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Characterization and quality control of recombinant adenovirus vectors for gene therapy

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Abstract

Highly purified recombinant adenovirus undergoes routine quality controls for identity, potency and purity prior to its use as a gene therapy vector. Quantitative characterization of infectivity is measurable by the expression of the DNA binding protein, an early adenoviral protein, in an immunofluorescence bioassay on permissive cells as a potency determinant. The specific particle count, a key quality indicator, is the total number of intact particles present compared to the number of infectious units. Electron microscopic analysis using negative staining gives a qualitative biophysical analysis of the particles eluted from anion-exchange HPLC. One purity assessment is accomplished via the documented presence and relative ratios of component adenoviral proteins as well as potential contaminants by reversed-phase HPLC of the intact virus followed by protein peak identification using MALDI-TOF mass spectrometry and subsequent data mining. Verification of the viral genome is performed and expression of the transgene is evaluated in in vitro systems for identity. Production lots are also evaluated for replication-competent adenovirus prior to human use. For adenovirus carrying the human IL-2 transgene, quantitative IL-2 expression is demonstrated by ELISA and cytokine potency by cytotoxic T lymphocyte assay following infection of permissive cells. Both quantitative and qualitative analyses show good batch to batch reproducibility under routine test conditions using validated methods.

Keywords: Gene therapy; Recombinant adenovirus

1. Introduction

The use and potential of recombinant adenovirus as a gene therapy vector has been widely reviewed among gene transfer technologies [28] specifically in

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the areas of cancer [39], including central nervous system tumors [8,29], as well as in cerebrovascular [38], cardiovascular [34], and genetic diseases [12]. The on-going gene therapy clinical trials using adenoviruses continue to promote extensive research of in vivo responses to recombinant adenovirus infection as well as to promote the development of large-scale adenovirus propagation methods and quality controls.

Adenoviruses are non-enveloped icosahedral structures of 60–90 nm in diameter, comprising 252 capsomers geometrically arranged with 12 fiber proteins protruding from the vertices and encapsidating approximately 36 kbp of DNA. Early information on adenovirus structure and assembly including the idea that the fibers are responsible for cell attachment during infection has been resumed [27]. Progress in understanding the generally complex adenovirus structure continues to be made, especially via X-ray crystallography [35] and electron microscopy [36].

Since adenovirus discovery in the early 1950s, there is a significant literature on their analyses and quantitation [39] which has been drawn upon for assay development for routine evaluation of the recombinant homologues. Today, there are guidelines which help to define quality controls for recombinant DNA products [1–4] and we have applied them to adenoviruses as gene therapy vectors. Here we describe some of the assay methods and their validation for both biological and physical parameters of recombinant adenovirus vectors for determining their quality prior to use in a clinical setting.

2. Experimental

2.1. Recombinant E1° E4° adenoviruses

The recombinant, E1- and E-4-deleted, E1°E4°, type 5 adenoviruses, ADTG5643 (containing the transgene cystic fibrosis transmembrane conductance regulator, CFTR) and ADTG6214 (containing the transgene human interleukin-2, huIL-2), were constructed with the transgene in the E1 deletion, produced as previously described using the 293-E4orf6+7 complementation cell line, 293TG5606 [25] and purified using CsCl density centrifugation according to classical methods [19].

2.2. Infectious units (iu) titration

Titers of infectious adenovirus were determined as infectious units (iu) by quantitative immunofluorescence of the DNA binding protein, DBP, an early adenoviral protein encoded by the vector [25]. Cultures of the 293-E4orf6+7 complementation cell line were grown to 70–90% confluence (24 h) on glass slides and were infected with serial dilutions of adenoviral suspensions of ADTG6214 or ADTG5643 Lot D (for an internal reference). The negative control was non-infected cells.

Cells were treated with acetone/methanol at 16–20 h post-infection, washed with PBS containing 1% fetal calf serum, and incubated with a solution of purified monoclonal antibody from the hybridoma α72K-B6-8 [31] directed against the DBP (a kind gift from Nancy Reich and Arnold Levine). Following the addition of a rabbit anti-mouse immunoglobulin (Dako, Trappes, France) for a 45 min incubation and washing, a subsequent goat anti-rabbit immunoglobulin labelled with fluoresceine isothiocyanate, FITC, (Sanofi Diagnostics Pasteur, Marnes La Coquette, France) was added and incubated 45 min in the dark. The cells were observed under a fluorescence microscope on a minimum of 20 fields and the fluorescent nuclei counted taking into consideration only those dilutions allowing the enumeration of 5 to 30 bright nuclei. The iu titer of a lot used for clinical or toxicological evaluation was the average of titers from three distinct tubes of the same production lot.

2.3. Adenovirus total particle (tp) count

Quantitation of total particles was performed by AIEX HPLC [32] with a 600S chromatography system equipped with a 717 plus autosampler and a 996 photodiode array (PDA) detector scanning from 210 to 300 nm (Waters, Saint Quentin en Yvelines, France). Millennium 32 software (Waters, Saint Quentin en Yvelines, France) was used to process the data.

Adenovirus samples were injected on a 1 ml Resource Q column (Pharmacia Biotech, Saclay, France) equilibrated in 300 m*M* NaCl, 50 m*M* Hepes, pH 7.5 (Buffer A) for 2 min at a flow-rate of 1 ml/ min. A linear saline gradient ranging from 300 m*M* to 600 m*M* NaCl in 50 m*M* Hepes, pH 7.5, was then applied over 10 min, followed by a 2 min wash at 600 m*M* NaCl. The column was then cleaned with 1.5 *M* NaCl, 50 m*M* Hepes, pH 7.5 (Buffer B) for 2.6 min and re-equilibrated in 300 m*M* NaCl, 50 m*M* Hepes, pH 7.5, for 9 min for the following injection.

Chromatograms at A_{260nm} were derived from PDA data and the peaks integrated. The typical retention time for adenovirus is approximately 11 ± 0.3 min depending on the specific column lot number and column age. A standard curve was calculated from the adenovirus peak areas at A_{260nm} of five ADTG5643 Lot C samples ranging from 6.0×10^9 to 10.2×10^{10} particles. The particle number for this lot was determined by the correlation for purified adenovirus as $A_{260nm}=1.1\times10^{12}$ particles [26]. Total particle concentration of adenovirus productions could thus be determined based on the standard surface areas.

Absolute adenovirus particle count values determined by A_{260nm} [26] correspond within approximately $\pm30\%$ of those determined using negative staining (uranyl acetate) electron microscopy performed using dilutions of adenovirus and known quantities of latex beads [14] (our own unpublished data). Purity of the lots was checked by analysis of the PDA data using triple-axis plots (time versus wavelength versus optical density) of injections made for tp analysis.

2.4. Negative staining electron microscopy

For negative staining of purified virus formvar carbon coated nickel grids (Structure Probe Inc., PA, USA) were used without submitting them to a glow discharge. Virus was deposited on the grid for 1 min and then stained for 20 s with uranyl acetate (2% in water). The observations were carried out using a CM120 Biotwin (Philips, Eindhoven, The Netherlands) electron microscope at 120 kV. Images were recorded on Kodak SO 163 films and developed using standard procedures.

2.5. Adenovirus genome characterization

2.5.1. DNA preparations

DNA was extracted from adenovirus-infected cells [22] following cell lysis by proteinase K. Chromosomal DNA was precipitated by 1 *M* NaCl treatment and viral DNA was recovered following phenol/ dichloromethane extractions and ethanol precipitation.

DNA was extracted from purified viral particles (about 5×10^9 pfu) after proteinase K lysis, phenol/ dichloromethane extractions and ethanol precipitation. DNA is quantitated spectrophotometrically by measuring the A_{260nm} and using the relationship of 1.0 $A_{260nm}=50$ μg/ml.

