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ARTICLE *in* BIOTECHNOLOGY PROGRESS · MARCH 2005

Impact Factor: 2.15 · DOI: 10.1021/bp049644r · Source: PubMed

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Development of a Purification Process for Adenovirus: Controlling Virus Aggregation to Improve the Clearance of Host Cell DNA

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The clearance of host cell DNA is a critical goal for purification process development for recombinant Ad5 (rAd5) based vaccines and gene therapy products. We have evaluated the clearance of DNA by a rAd5 purification process utilizing nuclease digestion, ultrafiltration, and anion exchange (AEX) chromatography and found residual host cell DNA to consistently reach a limiting value of about 100 pg/10^{11} rAd5 particles. Characterization of the purified rAd5 product using serial AEX chromatography, hydroxyapatite chromatography, or nuclease treatment with and without particle disruption showed that the residual DNA was associated with virus particles. Using a variety of additional physical characterization methods, a population of rAd5 virus in an aggregated state was detected. Aggregation was eliminated using nonionic detergents to attenuate hydrophobic interactions and sodium chloride to attenuate electrostatic interactions. After implementation of these modifications, the process was able to consistently reduce host cell DNA to levels at or below 5 pg/10^{11} rAd5 particles, suggesting that molecular interactions between cellular DNA and rAd5 are important determinants of process DNA clearance capability and that the co-purifying DNA was not encapsidated.

Introduction

The use of recombinant Ad5 (rAd5) vectors for gene therapy and vaccine applications continues to expand in scope (*1*–*8*) and presents to the bioprocess engineer the challenge to develop a readily scaleable manufacturing process capable of yielding rAd5 that consistently meets the expectations of regulatory agencies.

Recombinant E1-deleted rAd5 vectors have been most commonly propagated in the 293 cell line (*9*), though more recently, use of the PER.C6 cell line has grown in favor owing to the rational design of its E1 transgene (*10*), which precludes the generation of replication-competent adenovirus (RCA). The expression of the E1 gene in these cell lines transcomplements the E1 deficiency of rAd5 vectors and also is responsible for the immortalization of the cell lines. In addition, E1 expression may contribute to the tumorigenic phenotype when assayed in the nude mouse model (*11*). Therefore, clearance of host cell DNA, including the E1 gene, is an especially important consideration for process development. The specific target for residual host cell DNA in the product may depend on an analysis of clinical benefit versus hypothetical risk; however, regulatory guidelines have suggested residual DNA targets as low as 10 pg/dose for inactivated poliomyelitis vaccine produced in Vero cells (*12*, *13*). General guidelines issued for continuous cell lines suggested a target of 100 pg/dose (*14*). More recent discussion suggests a more relaxed regulatory posture, with limits of up to 10 ng/dose (*15*); however, recent adenovirus-specific regulatory guidance clarified that the limit of 10 ng would only be acceptable provided that the DNA was digested to less than 100–200 base pairs in length (*16*). For prophylactic vaccines, a target of 100 pg/dose (as measured by quantitative PCR) should be suitable to respect the exceedingly remote but theoretical possibility of the transduction of host cell E1 DNA by rAd5 vectors. Since adenoviruses are typically produced at about 10^4–10^5 viral particles (vp)/cell (*17*) and mammalian cells have a genome of about 10 pg (*18*), 7 logs of DNA clearance would be required in order to attain levels below 100 pg/dose for a high (10^{12} vp) dose of adenovirus.

Ad5s are large (80 nm), nonenveloped DNA viruses with a 35 kB genome enclosed in a capsid composed primarily of 240 trimers of the hexon protein. Each hexon monomer has a net charge of −23.8, resulting in an aggregate capsid negative charge of over 17,000. Despite this aggregate negativity, Ad5 particles are capable of self-association, forming paracrystalline arrays in the nucleus (*19*) during replication and assembly, and forming aggregates in solution, presumably mediated by hydrophobic interactions. For purified adenovirus, aggregation is a function of virus concentration, temperature, pH, excipient concentration, and storage container (*20*–*23*). Despite the growing literature on the relevance of aggregation for stability of purified rAd5, no literature exists on its impact on purification process development.

In this work, we first report our definition of a scaleable process for the purification of rAd5 virus, characterize a physical association of residual DNA with rAd5 aggregates, and finally report process modifications enabling the consistent clearance of host cell DNA to less than 5 pg/10^{11} viral particles.

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10.1021/bp049644r CCC: $30.25
Published on Web 02/18/2005

Materials and Methods

PER.C6 Cell Culture and Virus Propagation. PER.C6 cell growth and Ad5 propagation were conducted in serum-free suspension culture in roller bottles, as described in Youil et al. (*24*). An E1 deleted recombinant Ad5 vector expressing the HIV-1 *gag* gene was used for these experiments.

