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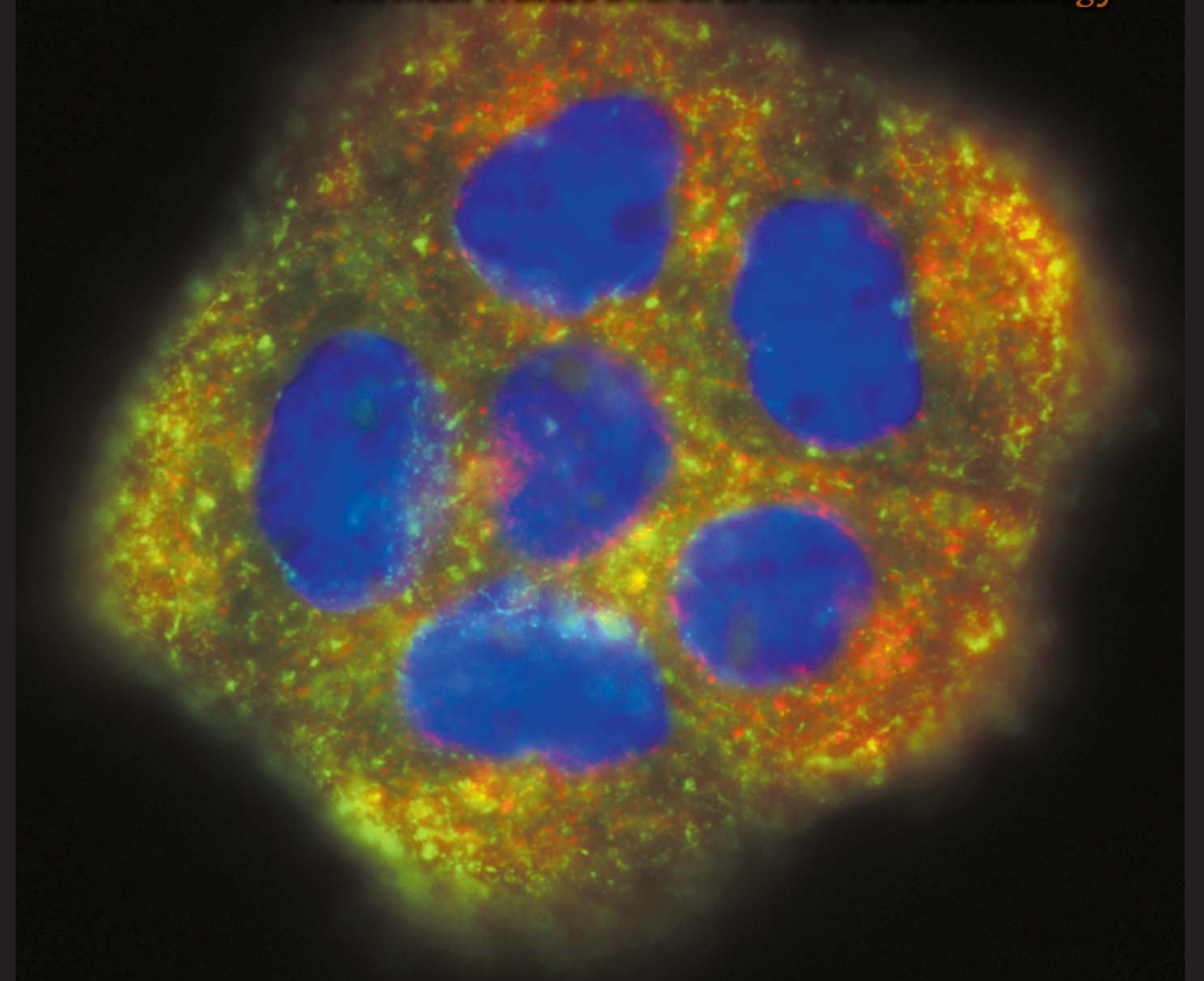

