albumin (cALB) (Fig. **4,** panel **D**), and essentially no affinity with bovine (Fig. **4,** panel **C**) under these conditions for the ABD viruses. No affinity with plastic was detected for these viruses (data not shown) indicating that the results seen with hALB and mALB were specific for the presence of these albumins adsorbed to the plates. In addition all plates were tested with albumin control antibodies to confirm adsorption to plates (data not shown). AdLucIX45A-ABD (solid triangles) performed the best with mALB (Fig. **4,** panel **B**) and was similar with AdLucIX75A-ABD (solid squares) on both hALB (Fig. **4,** panel **A**) and cALB (Fig. **4,** panel **D**). However, due to the hinted instability of AdLucIX75A-ABD it was decided that AdLucIX45A-ABD would be the ABD vector of choice to analyze in gene transfer experiments.

The ability of AdLucIX45A-ABD and control vector AdLucIX45A-Stop to infect cells was then analyzed following pre-mixing with a serial dilution of hALB, and after the initial incubation period, mixed with or without anti-Ad5 (used at the 1:2500 dilution) (Fig. **5**, panel **A**). AdCMVLuc, without hALB, but +/- antibody was used as a benchmark for

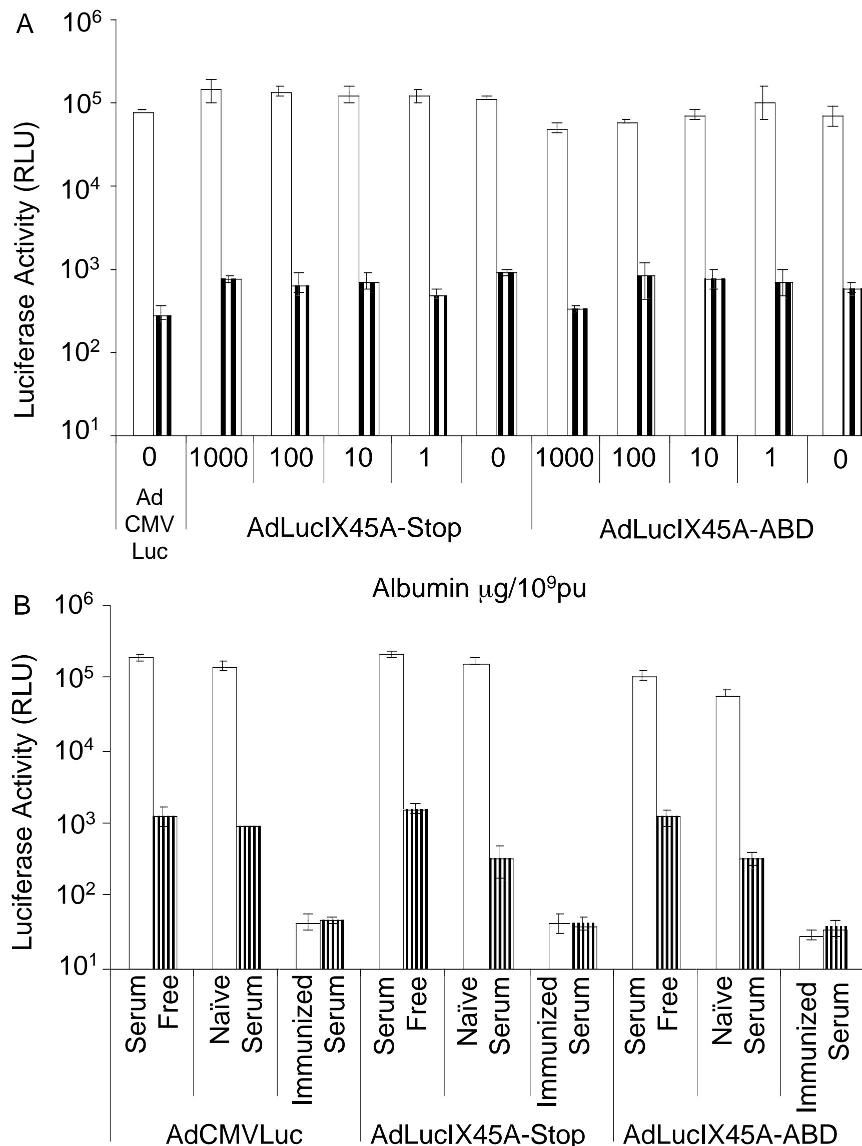

Fig. (5). Effect of anti-Ad5 antibody neutralizing capacity on gene transfer of adenovirus vectors with indirect incorporation of hALB conjugation (panel **A**) or mALB (panel **B**) was assessed 24 hours following infection of A549 cells. In panel A luciferase activity expressed as RLU is compared between vectors that were pre-mixed with varying quantities of hALB (Sigma) and then incubated with a 1:2500 dilution of anti-Ad5 antibody (striped columns) or no antibody incubation (white columns) (panel **A**). In panel **B** luciferase activity is compared between vectors that were pre-mixed with in serum free media, with 90% naïve murine serum or 90% serum from mice immunized with Ad5 and then incubated with a 1:2500 dilution of anti-Ad5 antibody (striped columns) or no antibody incubation (white columns) (panel **B**). In panel **A**, AdCMVLuc was not pre-incubated with hALB, but was pre-incubated +/- anti-Ad5 antibody. All of the measurements are shown as mean RLU (n=3, +/- standard deviation).