Virus Concentration Measurements. Virus particle concentrations were measured by anion exchange (AEX) HPLC essentially as described in ref 25, with minor modifications to the gradient. Particle measurements also were conducted by measuring the absorbance at 260 nm following denaturation with sodium dodecyl sulfate (SDS) and heat (hereafter referred to as UV/SDS) (*26*, *27*).

Assays of rAd5 Aggregation. The aggregation of rAd5 in purified samples was assayed by taking the A_{320}/A_{260} ratio, which normalizes light scatter to the nucleic acid content of the sample (*20*). Absorbances were measured against a buffer blank and were kept in the 0.1−1.0 range. Whenever feasible, multiple dilutions were tested. Samples also were analyzed by dynamic light scattering (DLS) using a Nicomp 380 DLS system (Particle Sizing Systems). Data fits were statistically assessed to choose between Gaussian and multimodal distributions.

Assay of Total Protein. Total protein was determined by the bicinchoninic acid (BCA) assay using a commercial kit (Pierce) with bovine serum albumin as a standard. Samples were analyzed in duplicate and absorbances were read with a microplate spectrophotometer.

SDS−PAGE. Purified rAd5 was denatured at 95 °C for 10 min in SDS sample buffer with 0.1 M dithiothreitol followed by fractionation on 10−16% polyacrylamide minigels (Novex). Gels were stained with colloidal Coomassie blue (Owl Scientific Pro-Blue Staining Kit) and imaged with a laser densitometer (Personal Densitometer SI, Molecular Dynamics).

Residual DNA Assay. DNA from various samples was purified using a Qiagen blood kit. Residual host cell DNA was assayed using Taqman quantitative polymerase chain reaction (QPCR) with an ABI 7700. Residual PER.C6 host cell DNA was amplified using primers and probes generated to a consensus human *alu* sequence and interpolated against a standard curve constructed with purified PER.C6 DNA. The limit-of-detection of the assay was determined to be 5 pg/10^{11} vp. Samples were analyzed at least in duplicate.

Adenovirus Purification. Cell-associated rAd5 was purified at laboratory scale using standard purification unit operations (Figure 1). After resuspension of the cell pellet in one-twentieth of the culture volume, rAd5 was released using three freeze/thaw cycles. Cell debris was removed by batch centrifugation (9250 × *g*, 10 min). The clarified lysate was incubated for 1 h at room temperature with Benzonase (EM Science) and, in most cases, a mixture of RNase T1 (Worthington) and RNase I (Epicentre) in order to digest nucleic acids. After holding overnight at 4 °C, the batch was concentrated approximately 3-fold and diafiltered into a buffer of 50 mM HEPES, 0.15 M NaCl, 2 mM $MgCl_2$, pH 7.5 using a 1000 kDa ultrafiltration membrane (Millipore Pellicon II). The concentration of NaCl of the retentate was adjusted to about 0.39 M and loaded at a linear velocity of 1.5 cm/min on to an AEX column sized for ca. 10^{12} vp/mL of resin (Source 15Q, Amersham Biosciences). The column was washed with five volumes of 50 mM HEPES, 0.39 M NaCl, 2 mM $MgCl_2$, pH 7.5 and eluted in a four column volume gradient to 0.47 M NaCl in the same buffer. In all cases, new resin was used for each run. The product peak was concentrated and diafiltered into formulation buffer using tangential flow ultrafiltration with a 300 kDa membrane (Millipore Pellicon II). Finally, the retentate was sterile-filtered using a 0.22 μm membrane filter (Millipore GV).

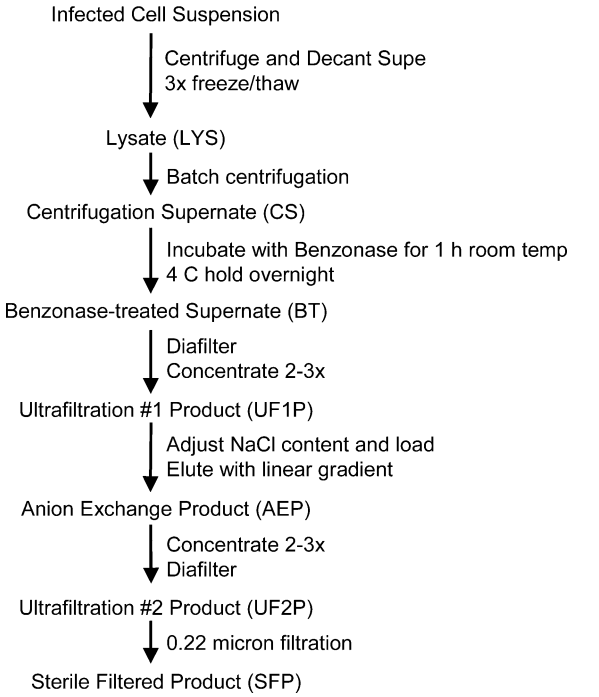

Figure 1. Prototype process for purification of Ad5. Details are described in Methods.