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Manufacturing and Testing of a Multi-Clade Adenoviral Vector-Based Candidate Vaccine Against Human Immunodeficiency Virus

By BRYAN T. BUTMAN, PHILLIP L. GOMEZ, REBECCA SHEETS, JASON GALL, DOUGLAS E. BROUGH, J. MICHAEL SOWERS, PERRY NEWTON, VICTORIA K. HAQUE, and ALENA LIZONOVA

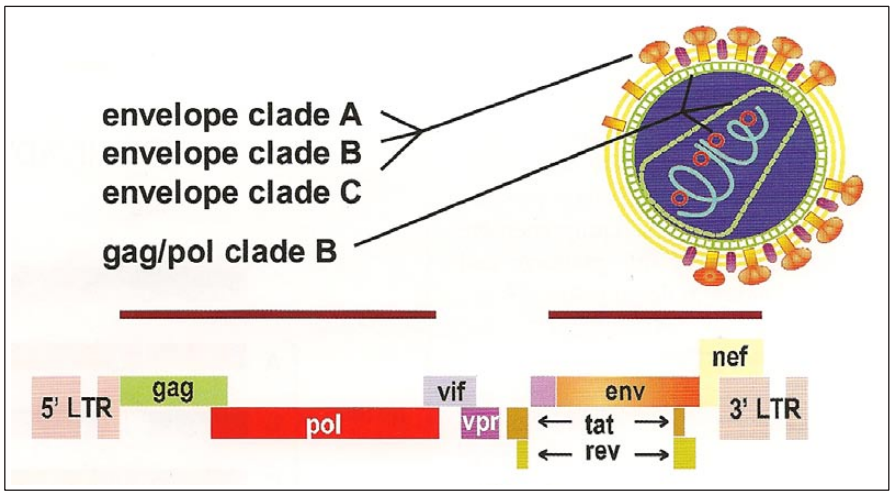

Figure 1. Human Immunodeficiency Virus.

Despite 20 years of intensive research, the development of an effective vaccine to combat the worldwide AIDS pandemic remains an elusive goal. Currently, more than 40 million individuals are infected with human immunodeficiency virus (HIV) and there have been more than 25 million related deaths. Globally, the rate of new infections is alarming, with ten new infections occurring every minute. Ninety-five percent of these infections occur in the developing world.

Several significant challenges face the development of an effective HIV vaccine. First, the HIV virus exhibits significant genetic variability with the evolution of distinct clades that possess significant antigenic diversity. Second, the protective levels of immunity remain undefined in the human population, preventing any clear understanding of the correlates of immunological protection with which to assess vaccine efficacy. Third, the current animal models are of limited value because they do not represent human disease progression. Clearly, the path forward involves clinical testing of vaccine concepts that incorporate an appropriate array of HIV antigens coupled with a potent system for antigen presentation and immunological stimulation.

We have developed a replication-deficient combination vaccine containing four recombinant adenovectors. The combination vaccine contains clade B HIV-1 genes that code for *in vivo* expression of *gag* and *pol*, as well as clade A, clade B and clade C *env* proteins (Figure 1). These immunogens are designed to induce a potent immune response against HIV. The primary objective is the induction of CD8+ cytotoxic T-lymphocytes (CTL) and the neutralization of antibody responses against HIV. This adenovector vaccine is currently being evaluated in Phase I and Phase II clinical trials in healthy volunteers.