determining success or failure of the experiment. As expected the control AdLucIX45A-Stop was neutralized under all conditions, in the same manner as AdCMVLuc. However, AdLucIX-45A-ABD was also neutralized under all conditions. This result indicated two possibilities. The first possibility is that the pre-incubation with hALB did not form an Ad-albumin complex which conflicts with the ELISA data where it was clear that AdLucIX45A-ABD could adhere to hALB coated plates. Secondly, if the virus was coated with albumin then the albumin was not providing a shield against neutralizing antibodies.

To further assess whether AdLucIX45A-ABD could bind albumin and attain a shielding affect, the virus was used in an experiment without prior coating to hALB. Instead the vector was mixed with serum from naïve mice (C57BL/6 murine model) and from mice that had been pre-immunized with wild type Ad5 vector. It was hypothesized that in naïve serum the vector would conjugate with mALB in the serum (based on the positive ELISA data, Fig. **4**) and the mALB would prevent neutralization by antibodies against Ad5 once the polyclonal antibody was added to the mix. It was a little less clear as to what would happen with immunized serum, as this already contains neutralizing antibodies, and there would potentially be competition between the albumin and antibodies in this serum. Due to the results from the control vector with 90% serum in section one of the results where a log decrease was seen in gene transfer it was decided to obtain serum from mice at a 28 day time period following wild type adenovirus administration. The 90% concentration of serum was again used in these experiments. Following pre-incubation of AdLucIX45A-ABD (and control vectors) in serum free media with naïve and immunized murine serum, the polyclonal antibody against Ad5 was added to the mix (Fig. **5**, panel **B**). In serum free control conditions AdLucIX45A-ABD was neutralized as expected, while in immunized serum, it was clear that murine anti-Ad5 antibodies are able to neutralize Ad vector infection. Furthermore the reduction of luciferase activity from AdLucIX45A-ABD in naïve serum conditions with anti-Ad5 illustrates that either insufficient mALB was bound to the vector or albumin conjugated to the capsid is not capable of preventing neutralization. The conclusion from this part of the work was that this particular binding motif, ABD and albumin (human or murine) were not capable of providing a shielding effect.