Additional Chromatographic Separations on the Purified Virus. Purified virus was refractionated using AEX or hydroxyapatite chromatography. For AEX chromatography using Source 15Q, 9.0 × 10^{11} vp were loaded onto a new 0.8 mL (0.5 cm × 4.1 cm) column, which then was washed with 6 column volumes of 50 mM HEPES, 0.39 M NaCl, 2 mM $MgCl_2$, pH 7.5 and eluted with a 10 column volume gradient to 0.47 M NaCl in the same buffer. Eluate was collected in 1 mL fractions and was analyzed for rAd5 using AEX HPLC and host cell DNA using QPCR, as described above.

For hydroxyapatite chromatography, a 0.9 mL (0.5 cm × 4.6 cm) column of Macroprep Ceramic Hydroxyapatite Type II (BioRad) was loaded with approximately 1 × 10^{12} vp of purified product. A 1 M NaCl background was maintained in both the wash and elution buffers to avoid irreversible binding, and elution was conducted with an optimized step gradient from 0 to 0.375 M phosphate at pH 7.0. As before, 1 mL fractions were collected across the elution and analyzed for rAd5 and host cell DNA.

Results and Discussion

Development of a Purification Process for rAd5. Adenovirus initially was purified as described in Methods. The purification process was designed with several objectives in mind: first and foremost, host cell DNA must be consistently reduced below 100 pg/10^{11} viral particles in order to facilitate clinical studies at or above doses of 10^{11} vp; second, the process should yield a product with a well-defined protein composition, leading to a protein purity target of 90%; and third, the process should be suitable for the purification of up to 10^{15} vp in a Good Manufacturing Practices (GMP) setting.

We reasoned that a single unit operation would never be sufficient to achieve the six logs of DNA clearance

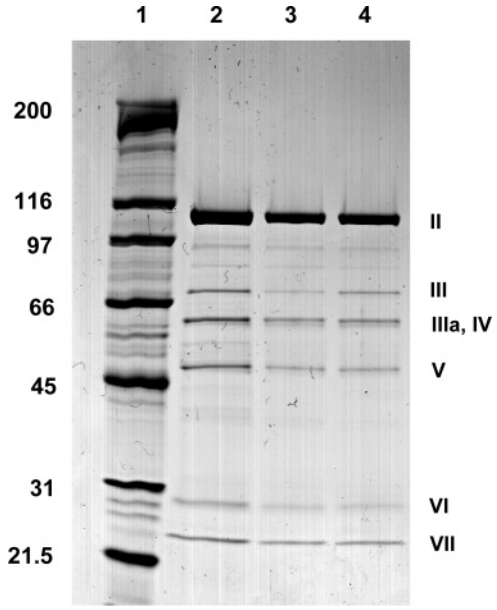

Figure 2. Protein purity of purified rAd5 products. SDS−PAGE gel was stained with colloidal Coomassie blue. Known viral proteins are identified by number. Lane 1: molecular weight markers; Lane 2: Lot E 1.7×10^{10} vp; Lane 3: Lot C 8.1×10^{9} vp; Lane 4: Lot F 9.3×10^{9} vp.

desired; instead, two unit operations were included, nuclease treatment and anion exchange (AEX) chromatography, which each were expected to result in substantial DNA reduction and which were likely to complement each other. Theoretically, digestion with nuclease should result in nucleic acid fragments small enough to permeate in a large pore (300−1000 kDa) ultrafiltration, and only a narrow size distribution of nucleic acid molecules should co-purify with adenovirus during preparative anion exchange. The ordering of nuclease treatment before AEX chromatography facilitated clearance of the nuclease and higher adenovirus loading on the resin since digested nucleic acid would appear in the nonbound fraction. In addition, the unique properties of rAd5 enable AEX chromatography to fractionate the virus from the majority of host cell proteins (*28*). The process is completed with a second ultrafiltration and diafiltration to concentrate the eluted rAd5 product and formulate the product in a buffer appropriate for administration in clinical trials.