2.5.2. Restriction patterns

Approximately 10 μg of adenoviral DNA was digested by four to six different restriction enzymes. For ADTG6214 the enzymes used were *Bsu*36I, *Hind*III, *Not*I, *Nru*I, and *Sph*I (Biolabs, St. Quentin, France). The DNA fragment mixtures (containing approximately 1 μg DNA) were separated by electrophoresis on a 1% agarose gel in the presence of ethidium bromide (1 μg/ml) in the gel and in the buffer and visualized by an ultraviolet lamp (312 nm). Gel images were analyzed using a Geldoc 1000 camera and GS-700 imaging densitometer (BioRad, Ivry sur Seine, France). Restriction patterns (fragment number and sizes) were compared with the theoretically predicted patterns.

2.5.3. Southern blot

Approximately 100 ng of digested DNA was put on a 1% agarose gel with ethidium bromide (1 μg/ml). Following electrophoresis, in situ denatured DNA was transferred and fixed via UV (312 nm) to a nylon membrane (Hybond N, Amersham, Saclay, France) which is hybridized (overnight, 68°C) either with a cDNA probe for the expression cassette (e.g. CFTR, huIL-2, etc.) labelled with digoxigenin (Roche Diagnostics, Meylan, France) or a digoxigenin-labelled adenovirus (ADTG9546, without transgene) DNA probe. Fragments are detected by chemiluminescence using CDP star, the substrate activated by the alkaline phosphatase coupled to a digoxigenin antibody (Roche Diagnostics, Meylan, France).

Prior to hybridization the filter was blocked from nonspecific adsorption by preincubation (1 h, 68°C) in the hybridization buffer. Following hybridization with the labelled probes, the filter was washed in saline solution containing 0.1% SDS first at room temperature (2×5 min) and then at 68°C (2×15 min) to eliminate excess free probe thus minimizing background. Autoradiographs (Hyper Film ECL, Amersham, Saclay, France) were exposed for 1 min.

2.5.4. Sequencing of the expression cassette and adenoviral deletions

The expression cassette (2629 base pairs) containing the RSV promoter, the rabbit betaglobin intron, the huIL-2 gene, and the rabbit betaglobin polyA as well as the flanking regions around the E3 and E4 adenoviral deletions (300 bp) were sequenced following PCR amplification of the genomic DNA extracted from the viral stock (Y9708) of ADTG6214 (Lark Technologies, Inc., Houston, TX, USA).

2.6. Replication competent adenovirus (RCA)

A bioassay based on a cell culture/cytopathic effect (CPE) method [21] was performed to detect the presence of replication competent adenovirus (RCA) in preparations of ADTG6214 Lot D (BioReliance, Rockville, MD, USA). For the primo-infection, appropriate numbers of roller bottles (850 cm^2) containing HeLa S3 (human cervical carcinoma, ATCC CCL 2.2) cells, permissive for RCA but not for E1° and/or E4° were inoculated with 1×10^9 iu or 1×10^{10} iu of ADTG6214 to achieve a multiplicity of infection (moi) of ≤10 iu/cell which avoids interference for the amplification of RCA. Wild type human adenovirus type 5 (huAd5) was used as a positive control at the limit of detection of the assay, one plaque-forming unit (pfu). Wild type huAd5 was also spiked into each test sample at one pfu to monitor vector interference with RCA growth.

Seven to nine days later, cells from each bottle were recovered, frozen and thawed twice and one-half of the volume of the resulting lysate was clarified by centrifugation and used to inoculate A549 indicator cells (human lung carcinoma, ATCC CCL185) for a second passage of 13–15 days. Subsequently, a third passage was performed and observations are continued for 6 to 8 days. Direct microscopic examination was used to screen for a CPE on monolayers of A549 cells during the final two incubation periods, indicative of the presence of RCA.

2.7. Preparation of culture supernatants for huIL-2 expression assays

Monolayers of 293 cells (human embryo kidney, ATCC CRL 1573) were obtained in GMEM/NT medium. The cells were counted on the day of adenoviral infection and infected with the virus to be analyzed at an moi of 10. In parallel, cells were infected at the same moi by ADTG9546 (containing an empty expression cassette) as a negative control virus. After 24 h of infection, the culture supernatants were harvested and used immediately or stored at −80°C for quantitative expression and functionality tests of huIL-2.

2.8. Expression of huIL-2 by ELISA

The expression of huIL-2 protein was measured using a sandwich ELISA (ELISA QUANTIKINE™, R & D Systems, Abingdon, UK) consisting of a monoclonal capture antibody specific for huIL-2 and an HRP-linked polyclonal anti-huIL-2 detection antibody. For this assay the enzyme-specific substrate tetramethylbenzidine, TMB, is transformed into a colored product of which the intensity is directly proportional to the quantity of IL-2 present in the samples tested. The kit was used both for the standard curve and unknown samples as instructed by the manufacturer. Two independent 2-fold dilution series of the culture supernatants and the standard huIL-2 solutions in the kit are made prior to each assay. The results were read at A_{450nm} using a Vmax or Thermomax microplate reader equipped with Softmax software (Molecular Devices, Biogenics, Maurin, France) and were analyzed using a 4-parameter logistic curve-fit as described in the kit instructions.

2.9. Cytotoxic T lymphocyte lysate (CTLL) assay

The biological activity of huIL-2 was measured by a proliferation test on an IL-2 dependent cell line,

CTLL-2 cells (mouse T cell, ATCC 214 T1B) [15] using recombinant human IL-2 (rhuIL-2) from *E. coli*, 0.1 μg/ml (Roche Diagnostics, Meylan, France) as the standard. This standard was calibrated against the international NIBSC standard (human Jurkat-derived, 86/504). A conversion factor, *x*, was thus generated which then allowed the values for the activity units, U, for the internal standard to be expressed in International Units, IU (not to be confused with iu, see above).

Three series of independent 1:2 dilutions of each culture supernatant were analyzed in RPMI medium (Life Technologies, SARL, Cergy Pontoise, France) with 10% fetal calf serum. Increasing amounts of rhuIL-2 or the culture supernatant samples to be tested were added to CTLL-2 culture medium deprived of IL-2. Following incubation, the cell proliferation was evaluated by a colorimetric assay, CellTiter 96™ AQ_{ueous} (Promega, Charbonnières, France), read at A_{490nm}, and the huIL-2 concentration of each sample was calculated using the 4-parameter logistic curve-fit. The color basis of the test is the conversion of tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(-sulfophenyl)-2H-tetrazolium, inner salt; MTS, Owen's reagent) into formazan by a dehydrogenase present in viable cells via an electron coupling reagent (phenazine methosulfate; PMS) present in the assay mixture. The quantity of formazan product is directly proportional to the number of viable cells and is quantitatively related to the amount of functional IL-2.

2.10. Reverse phase HPLC, (rpHPLC)

Adenoviral proteins were separated by rpHPLC (HP1090, Hewlett-Packard, Palo Alto, CA, USA) on a Jupiter C4 column packed with 5 μm, 300 Å particles (250×4 mm, Phenomenex, Le Mesnil le Roi, France) at 45°C and 1 ml/min using a 145 min gradient of aqueous acetonitrile (Carlo Erba, Val de Reuil, France) containing 0.1% TFA in a modification of a previously described method [23]. The solvents were as previously described (Solution A= 0.1% TFA; Solution B=0.1% TFA in acetonitrile); however, the gradient was 20–34% B from 0 to 37 min, 34–46% B from 37 to 85 min, 46–50% B from 85 to 114 min, 50–55% B from 114 to 134 min, and 55–80% B from 140 to 145 min. UV absorption was monitored at 215 nm. Peaks were collected manually and the solvent was evaporated by centrifugation under vacuum. The material contained in these peaks was either subjected to N-terminal sequencing or analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry after digestion by trypsin.

2.11. N-terminal peptide sequencing

Automated N-terminal peptide sequencing via Edman degradation was performed on a model 477A protein sequencer coupled to a model 120A on-line phenylthiohydantoin amino acid HPLC analyzer (PE Biosystems, Courtaboeuf, France).

2.12. MALDI-TOF mass spectrometry analysis

Protein identification by mass spectrometry and database searching was performed essentially as described previously [33]. Briefly, trypsin (sequencing-grade, modified, Promega, Charbonnières, France) digestion of dried protein samples was performed with sequence-grade, modified porcine trypsin in 25 m*M* ammonium bicarbonate, pH 8.2, overnight at 28°C. The mixture (10 μl) was then acidified with 5 μl of 2% TFA.