Vector/Production Cell Platform

GenVec has developed a proprietary adenovector/production cell platform that enables efficient production of rep-

Bryan T. Butman, Ph.D.* *(bbutman@genvec.com), is vice president of vector operations; Jason Gall, Ph.D., is director of vector core; Douglas E. Brough, Ph.D., is senior director of vector sciences; J. Michael Sowers is manager of quality assurance; Perry Newton is associate director of manufacturing science; Victoria Kulesa Haque is senior project manager; and Alena Lizonova, Ph.D., is director of analytical sciences; GenVec, Inc., Gaithersburg, MD. Phil Gomez, Ph.D., is director of vector production; and Rebecca Sheets, Ph.D., is vaccine scientific and regulatory specialist; Vaccine Research Center, NIAID, NIH, Bethesda, MD.*
**Corresponding author.*

lication-incompetent adenovirus vectors. The "GV11" adenovector backbone contains deletions of two regions essential for adenovirus replication, E1 and E4, as well as a partial deletion of the E3 domain. These deletions essentially eliminate the generation of replication-competent adenovirus (RCA) while also providing sufficient genomic space for insertion of transgenes of interest. The complementing production cell line, "293-ORF6," contains a full set of E1 genes as well as open reading frame #6 from the adenoviral E4 domain under the control of a zinc-inducible promoter (sheep metallothionein promoter). Together, these genetic insertions within the chromosomes of the cell line enable controlled complementation of the deleted adenovirus genes to facilitate potent vector production (Figure 2). The comprehensive characterization of this platform cell line is discussed in detail below. [1,2]

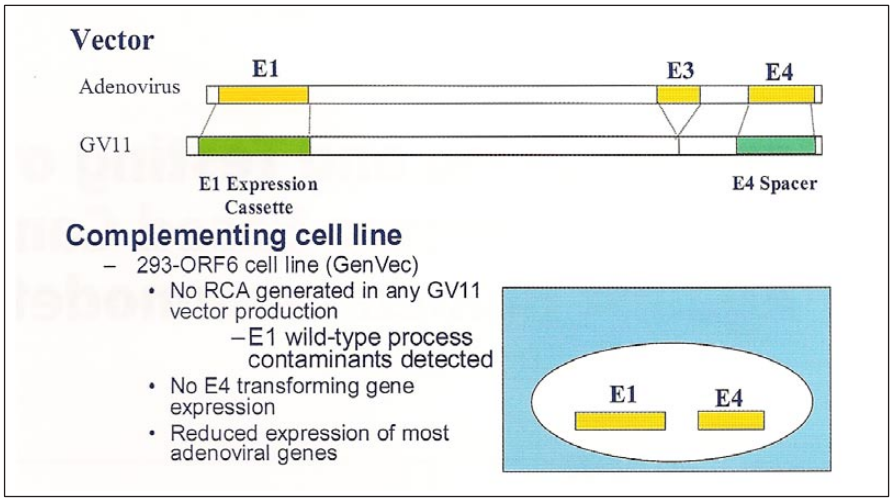

Figure 2. GV11 vector cell line system.

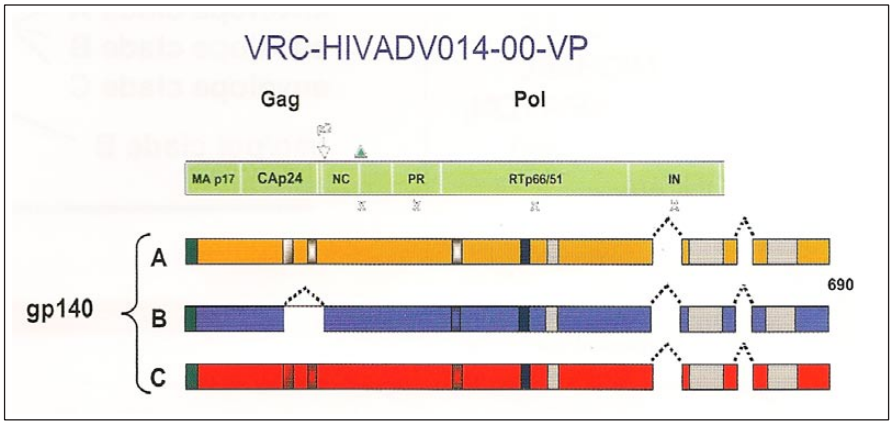

Figure 3. Multi-clade, multi-valent adenoviral vector vaccine candidate.