The process described above was operated several times, with only minor modifications in cell culture or purification conditions and was found to yield a product that was >90% pure by SDS/PAGE as shown in Figure 2. Protein clearance data from a representative lot are shown in Table 1, with the specific protein content of the final product approximately consistent with the theoretical value for adenovirus structural proteins (26 $\mu g/10^{11}$ particles). DNA clearance profiles for four representative lots presented in Figure 3 reveal that host cell DNA was cleared effectively in the early process steps, with greater than 4 logs of clearance across the unit operations of centrifugation, nuclease treatment, and ultrafiltration. Surprisingly little clearance was observed across AEX chromatography and the final ultrafiltration, with residual host cell DNA consistently reaching a minimum of about 100 pg/10^{11} vp. Examination of a larger number of AEX runs (Table 2) suggested that reasonably good purification factors were achieved when the input was high but, as the input approached approximately 100 pg/10^{11} vp, little to no additional clearance was obtained.

Table 1. Typical Protein Clearance for Ad5 Purification; Total Protein Measured in Duplicate by BCA Assay, rAd5 by AEX-HPLC

intermediate	vol (mL)	Ad (10^{11} vp/mL)	protein (μg/mL)	specific protein ($\mu g/10^{11}$ vp)	purification factor[a]
LYS	105	3.0	2970	998	
CS	98	3.0	2560	860	1.2
UF1P	44	7.2	2280	316	2.6
AEP	67	2.3	64	27	12
SFP	21	6.8	105	15	1.8

[a] Purification factor for a step is defined as (specific protein input/specific protein output).

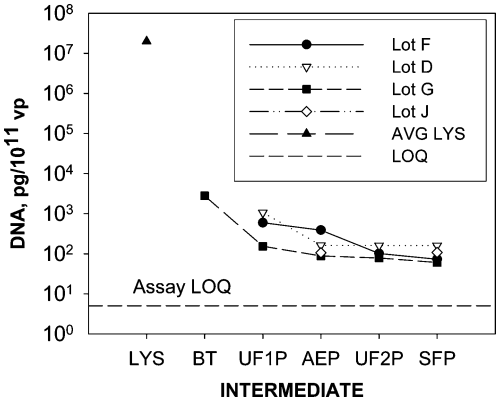

Figure 3. Profiles of the clearance of host cell DNA by the prototype process. For simplicity, the mean DNA value for lysates is shown (individual lots ranged from 0.1 to 3.6×10^{7} pg/10^{11} vp).

Table 2. Clearance of Host Cell DNA across AEX Chromatography

lot	DNA in feed (pg/10^{11} vp)	DNA in eluate (pg/10^{11} vp)	purification factor[a]	yield (%)
C	6540	635	10.3	68
D	1060	162	6.6	58
E	780	224	3.5	76
F	592	388	1.5	62
G	154	88	1.8	78
H	154	66	2.3	83
I	84	86	1.0	60
L	n/a	107	n/a	56

[a] Purification factor for a step is defined as (specific DNA input/specific DNA output).

Hypotheses for the clearance limitation included (1) a physical association of the host cell DNA with rAd5 virus capsids, (2) a promiscuous encapsidation of the host cell DNA within rAd5 capsids and (3) a simple co-purification of both the DNA and rAd5. Our experimental attempts to distinguish among these alternative hypotheses are reported in the sections below.

Effect of Serial AEX Chromatography on Residual DNA in rAd5 Product. Final product from the representative Lot L was run on a second Source 15Q chromatography column to understand the physical relationship between rAd5 virus and co-purifying DNA. AEX chromatography conditions, described in Methods, were more rigorous than the initial purification. Fractions were analyzed for rAd5 using AEX HPLC and host cell DNA using QPCR. The elution profile shown in Figure 4 reveals that 64% of the rAd5 and 33% of the DNA were recovered in the five center fractions, resulting in only a 50% reduction in the specific DNA content. The DNA concentration at the rAd5 peak center was substantially higher than in adjacent fractions and also was enriched in the peak tail. The bimodal DNA elution

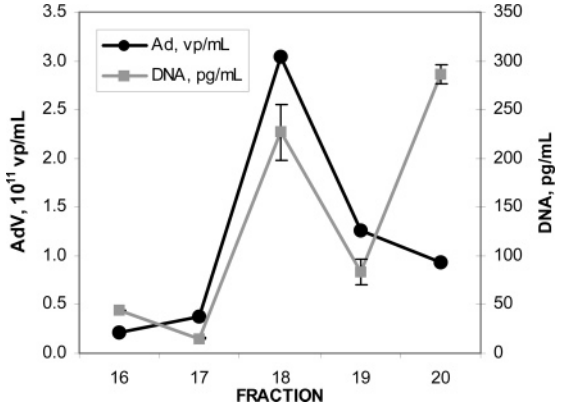

Figure 4. Second AEX chromatography on a purified product. Ad concentrations were measured by AEX HPLC and confirmed by A_{260} analysis. Residual DNA samples were measured in duplicate with mean and standard error reported.