For MALDI-TOF mass spectrometry a saturated solution of the matrix, 4-hydroxy-α-cyanocinnamic acid, (Sigma–Aldrich, St. Quentin Fallavier, France) in acetone (0.5 μl) was deposited onto the sample probe followed by 0.5 μl of 2% TFA. Subsequently, the tryptic digest (0.5 μl) was mixed with the TFA solution on the matrix surface, whereupon 0.5 μl of a saturated solution of the matrix in acetonitrile/water (50:50) was added and allowed to dry at room temperature. Spectra were obtained on a Biflex time-of-flight instrument (Bruker, Bremen, Germany) operated in the reflector mode. Spectra were internally calibrated using known trypsin autodigestion peptides. Monoisotopic peptide masses obtained were used for database searching with the program MS-Fit, accessible at http://prospector.ucsf.edu/, interrogating the NCBI non-redundant database.

2.13. Cesium analysis

Quantitative analysis for the presence of cesium in adenoviral samples was performed by energy dispersive X-ray fluorescence spectrometry [30] with cadmium as the internal standard. The standardization is performed with a known concentration of cesium mixed with a known concentration of cadmium at 500 ppm. The coefficient used for calculation is 1/slope of the standard curve. Samples for analyses are mixed volume/volume with the internal standard. Each sample was analyzed in triplicate at 50 keV and 6 mA during 2000 s per deposit on a polypropylene (or mylar) film making certain that the dried samples avoid dust contamination (ECPM, Laboratoire de Chimie Analytique et Minérale du Professeur Leroy, Strasbourg, France).

3. Results and discussion

Recombinant adenoviruses destined for clinical use undergo quality controls at five different stages in their preparation, including the complementation cell banks, virus seed, the crude harvest, purified bulk, and the final product in accordance with the regulatory authorities implicated (Fig. 1). The qualitative and quantitative analyses described in this text focus primarily on recombinant adenovirus vector

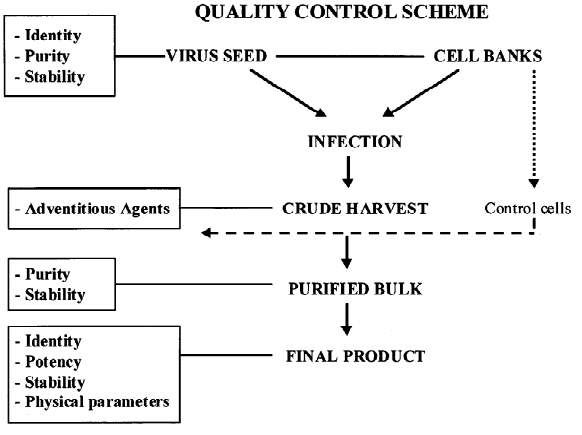

Fig. 1. Quality control scheme. The principle steps of adenovirus production are outlined between the arrows. Quality controls for various aspects of the active principle are in boxes.

identity, purity, and potency [11] and are only a fraction of the complete battery of quality controls detailed in the final dossier submitted for clinical trial approval of a given recombinant adenovirus. Genetic stability of the adenovirus construct is assured prior to manufacturing of the virus seed. Extensive analyses for adventitious agents including assays to detect bacteria, fungi, and mycoplasma [7] and adventitious agents [3,4,7] are performed in both the cell banks and crude adenovirus harvests. Thermal stability studies on purified virus are undertaken as early as possible in the development process and are followed throughout the life of the product through manufacture, clinical evaluation and eventually market. And while detailed discussions of the various analyses required for virus stability and adventitious agents are beyond the scope of this article, these analyses are nonetheless critical to the control of adenovirus active principles.

However, in summary, recombinant adenovirus products are shown to be stable genetically over the prerequisite number of generations (including a safety margin) previewed for large-scale manufacturing and their thermal stability is assured over the physical parameters of the final product environment (including storage time and temperature in a defined formulation buffer). They must pass the test for bacterial and fungal sterility, be mycoplasma-free and indemn of adventitious viruses prior to human use.

The quantitative validation data of some of the specific identity, potency and purity controls is presented as well as its application to four lots of the E1°E4° recombinant adenovirus, ADTG6214 (Fig. 2a and Table 1).

3.1. Identity

At the virus seed and final product levels, the active principle identity consists of the adenovirus genome characterization as well as the demonstrated transgene expression.

3.1.1. Genome

Adenoviral genome analysis via five restriction enzyme digests reveals 36 of the 40 DNA fragments theoretically present (Fig. 2a and Table 2) via an ethidium bromide-stained agarose gel (Fig. 3a).

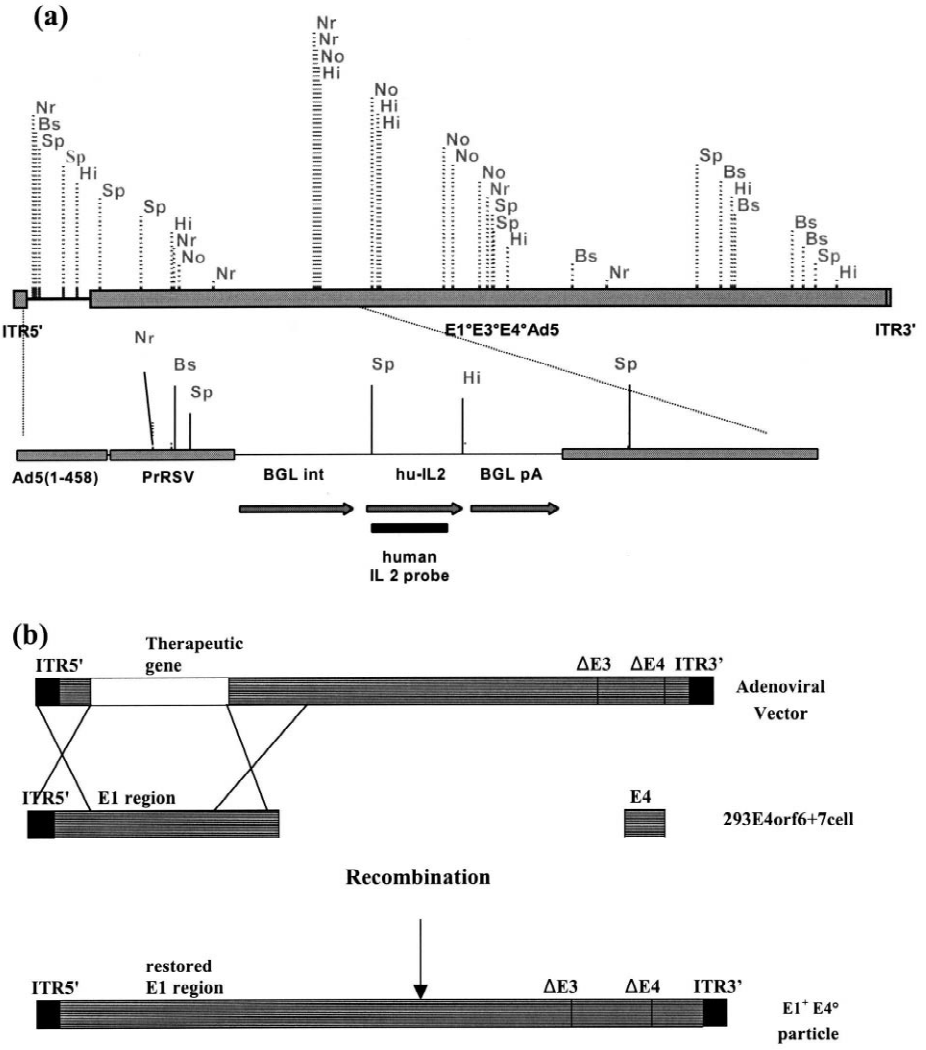

Fig. 2. (a) Structure of E1-E3-E4-deleted adenovirus ADTG6214 expressing the huIL-2 gene. The therapeutic gene (huIL-2) inserted in the deleted adenovirus E1-coding region is transcriptionally regulated by the RSV promoter (PrRSV) and the rabbit beta-globin polyA (BGLpA) sequences. The adenovirus E4-coding region as well as the E1 and E3 regions have been deleted in this vector. The restriction enzymes: *Nru*I=Nr, *Bsu*36I=Bs, *Sph*I=Sp, *Hind*III=Hi, *Not*I=No have been used to cleave the adenovirus genome into 7–8 easily recognizable overlapping fragments (see Table 2) per enzyme for Southern analysis (see Fig. 3). (b) Homologous recombination. Schematic diagram representing hypothetical recombination between the adenovirus vector and the viral DNA present in the complementation cell line 293-E4orf6+7, generating a recombinant, $E1^{+}E4^{\circ}$ virus with a functional E1 region.