Vector Construction

The key steps to construction of the HIV antigen expression adenovectors include: 1) construction of a shuttle plasmid that contains the expression cassette with the specific HIV antigen(s); 2) construction of the full-length adenovector genome containing HIV antigen expression cassettes using GenVec's AdFAST procedures (bacterial recombination system to allow rapid construction and selection); 3) construction of the HIV antigen adenovector by conversion of the AdFAST genome into a viral vector (GV11) on complementing mammalian cells (293-ORF6); and 4) expansion of the HIV antigen adenovectors by serial passaging to generate high titer stocks.

Four adenoviral vectors were constructed: *Adt.GagPol(B).11D, Adgp140(A).11D, Adt.gp140dv12(B).11D* and *Adgp140(C).11D.* Each vector contains human HIV antigen open reading frame (ORF) expression cassettes (EC) that replace the deleted adenovirus E1 region. These EC are driven by the *cytomegalovirus* (CMV) immediate early promoter and are terminated by SV40 poly-A stop sequence. The deleted E4 region has been replaced with a transcriptionally inert spacer (TIS1) element (Figure 3).

In the development of the HIV antigen expression constructs by the NIH Vaccine Research Center (VRC), the codons were optimized for expression in human cells. The truncated *env* genes lack the fusion and cytoplasmic domains but regions important for oligomeric formation are retained. In addition, for the gp140(B) construct, the V1 and V2 loops were deleted to improve vector yield during production, and to improve antigen presentation *in vivo*. The gp140(A) and gp140(C) vectors had adequate stability and yield without the deletion of these loops. The synthetic *gag* gene contains all of the mature *gag* proteins except for the last two (p1 and p6) that are normally cleaved from the carboxy-terminus of the *gag* polyprotein. The synthetic *gag* gene was ligated in-frame with sequences encoding the *pol* polyprotein, again with codons optimized for expression in human cells. The *pol* gene product is nonfunctional because it is present as a fusion protein. Furthermore, mutations were introduced into the synthetic protease and reverse transcriptase genes to render them nonfunctional also.

293-ORF6 Master Cell Bank

The 293-ORF6 cell line was adapted to growth in serum-free suspension and a master cell bank (MCB) was established at passage 27. In accordance with the relevant regulatory guidelines, demonstration of the absence of bacteria, fungi, yeasts, mycoplasmas and a range of potential adventitious agents including viruses originating from bovine, porcine, simian and human sources has been achieved. (Details of this safety testing are described.) [3-7]

In addition to the creation of the MCB, an "aged cell bank" was established

by further passage of 293-ORF6 cells in serum-free medium to passage 43. This was done to address the potential issues of the MCB undergoing changes with sequential passages through the tiers of cell banks to beyond the projected scale for production runs. This aged cell bank was then characterized in several cell line studies to demonstrate phenotypic and genotypic stability. These studies include quantitation of E1 and E4 copy number, E1 and E4 chromosome localization, cytogenetic analysis for ploidy, DNA sequencing of the PrP gene, demonstration of protease sensitivity of the PrP gene product, and demonstration of cell identity by PCR. These studies are summarized in Table 1 (and in reference).[8]

Table 1. Summary of additional characterization data for 293-ORF6 MCB and aged cell bank.