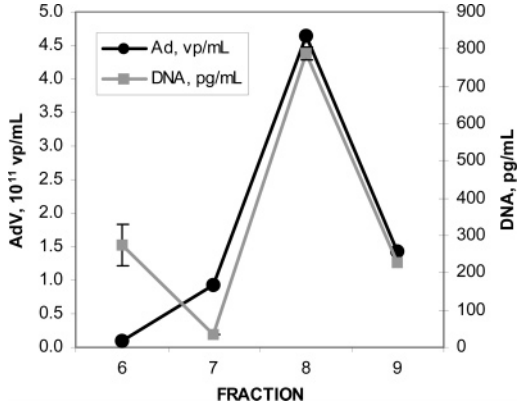

Figure 5. Hydroxyapatite chromatography on a purified product. Ad concentrations were measured by AEX HPLC and confirmed by A_{260} analysis. Residual DNA samples were measured in duplicate with mean and standard error reported.

profile was incompatible with a simple coelution hypothesis, as free nucleic acids elute in a broad peak across the gradient due to differences in DNA size following nuclease (Benzonase) digestion. These results are consistent with a fraction of the DNA being associated with rAd5 particles, with the remainder free in solution or associated with other molecular species.

Effect of Hydroxyapatite Chromatography for Orthogonal Purification on Residual DNA. A similar experiment was conducted using hydroxyapatite chromatography. Hydroxyapatite, a resin of crystalline calcium and phosphate, presents both positively and negatively charged binding sites for adsorption and consequently has differing selectivity for protein and DNA than AEX (*29*, *30*). Specifics of the chromatography are found in Methods. Again, eluted fractions were collected and analyzed for rAd5 and host cell DNA with the results shown in Figure 5. The recovery of rAd5 across the center three fractions was 71% with 45% of the host cell DNA recovered, for a 37% reduction in the specific DNA content. These results were similar to the results of the serial AEX chromatography, where DNA elution was again bimodal, with some DNA eluting before the rAd5 peak and some in a rAd5 concentration-dependent manner. These results were consistent with an association of a fraction of the host cell DNA with rAd5 particles.

Effect of Benzonase Digestion on the Residual DNA. A third experiment was used to confirm the association of residual host cell DNA with rAd5 particles. Specifically, Benzonase treatment, with and without particle disruption, was used to test if a fraction of the DNA was protected from nuclease activity. To that end, final product from Lot C was either (1) directly treated with Benzonase, (2) disrupted by heating to 56 °C in the presence of 0.1% SDS, or (3) disrupted by heating to 56 °C in the presence of 0.1% SDS followed by treatment with Benzonase. Benzonase-treated samples were dialyzed prior to residual DNA testing to avoid assay interference. The recovery of rAd5 was quantitative by AEX HPLC for the Benzonase-treated and dialyzed sample. The experiment was controlled by parallel analysis of these samples with a DNA spike to test for matrix interferences in the DNA assay; in all cases, spike recoveries were within the range expected for assay variability. Figure 6 shows that the process of disruption clearly facilitates elimination of DNA by nuclease treatment and dialysis; neither disruption alone nor nuclease treatment/dialysis is sufficient. These results are consistent with the notion that DNA is associated with rAd5

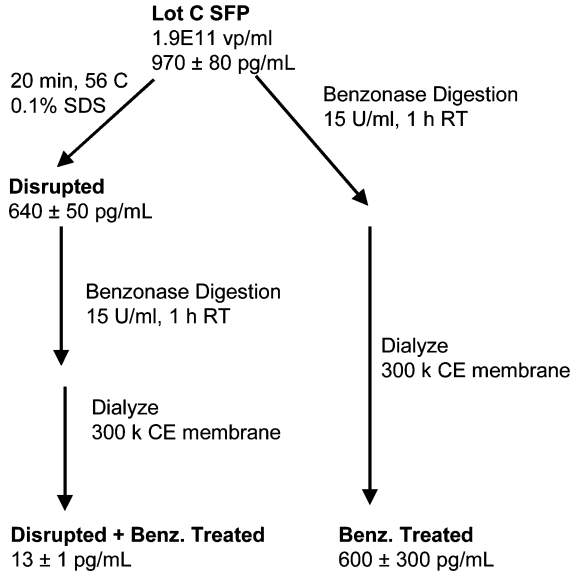

Figure 6. Effect of rAd5 disruption on residual DNA digestion by Benzonase. Residual DNA samples were analyzed in duplicate with mean and standard error reported.

particles and allowed the model to be refined to suggest that the DNA is protected from digestion by Benzonase (a dimer of 30 kDa subunits), either by association with rAd5 aggregates or perhaps as a result of the promiscuous encapsidation of host cell DNA within rAd5 particles. Given the tendency of Ad5 particles to form paracrystalline arrays in the nucleus (*19*), we explored the possibility that the DNA was entrapped within rAd5 aggregates.