Three of the four bands not visible with this technique are very small, ≤81 bp, and thus either elute off the gel or like the 326 bp band are at the limit of detection in DNA amount. Thus, each of these five restriction enzymes provide a routine control of ≥99.9% of the adenoviral genome (in ADTG6214 consisting of 31 475 bp) and their concerted use provides the assurance of complete genome coverage.

Southern analysis using an adenoviral probe carrying no transgene (not shown) reveals a banding pattern similar to that of the agarose gel for the

Table 1
Quantitative characteristics of ADTG6214 adenoviral vectors

Adeno huIL-2 ADTG6214 LOTS	tp/ml	iu/ml	Specific particles tp/iu	ng IL-2/ml/24 h	IU IL-2/ml/24 h	Specific activity IU/ng IL-2
B	1.9E+12	4.0E+10	47.3	54	2.5E+03	46.7
C	1.3E+12	1.6E+10	82.5	231	4.9E+03	21.1
D	2.0E+12	5.8E+10	34.1	98	2.7E+03	27.4
E	9.0E+11	2.4E+10	37.5	62	2.4E+03	38.6
Average			50.3			33.4
SD			22.1			11.4
RSD, %			44.0			34.2

majority of the bands with difficulties in revelation for the lower molecular mass fragments (Table 2). However, the *Sph*I fragment of 868 bp is part of the expression block comprising the 3′-segment of the RSV promotor, the beta globin intron and 15 bp of the huIL-2 gene, and thus was unable to hybridize to the adenovirus backbone probe due to a complete lack of homology. The presence of the huIL-2 gene via Southern analysis (Fig. 3b) is shown in each digest as a single fragment (see Table 2). The 868 bp *Sph*I fragment does not hybridize with huIL-2 probe as the 15 bp are insufficient homology.

The expression cassette containing the huIL-2 gene was completely nucleotide sequenced and was found to differ from that published by Tanigushi [37] in positions 35 (G→T) and 387 (G→A). Nonetheless, the ultimately translated huIL-2 amino acid sequence expressed corresponds exactly to the one described in GENBANK (X01586) as these two nucleotide differences are silent.

Table 2
Theoretical sizes of ADTG6214 restriction fragments

*Bsu*36I	*Hin*dIII	*Not*I	*Nru*I	*Sph*I
19 277[a,b]	8010[a]	14752[a]	10187[a]	12635[a]
5298[a]	5321[a]	5916[a,o]	6163[a]	7277[a]
3165[a]	4597[a]	5001[a]	5043[a,b]	4248[a]
2065[a]	3787[a]	2589[a]	4283[a]	2716[a]
778[a]	3398[a]	1931[a]	3613[a]	1476[a]
502[a]	2256[a,b]	960[a]	1419[a]	1300[a,b]
390[a]	2081[a]	326[a]	686[a]	910[a]
	1947[a]		81	868
	75			45

[a] Fragment detected by adenovirus (without transgene)-labelled probe.
[b] Fragment detected by the huIL-2-labelled probe.

3.1.2. Transgene expression

The quantitative expression of the huIL-2 transgene carried by ADTG6214 was measured by a commercially supplied ELISA specific for huIL-2 with an assay range of 0.015 to 2 ng/ml (correlation coefficient of >0.995). Comparison of IL-2 ELISA kits from three suppliers was undertaken prior to test validation as there can be significant differences among the kit's ease of use and responses [9]. For the chosen kit, the inter- and intra-kit precision data revealed an RSD<5% for 20 analyses of three samples of known concentration. However, the results of 13 completely independent (including viral infection) assays for reproducibility yielded a higher RSD of 20% with the results expressed as ng/ml/24 h, indicative of the variation due to biological systems.

3.2. Potency

Potency of the active principle is measured by the functionality of the transgene expressed, i.e. its specific activity, as well as the quantitative analyses of the number of viral particles, tp, and their infectivity, iu.

3.2.1. Virus

Even in highly purified adenovirus suspensions at least two subsets of viral particles are present and are generally distinguishable by two distinct assays for tp and iu. The total number of particles, tp, is the