DISCUSSION AND CONCLUSION

The data demonstrated several issues with pIX fusions and the potential of genetic shielding. This is the first study to show that not all molecules fused to pIX can be successfully incorporated into the Ad vector capsid. In this case hALB was deemed incompatible with direct capsid incorporation and it could be speculated that the 83 kDa pIXFlag-hALB fusion appeared to have inhibitory actions on the formation of virions. While capsid assembly is supposedly not affected by the absence of pIX [51], it is thought to play a role in packaging full length genomes [52]. Furthermore, while the product of the adenovirus intermediate gene IX is a transcriptional activator it is not absolutely required [53]. Albumin is not known to bind DNA and therefore this should not be a cause for the problems seen here. Although pIXFlag-hALB fusions could be detected in an early round of propagation, perhaps the disulphide complexity of the albumin and/or some other factor makes this protein unstable and ultimately unsuitable for capsid incorporation leading to inefficient packaging of genomes. Based on the western blot results we concluded that the pIXFlag-hALB was not aggregating. It would seem virions with full length genomes are attained in early rounds of propagation, but empty capsids become more predominant as the number of rounds of propagation increase. The ABD ELISA data also indicates that there may be spatial factors playing a role with albumin, in that longer linkers were required to see good conjugation to albumin, thus suggesting the natural globular configuration of albumin may prevent it from fitting into the pIX cavity. Currently the classical configuration of the Ad capsid and position of pIX is being questioned [54] and therefore spatial elements of the capsid may play a bigger role in determining suitable large pIX-protein fusions to develop as pIX-modified vectors. Despite the failure with albumin a smaller self-protein alpha-1-antitrypsin was successfully fused to the pIX protein and virus was generated with this fusion capsid incorporated. This is the first report of a large protein, not functionally limited to imaging applications, fused to the pIX and broadens the scope of proteins that can be fused to pIX.

The data obtained from the *in vitro* shielding experiments showed that (a) direct incorporation of proteins of ~60 kDa (A1A) or less cannot provide sufficient coverage of the highly immunogenic hexon regions of the adenovirus capsid and (b) indirect incorporation of albumin to the adenovirus capsid through the ABD protein did not provide shielding to the vector. This is based on the significant reduction in adenovirus infection of the pIX-modified vectors in the presence of anti-Ad5 antibody (for all viruses) and in the presence of anti-hexon antibody (for sr39TK and A1A(P1-P1')). Certainly the hexon data indicates our molecules are not large enough to cover the immunogenic epitopes of hexon. The data for sr39TK is contradictory to our initial report that a pIXFlag-sr39TK virus can provide a shielding effect [28]. The first report was based on a static ELISA method and the results illustrated here are based on a 3 dimensional experiment with serum mixed with virus prior to gene transfer. Therefore this study indicates the need to use suitable methodologies when examining concepts.

Despite the problems with pIXFlag-hALB, a larger protein, a TK-luciferase fusion (over 90 kDa) has been successfully incorporated into pIX-modified virions with just the standard FLAG motif [36] indicating larger self-proteins could be explored as shielding agents. The absolute size limitations for pIX-fusions have yet to be determined but other considerations such as disulphide complexity, and post-translational modifications will need to be accounted for when looking for a suitable self-protein. Even if a suitably large self-protein was determined and could show hexon coverage, then modifications to the fiber may also be required to block neutralizing antibody sites on the fiber due to the extension away from the capsid of this protein. Two recent reports have indicated that fiber modifications may help Ad vectors escape the immune response [55, 56], and therefore this would certainly be something that requires further investigation in combination with finding suitable alternate shielding molecules.

ABBREVIATIONS

A1A	=	Alpha-1-antitrypsin
ABD	=	Albumin binding domain 3
Ad	=	Adenovirus
cALB	=	Canine albumin
CPE	=	Cytopathic effect
CRAd	=	Conditionally replicative Ad vectors
CsCl	=	Cesium chloride
ds cDNA	=	Double stranded cDNA
ffu	=	Focus forming unit
hALB	=	Human albumin
mALB	=	Murine albumin
pu	=	Particle unit
rHSA	=	Recombinant human serum albumin
RLU	=	Relative light units

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CONFLICT OF INTEREST

David T. Curiel is an equity holder in VectorLogics, Inc.

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