Detection of Aggregated rAd5 in Purified Virus Product. In parallel with the experiments described above, the aggregation state of the purified rAd5 products was analyzed using the absorbance ratio, A_{320}/A_{260} (*20*). In addition, this assay was used in tandem with the denaturing UV/SDS assay to prove that the AEX HPLC assay does not detect aggregated rAd5. Conclusive proof that AEX HPLC is an effective tool for aggregation monitoring would then facilitate the study of more complex streams where direct physical methods cannot be applied. Specifically, by measuring three properties, A_{320}/A_{260}, AEX HPLC, and UV/SDS, for a large number of preparations, we examined the relationship in more detail. Since the UV/SDS assay measures the absorbance of rAd5 at 260 nm after denaturing in SDS solution, it is unbiased by the aggregation state of the sample and can be interpreted as a measure of total rAd5 (as long as the

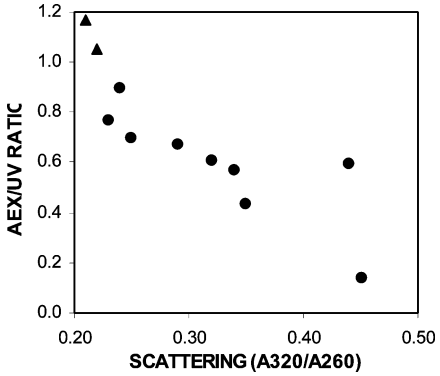

Figure 7. Relationship between AEX-HPLC, UV/SDS, and A_{320}/A_{260} assays. Triangles indicate products made following process changes that reduced aggregation.

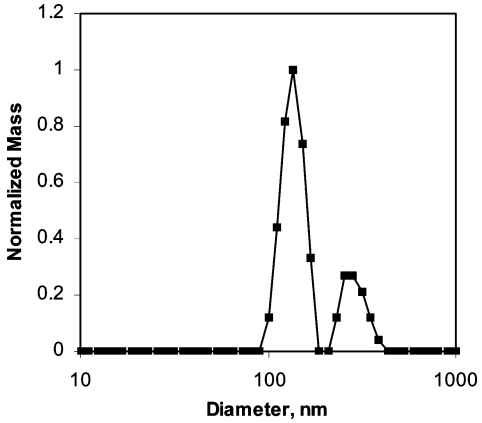

Figure 8. Dynamic light scattering analysis of lot B SFP.

mass of impurity nucleic acid is small relative to the mass of rAd5 genomes). Assuming that the AEX HPLC assay does not measure aggregated particles, the ratio of the concentration measured by AEX divided by the concentration measured by UV/SDS represents the fraction of rAd5 which remains unaggregated. A plot of this fraction against the A_{320}/A_{260} ratio, therefore, should result in an inverse relationship as demonstrated in Figure 7 for 11 different preparations with varying A_{320}/A_{260} ratios; the relationship is confirmed as significant by an F-test of a linear regression model (p=0.001), proving that AEX HPLC does not appreciably measure aggregated rAd5.

Several of the 11 lots of purified rAd5 analyzed above were explored in greater detail using dynamic light scattering (DLS). The unfiltered product of Lot B (UF2P, see Figure 1), which had a A_{320}/A_{260} ratio of 0.29, required a multimodal distribution to obtain a satisfactory fit. The resulting bimodal distribution had peaks centered around 120 and 500 nm, corresponding to monomeric and aggregated virus, respectively. When the distribution was transformed to correspond to the "volume" of particles (equivalent to mass for particles of equivalent density), 58% of the sample was monomeric, a result consistent with the value predicted from the AEX/UV ratio in Figure 7 (67%). A similar analysis for the sterile-filtered rAd5 product from the same Lot B, having a A_{320}/A_{260} ratio of 0.23, resulted in a bimodal distribution with peaks at about 130 and 260 nm (Figure 8). In this case, 77% of the sample was monomeric, which was identical to the AEX/UV ratio. This Lot B was of particular interest because the A_{320}/A_{260} ratio of 0.23, which is at the low end of the "unaggregated" range specified by Bondoc and Fitzpatrick (*20*), still contains substantial aggregated product. It is unclear if this conflict is the result of subtle analytical differences or if the methods described here (AEX and DLS) are more sensitive to low levels of aggregation than disc centrifugation.