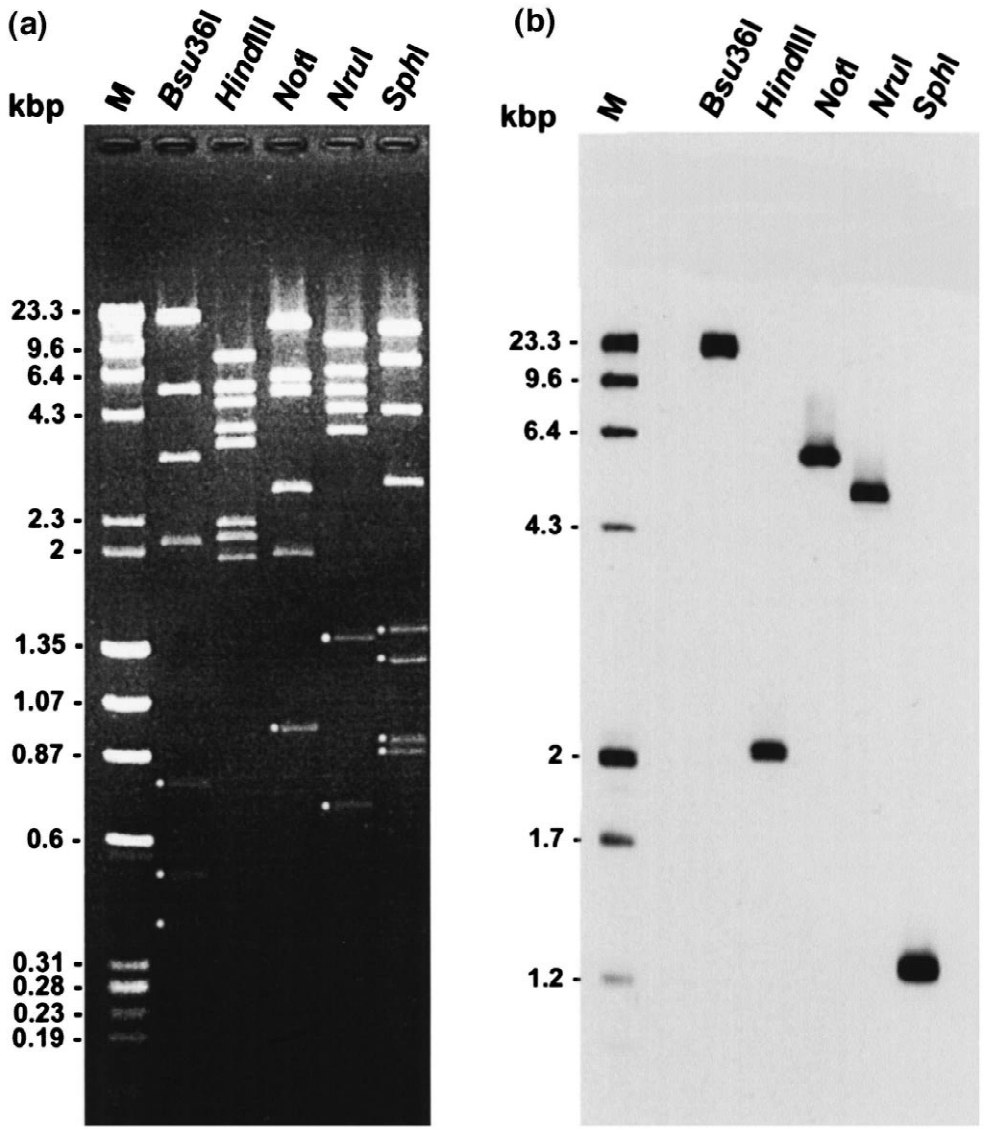

Fig. 3. Recombinant adenovirus (ADTG6214) genomic analysis (a)Electrophoretic analysis of DNA restriction fragments from five different enzyme digests (approximately 500 ng per lane) from Lot D on an ethidium bromide-stained agarose gel shows 36 of 40 theoretical DNA fragments (see Table 2). White dots, °, denote fragments smaller than 1500 bp. Molecular weight markers (M) are a mixture of λ phage cleaved with *Hind*III and ϕX 174 phage cleaved with *Hae*III. (b) Southern blot analysis of DNA restriction fragments (100 ng) of Lot D electrophoresed and blotted onto a Hybond N nylon membrane shows hybridization with the digoxenin-labelled probe for huIL-2. Theoretical restriction fragments are found in Table 2.

largest population and today, the Center for Biologics Evaluation and Research, CBER, recommends that patient dosing be based on particle number [10]. The tp quantitation by anion-exchange HPLC was evaluated for its accuracy, specificity, linearity, precision and range in validation studies [5,6]. The values determined by the HPLC method [32] proved to be within 90% of those obtained using the standard A_{260nm} method of Maizel [26] which gives a direct correlation between A_{260nm} after SDS

lysis and the particle number. The A_{260nm} peak at 11 min possesses both the same characteristic 260 nm/280 nm absorbance ratio and UV scan (210–300 nm) of adenovirus in solution (Fig. 4a–b) and, in addition, electron microscopy revealed intact adenoviral particles (Fig. 5). There was no interference of the assay observed upon sample dilution and formulation buffer. The relationship of the A_{260nm} surface area to

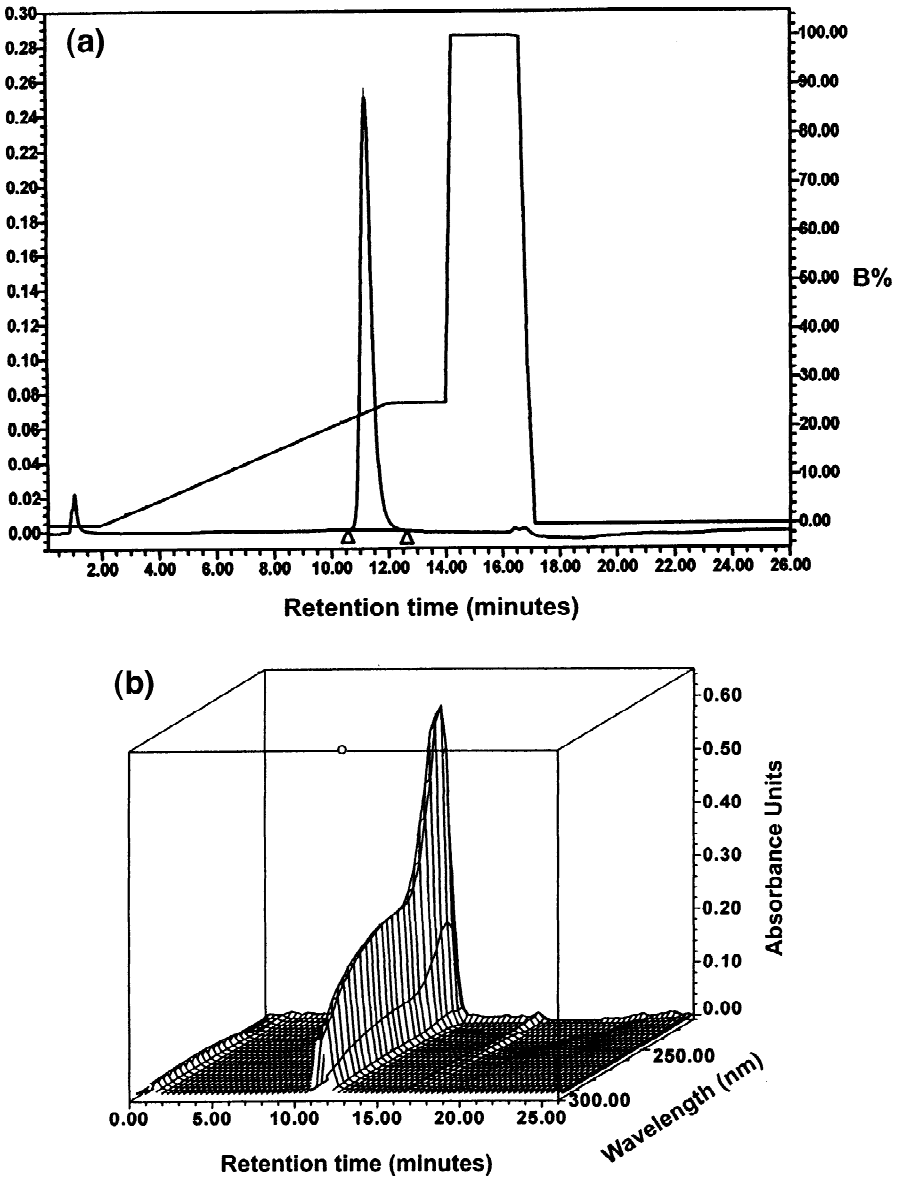

Fig. 4. Total adenovirus particle (tp) analyses. Anion-exchange HPLC (Resource Q column) chromatogram monitored at A_{260nm} of purified adenovirus ADTG6214 Lot B for total particle, tp, analysis, 5.9×10^{10} tp injected (a) and its 3-dimensional (time; wavelength, 8; and absorbance units, AU) chromatogram (b) demonstrating the product quality, spectrophotometrically. The A_{260nm} peak at approximately 11 min is integrated and the tp value determined by comparison to the standard curve as described in the Experimental section. Repeatability and intermediate precision studies of the tp analysis (c) show the technique to be highly reliable at the values analyzed.

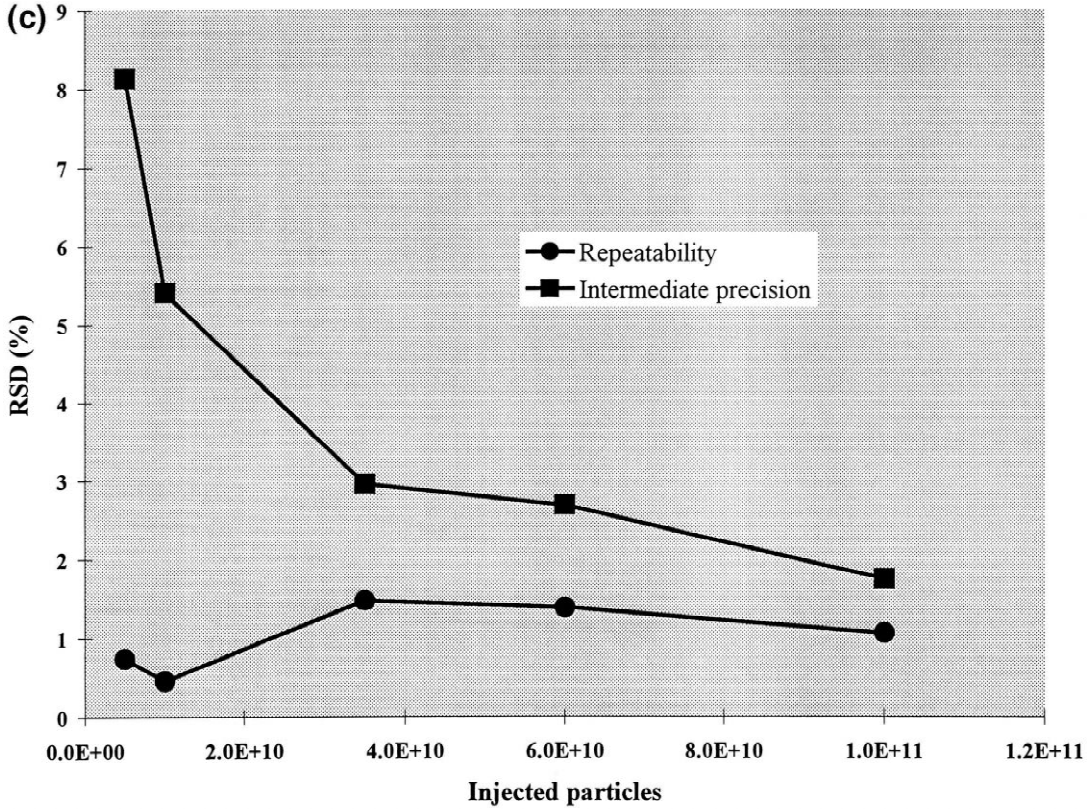

Fig. 4. (*continued*)

the number of particles was linear between 0.5×10^{9} and 1.0×10^{11} injected particles with a correlation coefficient of >0.99.