Analysis Performed	Cells from the Master Cell Bank (P27)	Cells from the Aged Cell Bank (P43)
E1 Copy Number	6.5 ± 0.8 copies per diploid cell	5.1 ± 0.7 copies per diploid cell
E4 Copy Number	2.6 ± 0.2 copies per diploid cell	2.4 ± 0.4 copies per diploid cell
E1 Chromosome Localization	Chromosome 19 at 19q13.3 locus	Chromosome 19 at 19q13.3 locus
E4 Chromosome Localization	Chromosome 6 at 6q16-21 locus	Chromosome 6 at 6q16-21 locus
Cytogenetic Analysis for Ploidy	Median = 72 chromosomes per metaphase (n = 100 metaphases); Range = 66 to 80 chromosomes per metaphase	Median = 72 chromosomes per metaphase (n = 100 metaphases); Range = 54 to 78 chromosomes per metaphase
DNA Sequence for PrP Gene	No evidence of infectious PrP^{SC}	No evidence of infectious PrP^{SC}
Protease Sensitivity of PrP Gene Product	No detectable PrP-res by Western blot	No detectable PrP-res by Western blot
Cell Identity PCR Assay	E1 and E4-ORF6 sequences detected	E1 and E4-ORF6 sequences detected

In addition, the tumorigenic potential of 293-ORF6 cells was evaluated using the aged cell bank. The purpose of the study was to determine whether the genetic manipulations that gave rise to the 293-ORF6 cells (insertion of E4-ORF6 expression cassette) resulted in enhanced tumorigenic potential in nude mice relative to the parental 293 cells. Both the parental 293 and 293-ORF6 aged cell bank generated tumors at 107 cells per animal, demonstrating comparability. In order to further assure the safety of vaccines produced in the 293-ORF6 platform, we are currently conducting oncogenicity studies using both cell lysate and purified cellular DNA as test articles in neonatal animals.

Manufacturing and Purification of Phase I/II Bulk Vaccine

GenVec has developed two serum-free, animal protein-free manufacturing processes for the generation of clinical trial vectors (summarized in Table 2). Because the key goal for this program was to move rapidly into manufacture of the four monovalent vectors to initiate clinical trials, the Phase I/II process was employed. The bulk clinical monovalent vector lots were generated through two tiers of serum-free manufacturing using the Phase I/II process described in Table 2. Research vector stocks were used to seed the first tier of production (master virus bank), and this purified vector material was subsequently used to seed the second tier of production (bulk clinical monovalent vector lots). The bulk purified vectors were filtered through a 0.22 µm filter and stored at -60° C until formulation and filling of the combined tetravalent final vaccine product.

Table 2. Comparison of early stage and late stage clinical vector manufacturing processes.

	Phase I/II	Phase II/III
Cell Culture	Serum-free suspension of 293-ORF6 cells in shaker flasks	Serum-free suspension of 293-ORF6 cells in stirred tank bioreactor
Infection	100 PU/cell with Zn-induction of E4 expression	100 PU/cell with Zn-induction of E4 expression
Lysis	3 freeze/thaw cycles	Detergent lysis (Triton X-100)
Initial Processing	Benzonase digestion and Freon® extraction	Ultrafiltration, Benzonase digestion and diafiltration
Purification	3 cycles of density gradient centrifugation on CsCl	Column chromatography
Buffer Exchange	Dialysis into final formulation buffer	Size exclusion chromatography into final formulation buffer

Vialing of Phase I/II Vaccine Product

Each of the four bulk vector lots was thawed and then pooled together in the following ratio: 50% *Adt.GagPol(B).11D*, 16.7% *Adgp140(A).11D*, 16.7% *Adt.gp140dv12(B).11D* and 16.7% *Adgp140(C).11D*. The bulk formulated multi-clade vaccine was subsequently filtered through a 0.22 µm filter and dispensed into borosilicate glass vials fitted with butyl rubber stoppers and aluminum crimp seals. Vialed final vaccine product was frozen and stored at -20° C.