These assays were applied to products before and after 0.22 μm absolute filtration. Table 3 shows the yield across filtration calculated by both the AEX HPLC assay and the UV/SDS assay, as well as the A_{320}/A_{260} ratio pre- and post-filtration. The significant decline in the A_{320}/A_{260} ratio demonstrates a reduction in aggregation across the filtration. As expected, yields by UV/SDS correlate with the A_{320}/A_{260} ratio of the filtration feed, with more aggregation causing a lower yield. Higher yields using the AEX assay are not unexpected insofar as the assay measures unaggregated virus which is less likely to be removed during filtration. Despite the removal of some aggregates, the DLS results described earlier, and the ratios for certain lots (e.g., Lot J) demonstrate that

Table 3. Reduction of rAd5 Aggregation by 0.22 μm Filtration

	scattering ratio (A_{320}/A_{260})		yield across filtration (%)	
lot	feed (UF2P)	filtrate (SFP)	AEX-HPLC	UV/SDS
A	0.31	0.23	89	60
B	0.29	0.23	86	75
J	0.45	0.34	77	19
K	0.44	0.25	57	49
M	0.22	0.21	97	87

smaller aggregates do pass through the 0.22 μm filter. The DLS results suggest that the mean hydrodynamic diameter of the aggregates post-filtration is slightly in excess of the filter pore size, suggesting that some aggregates may be somewhat cylindrical in shape, requiring them to orient along the streamlines to pass through the pores. Assuming that the aggregates are well-packed, the mass-weighted-mean aggregate contains roughly 15 particles.

In sum, the results of the UV/SDS, AEX-HPLC, A_{320}/A_{260} and DLS assays argue consistently for the presence of rAd5 aggregates in most of the purified products and suggest that yield losses measured by AEX HPLC may be the consequence of rAd5 aggregation. These data fail, however, to allow us to distinguish between an extracapsid association with rAd5 aggregates or encapsidation in rAd5 particles. Further efforts explored the proposed association of DNA with Ad5, and attempted to minimize aggregation in order to improve yield and potentially eliminate the co-purification of host cell DNA.

Association of DNA and Ad5 in Crude Samples. During the nuclease incubation step after clarification, substantial yield losses often were observed by AEX HPLC. Reasoning that these losses were caused by association of rAd5 in complexes with DNA, attempts were made to dissociate DNA and rAd5 using the nonionic detergent Polysorbate-80 (PS-80). Varying PS-80 concentrations to 1% (v/v) were used to attenuate what we postulated were interactions based on hydrophobicity. The addition of PS-80, however, did not mitigate the observed yield losses. Though electrostatic interactions seemed less likely (both rAd5 capsids and DNA have high overall negative charge), it was plausible that DNA could bind to local positive charge patches on the capsid; sodium chloride was added to test this possibility. Benzonase-treated supernatant (Figure 1) was incubated for 1 h with 1% PS-80 and NaCl concentrations ranging from 75 mM to 1 M, after which centrifugation (10,000 $\times$ g, 4 min) was used diagnostically to pellet large complexes. Using these centrifugation conditions, negligible monomeric virus in the tube would pellet (calculation using sedimentation coefficient from Nyberg-Hoffman (*31*)).

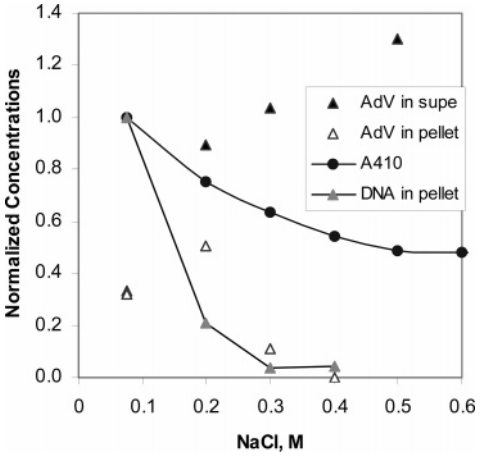

Figure 9. Centrifugation of BT intermediate after incubation with NaCl. Ad5 concentrations by AEX HPLC in supe and pellet are normalized to initial sample at 75 mM NaCl (before centrifugation). DNA concentration in pellet normalized to pellet of initial sample at 75 mM NaCl.