For repeatability studies of the tp assay on the same day by the same operator, with no changes of buffers and column, the RSD of the AIEX HPLC analysis was $<2\%$ (Fig. 3c). For intermediate precision studies, three different operators performed five independent assays using three different lots of eluting buffers and three different columns. The intermediate precision was found to be $<5\%$ RSD for 2.0×10^{10} to 1.0×10^{11} injected particles and between 8.5 and 5% RSD for 5.0×10^{9} to 1.4×10^{10} injected particles (Fig. 4c). The routine injection was thus designated to be approximately 2.0×10^{10} particles.

The second subset of adenovirus populations is the iu. The specificity of the immunofluorescent iu titration (Fig. 6b–d) was demonstrated by the absence of fluorescent nuclei in the negative control cultures (Fig. 6a). The background of this assay was largely improved over the initial description [25] by the signal amplification achieved using the second and third antibodies as well as by blocking aspecific binding using fetal calf serum. Furthermore, the assay was validated for repeatability and reproducibility involving 5 different operators performing 26 titrations on 18 separate days (data not shown). Four conditions of cell culture and 3 to 6 lots of three key reagents were varied in the course of these studies. The RSD for the repeatability of this assay proved to be $<5\%$. However, for the reproducibility, the RSD was 29.4% indicative of a biological assay where the state of the cells to be infected remains a parameter generating a relatively high variability. The routine use of an internal reference standard assures that the assay is performing normally, and now with 2 years of experience the RSD=32% for 50 assays of

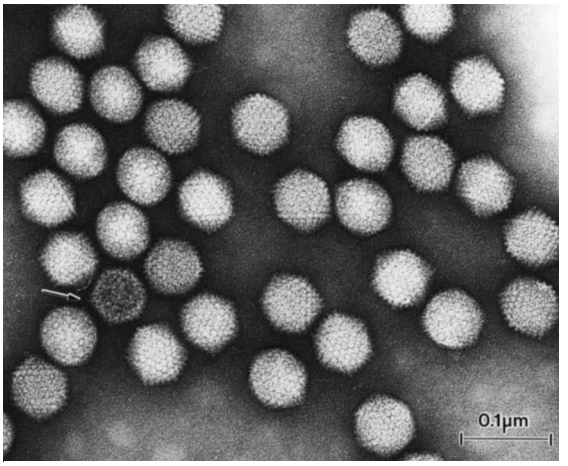

Fig. 5. Negative stained electron micrograph of adenovirus (ADTG6214, Lot D) particles eluted from anion-exchange HPLC analyses. The majority of the particles are intact and display their characteristic icosahedral structure. Hexon subunits are clearly visible. The arrow designates a damaged particle where the uranyl acetate stain has penetrated the particle.

clinical-grade material. While the iu titer of production lots remains a critical parameter for characterizing the function of recombinant adenovirus, it is rather its use in a ratio in conjunction with the total number of viral particles in the specific particle measurement which is a key quality indicator. Table 1 shows this value to be 50.3 on the average for the four lots of ADTG6214 with each lot conforming to the FDA recommendation of having less than or equal to 100 tp/iu [10].

3.2.2. Transgene functionality

That the huIL-2 expressed was biologically functional was demonstrated using a proliferation test on an IL-2 dependent cell line. The standard curve from 2.9 to 50 U IL-2/ml showed a correlation coefficient of >0.98. The specificity was shown by infection with an adenovirus carrying no passenger gene as the negative control. Reproducibility studies of this analysis involved the preparation of 14 independent cultures (12 different CTLL passages) by three different operators in two laboratories over 11 days. At moi=10, an RSD of 33% was obtained for IL-2 functionalities expressed as IU/ml/24 h.

The huIL-2 specific activity, defined as the ratio between the IL-2 functionality (CTLL) and IL-2 expression (ELISA), for 12 supernatants of ADTG6214 Lot A (infections at moi = 10) was 34.7 IU/ng huIL-2 with an RSD=18%. The average value of Lots B–E (Table 1) was 33.4 IU/ng huIL-2 which agrees very well with the validated value. The RSD=33% for the specific activity of four independent lots of the same virus shows them to be similar.

3.3. Purity

A critical analysis for recombinant adenovirus purity and safety is RCA evaluation measuring the presence of either wild type virus or viruses which have regained the capability of replication resulting from recombination events. In addition, rpHPLC analysis gives a good evaluation of the relative amounts of component adenoviral proteins as well as an identification/quantitation of any residual protein impurities making this a useful measure of lot-to-lot reproducibility. Residual cesium analysis from the CsCl gradient ultracentrifugation is also monitored in the final product to assure minimal carryover.

3.3.1. RCA

The AdE1°E4° are doubly deleted, each deletion being sufficient to prevent virus replication, and thus for propagation they require a complementation cell line containing critical segments of both deleted segments. As for the parental 293 cell line, the complementation cell line, 293E4orf6+7, can undergo a recombination event involving the region between the ITR and the adenoviral encapsidation site, at one point, and by the region coding for the polypeptide IX on the opposite side of the expression cassette generating a particle with a restored E1 region [24], $E1^{+}E4°$, schematically represented in Fig. 2b. However, at the E4 region there is only a single homology (limited to 194 bp) between the segment, E4orf6,6/7, contained both in the cell line and in the adenoviral genome, greatly minimizing the probability of any potential recombination event at this position. As each of these two regions, E1 and E4orf6,6/7, has been independently incorporated into the cell line [17,18,25], it is likely that they are not incorporated into the same locus. Thus, the likelihood of a particle being derived from the recombination at the E1 region as well as at the E4 region, is extremely small and the probability of