These titrations of NaCl yielded several important observations in Figure 9. First, the clarity of the solutions, as measured by the absorbance at 410 nm, improved substantially with increasing NaCl concentrations up to 0.5 M. Second, assay of virus in the pellet declined from about 40−50% to below the limit-of-quantitation over the same range of NaCl concentration. As expected, the virus in the supernatant increased 4-fold, from 33% to 130% of the control without centrifugation. This result indicates that only one-quarter (33%/130%) of the virus was not complexed in the initial sample, though three-quarters (100%/130%) was measurable by AEX HPLC. The DNA content in the pellet followed a similar trend, suggesting that the DNA and rAd5 were associated in complexes. The increase in rAd5 concentration following an incubation with 1 M NaCl and 1% PS-80 was reproduced in several lots, indicating that virus was released from complexes during salt/PS-80 treatment.

An additional purification was run including incubation of the Benzonase-treated clarified lysate (BT) in 1% PS-80 and 1 M NaCl as outlined. Following this incubation, ultrafiltration and anion exchange chromatography were completed. Despite this modification, the anion exchange product still was partially aggregated when analyzed using DLS, with a mean diameter of 162 nm and polydispersity of 0.53. As a result of this observation, it was apparent that additional measures were needed to eliminate aggregation.

Addition of Polysorbate-80 to Chromatography Buffers. In the prior run, PS-80 was present during all processing steps except for anion exchange chromatography. During chromatography, the PS-80 that remained in the ultrafiltration retentate was readily washed out (data not shown). Then, the rAd5 was eluted from the column, achieving a concentration greater than 10^{12} vp/mL at the peak center. Since rAd5 aggregation is concentration-dependent *(21)*, we reasoned that the aggregates detected in the prior experiment may have formed during elution. To test this hypothesis, 0.1% PS-80 was added to the chromatography buffers. Five subsequent purification runs were completed with this change (as well as including the NaCl incubation described above). There was no evidence of aggregation in the products of these lots. In addition, the yield across the anion exchange chromatography was markedly improved (mean ± standard deviation of 97 ± 9% with PS-80 compared to 68 ± 10% without PS-80).

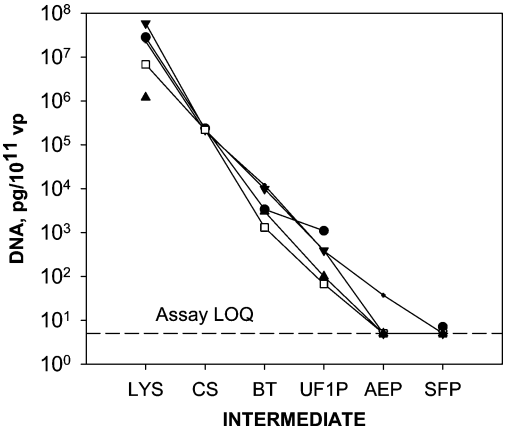

Figure 10. DNA Clearance for five successive purifications using the modified process.

Performance of the Modified Process. Residual host cell DNA concentrations were measured for the five lots incorporating the process changes described in the prior two sections, namely, incubating in 1 M NaCl and ensuring the presence of PS-80 throughout the process. The clearance data are presented in Figure 10, which reveals in all five rAd5 products a level of residual host cell DNA at or below the limit of quantitation (<5 pg/10^{11} vp) of the QPCR-based assay. The coincident reduction in aggregation and improvement in DNA clearance strongly supports the hypothesis that co-purification was due to association with rAd5 aggregates rather than encapsidation of host cell DNA into rAd5 particles.

Conclusion

Co-purification of host cell DNA was identified in a chromatography-based purification process for adenovirus. The inability of the process to clear residual host cell DNA below 100 pg/10^{11} particles was shown to be due to association between DNA and viral particles using three independent experimental approaches: anion exchange chromatography, hydroxyapatite chromatography, and nuclease treatment with and without particle disruption. In addition, aggregates were encountered in various degrees over a large number of process operations. Two process modifications were made to eliminate aggregation and DNA co-purification. First, Polysorbate-80 levels were maintained above a critical threshold throughout the purification. Second, NaCl was spiked into two process intermediates to reverse DNA/rAd5 complexes. These modifications allowed consistent clearance of PER.C6 cell DNA to below a detectable limit. This accomplishment will be an important component of future efforts to license recombinant adenovirus-based products.

Acknowledgment

The authors thank the following individuals/groups for their assistance with this work: Jedd Taylor, Andre Johnson, and Susan Dusenbery for assisting in the purification of these lots and in running of the UV assays; Bill Mathis and Valarie McGarty-Dugan for analyzing residual DNA samples; and the department of Fermentation and Cell Culture for providing infected cell cultures.

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Accepted for publication December 9, 2004.

BP049644R