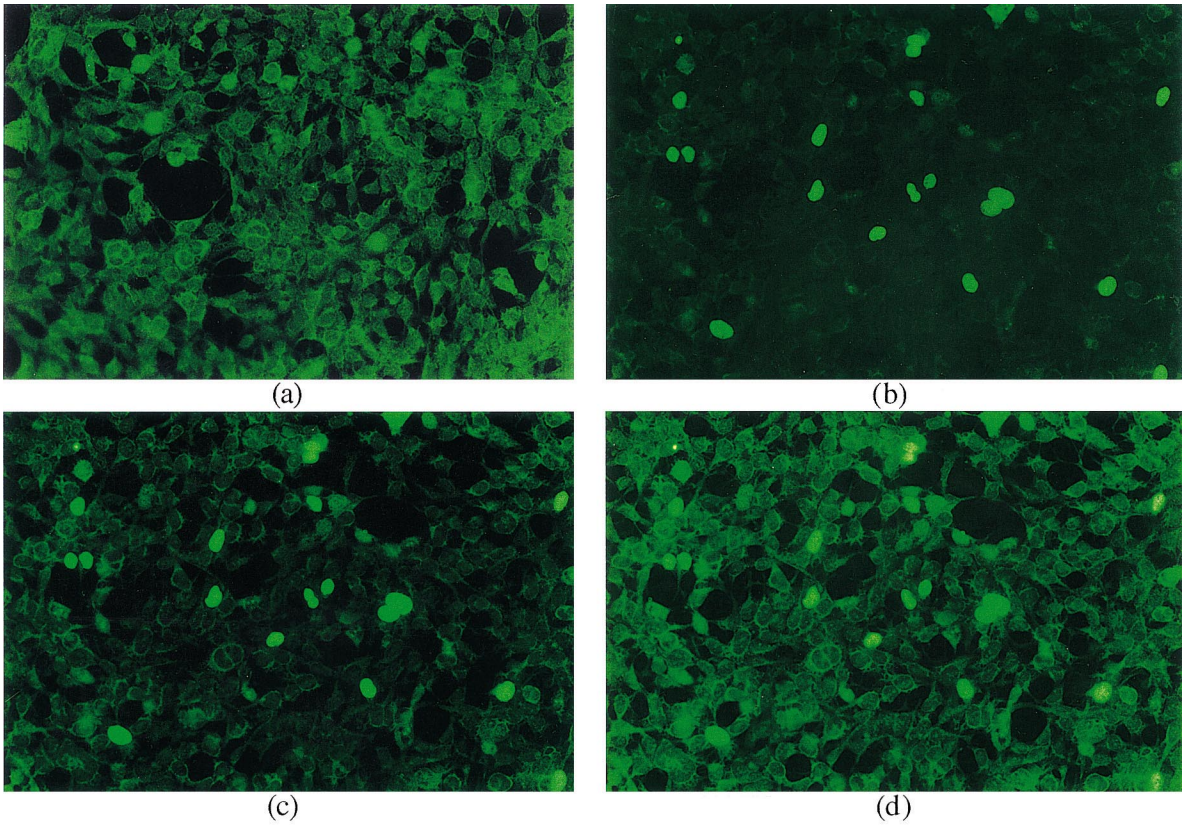

Fig. 6. Immunofluorescent assay of adenovirus infectious units (iu). Permissive cells are either non-infected (a) or infected with adenovirus ADTG5643 LotA (b–d) as described in the Experimental section. Quantitation of the adenovirus iu relies on counting the FITC-labelled nuclei resulting from the initial monoclonal antibody recognition of the adenovirus DBP early protein expression being amplified via two subsequent antibodies, the latter one being FITC-labelled. Exposure times were (a) 5 s for the negative control and (b) 1 s, (c) 2 s, or (d) 5 s for the infected samples.

generating an RCA is significantly inferior to that subsisting with E1°E4+ vectors in 293 cells. Assay for RCA is nonetheless a prerequisite for material destined for human use.

Quantitative assay methods for RCA include biological assays [21] as well as PCR analyses [13,40,41]. The bioassay based on an RCA-induced cytopathic effect on the A549 cell lines is sensitive to one RCA/10^{10} iu. No RCA was detected in a total of 1×10^{10} iu of Lot D.

3.3.2. Viral protein and residual impurities

The designation of the component adenoviral proteins originally separated electrophoretically have been given Roman numeral designations by convention [26] (Fig. 7a). Dissociation and separation of adenoviral capsid proteins can also be accomplished using rpHPLC [23]. Such a proteome analysis of the standard, recombinant adenovirus, ADTG5643 Lot B, is shown in Fig. 7b.

Baseline resolution of the viral proteins which was particularly sensitive to temperature was accomplished on the C4 Jupiter column using the especially-designed 145 min acetonitrile gradient at 45°C as described in the Experimental section. N-terminal sequence and mass spectrometry analysis were combined with database searching methods to identify the material contained in the peaks numbered 1–14 in Fig. 7b. All the detectable peaks obtained in this reverse phase separation contained

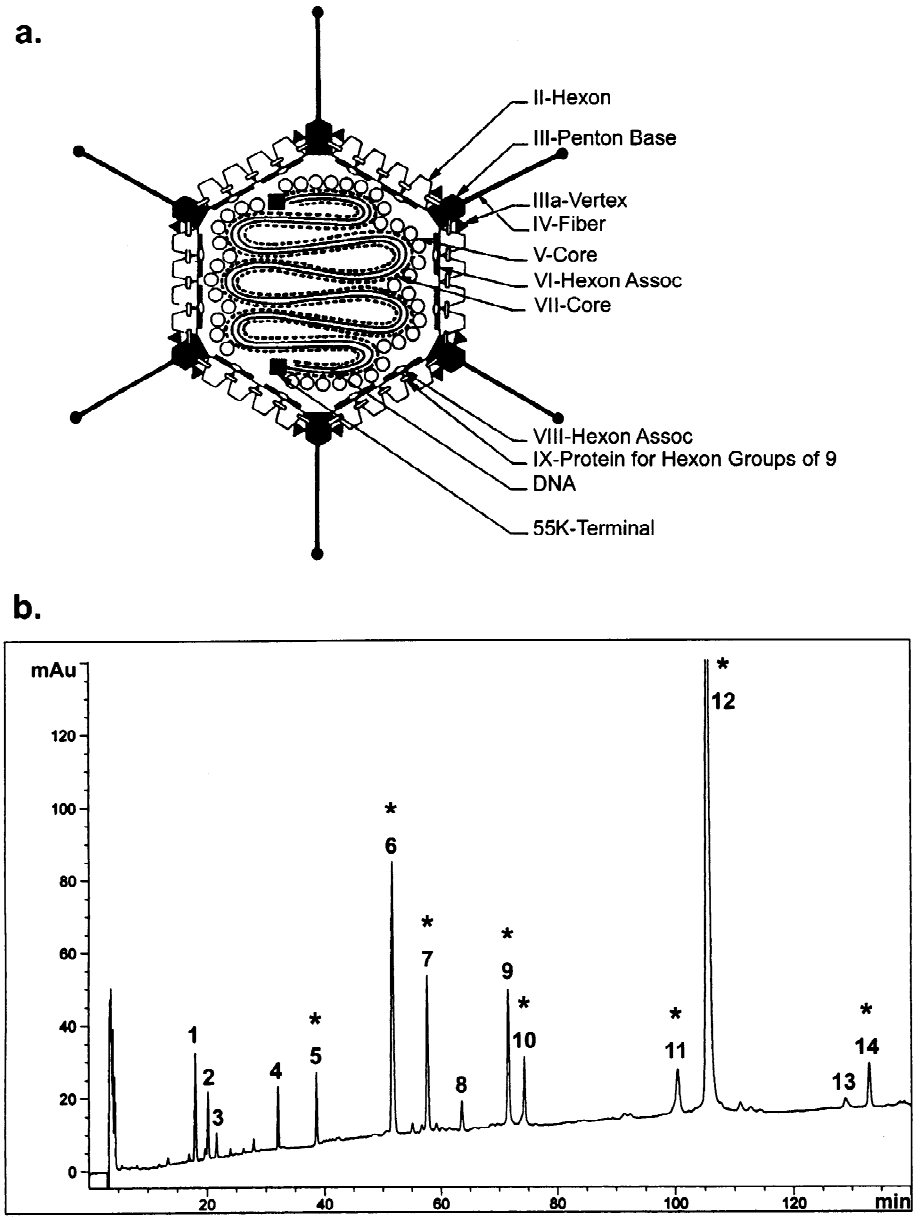

Fig. 7. (a) Schematic cross-section of adenovirus protein components and DNA (with permission from Ginsberg [16]). Roman numeral designation of proteins is by convention [26]. (b)Reverse phase HPLC chromatogram showing the component adenovirus polypeptides. 2×10^{11} total particles of ADTG5643 Lot B were chromatographed (8=215 nm) in an acetonitrile (0.1% TFA) gradient on a Jupiter C4 column at 45°C generating 14 adenoviral polypeptide (using the Roman numeral convention) peaks (designated in arabic numbers) with the following correspondence: **1**=VII (N); **2**=X (N)+VI (C); **3**=X (N), dimerized; **4**=VI (N); **5**=VIII (N); **6**=VII; **7**=V; **8**=VIII (C); **9**=VI; **10**=III; **11**=IIIa; **12**=II; **13**=X (C); and **14**=IX. N=N-terminal fragment/segment/propeptide; C=C-terminal fragment/segment; Black star,★, indicates identification using mass spectrometry. See sequence and mass determinations in text.

adenoviral proteins or fragments thereof. No cellular protein was identified.

Eight peaks, 5, 6, 7, 9, 10, 11, 12 and 14, were identified as adenoviral polypeptides VIII, VII, V, VI, III, IIIa, II, and IX, respectively, by mass spectrometry of their tryptic peptide mixtures and database comparison. The material contained in the remaining peaks could not be similarly identified unambiguously as insufficient mass signals were obtained. This occurs frequently when the tryptic peptides generated are (1) few, (2) unexpected due to internal cleavages or (3) simply very small in size. Thus, N-terminal sequencing was performed on these peaks without trypsin treatment.

N-terminal sequencing of Peak 1 gave an internal sequence (GLRFPSKMFGG) of the propeptide of protein VII (G_{14}–G_{24}). [Amino acid numbering takes into account the initial methionine.] The mature protein VII is found in Peak 6 starting at A_{25}.

Edman degradation of Peak 2 gave two sequences: one of them (ALTCRLRFPVPGFRGRMHR) corresponding to the N-terminal sequence of protein X and representing the propeptide A_2–G_{32}, and the second (GVQSLKR) corresponding to an internal fragment of protein VI starting at $G_{240,}$ thus probably representing the C-terminal peptide G_{240}–F_{250}, also called the protease cofactor.

Edman degradation of Peak 3 gave the N-terminal sequence of protein X as in Peak 2 except that cycle 4 revealed a tyrosine rather than a cysteine. This could be indicative of a dimerization via a disulfide bond at C_5 as PTH-cystine migrates chromatographically similar to PTH-tyrosine [20].

Peak 4 was N-terminally blocked and was thus analyzed via cyanogen bromide cleavage. The two sequences obtained (XXINFASLAPXXGSXPFMG and GNWQXIGTXNM) identified the material contained in this peak as the N-terminus of protein VI, probably the propeptide M_1 to G_{33}. The remaining of the protein is found in Peak 9 starting at A_{34}.

For Peak 8, an internal sequence (GRSSFTPRQAILTLQTS) of protein VIII was identified starting at G_{158}. It is noteworthy that at position 160, a serine was determined instead of the proline recorded in the sequence databases. The presence of a monoisotopic mass, 1530.71 Da, in the tryptic digest, correlates well with a peptide corresponding to Q_{166}–R_{179} of protein VIII, thus verifying the presence of a minimum protein VIII fragment length of amino acids G_{158}–R_{179}. In addition, the average mass measured (7640 Da) for the Peak 8 material without trypsin digestion was in good agreement with the calculated average mass (7642 Da) of the entire fragment (G_{158} to the C-terminal position D_{227}, with S_{160} substituted for P_{160}). The N-terminal part of protein VIII (1–157) is attributed to Peak 5 where peptide masses covering the sequence from position E_4–R_{131} were obtained. No peptide masses from the C-terminal part of the protein (G_{158} –D_{227}) were found in Peak 5.

N-terminal sequencing of Peak 13 gave an internal sequence (ILPLLIPLIAAAIGAVPGIASVAL) of protein X corresponding to I_{52} and following. Monoisotopic masses, 2886.76 and 2749.70 Da, were obtained after trypsin treatment corresponding to the intact C-terminus (I_{52}–H_{80}) and the fragment I_{52}–$R_{79,}$ respectively, upon removal of the C-terminal histidine by trypsin.

In addition to there being no cellular components identifiable, a comparison of ADTG6214 Lots B, C, D and E with each other showed that the 14 peak surfaces (Table 3) are very similar with RSDs of $<$30% indicative of highly reproducible production and purification procedures for this vector.

Residual routine cesium analyses by dispersive energy X-ray fluorescence spectroscopy on the final product shows the level to be $<$10 ppm unless there is failure of the final purification step of gel filtration for the CsCl removal. The RSD for the cesium determination is less than 1%.

3.4. Clinical application

The four lots of ADTG6214 to which these specific analytical methods of identity, potency and purity have been applied are representative of clinical-grade material as Lots C and Lot D are the toxicological and clinical lots, respectively, of an on-going study in hepatic metastases of colon cancer in France (Hôpitaux Universitaires de Strasbourg with J. Marescaux, investigator/coordinator). The quality of these lots complies with the international standards which exist and to those specifications given internally.

Table 3
Comparative ADTG6214 adenoviral protein peak ratios from rpHPLC

Peak number	Area%				Average	RSD,%
	Lot B	Lot C	Lot D	Lot E		
1	1.3	1.0	1.1	1.2	1.2	11.2
2	1.6	1.5	1.7	1.6	1.6	5.1
3	0.5	0.7	0.6	0.8	0.7	19.9
4	1.4	1.3	1.5	1.5	1.4	6.7
5	1.5	1.4	1.7	1.5	1.5	8.3
6	11.0	10.7	10.6	11.5	11.0	3.7
7	6.2	5.7	6.4	6.4	6.2	5.4
8	1.0	1.0	1.1	1.2	1.1	8.9
9	5.1	5.0	5.9	5.2	5.3	7.7
10	2.9	2.7	3.3	1.6	2.6	27.7
11	2.0	2.0	2.4	1.1	1.9	29.3
12	62.1	63.6	60.0	62.8	62.1	2.5
13	0.8	0.9	0.9	0.9	0.9	5.7
14	2.6	2.5	2.8	2.7	2.7	4.9

4. Conclusions

As this paper has demonstrated, comprehensive quality control and characterization of adenovirus vectors requires numerous tests at different stages of the production process. Some tests are non-specific for adenovirus vectors and are based on existing monographs on vaccines for human use, vectors for gene therapy or biotech products.

Other tests are specific for adenovirus vectors. Results and some validation data were given for the molecular biology characterization, in vitro transgene expression, potency, and protein characterization. For some of the assays, for instance, titration, total particles, or rpHPLC, an international harmonization of the methods and reference standards would indeed be welcomed to allow improved comparisons between pre-clinical or clinical studies as well as among different laboratories world-wide.

Method validation provides at a minimum the assurance that the assay developed is specific and gives indications of its precision both with respect to repeatability and reproducibility. Extensive in vitro characterization with validated methods of active principle components and residual impurities is a way of assuring (a) increased safety for the patient (b) thorough evaluation of lot-to-lot consistency for both vector and residual impurities, (c) reductions in in vivo testing and (d) minimization of time-consuming and costly bridging studies.

5. Nomenclature

ADTG	transgene adenovirus
Ad5	adenovirus type 5
AIEX	anion-exchange
ATCC	American Type Culture Collection
bp	base pair
CBER	Center for Biologics Evaluation and Research
CDP star	disodium 4-chloro-3-(methoxyspiro {1,2-dioxetane-3,2′-[5′-chloro]tricyclo[3.3.1.1]decan}-4-yl]phenyl phosphate
CFTR	cystic fibrosis transmembrane conductance regulator
CPE	cytopathic effect
CTLL	cytotoxic T lymphocyte lysate
DBP	DNA binding protein
E1-E3-E4	early adenovirus genes 1, 3 and 4
ELISA	enzyme-linked immunosorbent assay
FITC	fluoresceine isothiocyanate
HRP	horseradish peroxidase

HPLC	high-performance liquid chromatography
iu	infectious unit
IU	international unit
kbp	kilobase pair
moi	multiplicity of infection
MALDI-TOF	matrix-assisted laser desorption-time of flight
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(-sulfophenyl)-2H-tetrazolium, inner salt, Owen's reagent
NCBI	National Center for Biotechnology Information
NIBSC	National Institute for Biological Standards and Control
orf	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDA	photodiode array
PMS	phenazine methosulfate
RSD	relative standard deviation
RSV	Rous sarcoma virus
rhuIL-2	recombinant human interleukin 2
RCA	replication competent adenovirus
rp	reverse phase
TMB	tetramethylbenzidine
tp	total particles
U	activity units

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