Characterization of the Monovalent Vector Lots and the Combined Final Vaccine Product

Safety

A full panel of microbial and viral safety tests (in accordance with relevant regulatory guidelines) was performed in order to assure absence of adventitious agents. A cell culture method to determine the presence of RCA was performed on each lot and demonstrated absence of RCA (50 billion particle units [PU] tested). In addition, the final vaccine product was satisfactorily tested in a general safety assay to detect the presence of unknown toxic substances for which specific assays are not performed (as prescribed in 21 CFR 610.11). [4-7]

Potency

Vector particle concentration for the bulk vector lots was determined by UV spectrophotometry at 260 nm, (using an extinction coefficient of 1.1 x 10^{12} PU/ml). For final vaccine product, vector particle concentration was determined using a proprietary anion exchange high pressure liquid chromatography (HPLC) method with fluorescence detection. Infectivity was determined using an immunofluorescence-based focus-forming unit (FFU) assay that targets an adenovirus DNA-binding protein. Typical PU:FFU ratios for the individual vector lots were in the range of 8–12 PU:FFU. Expression of the specific transgene products was demonstrated by infection of 293 cells followed by Western blot analysis of the 293 cell lysates. The detector antibody for the immunoassay was polyclonal human anti-HIV anti-serum. A typical Western blot is shown in Figure 4.

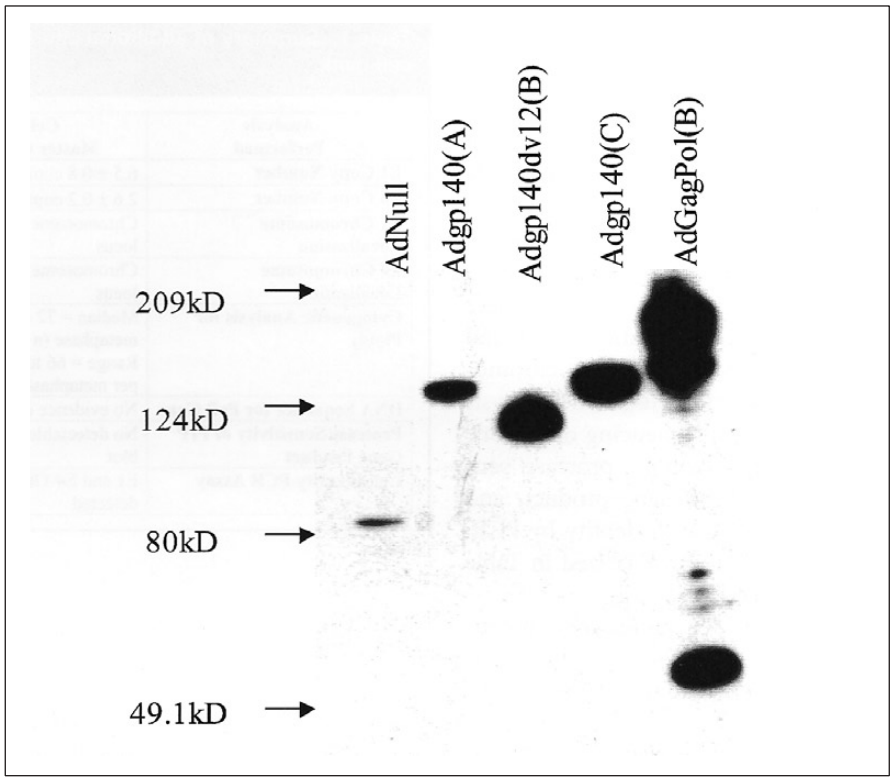

Figure 4. Western blot analysis for transgene expression of HIV proteins.

Purity

SDS-PAGE analysis with silver staining was employed to demonstrate the purity of the adenovirus lots. Assays for quantifying process impurities included methods for residual CsCl by atomic absorption, host cell DNA by quantitative polymerase chain reaction (PCR), and endotoxin by the Limulus amebocyte lysate (LAL) chromogenic assay.

Identity

The genetic structural identity assay employs qualitative PCR to determine the integrity of distinct vector genome regions and confirm the identity of the vector. In addition, each master virus bank was subjected to full-length DNA sequencing of the 32 KB adenovector genome.

Quality

Additional assays for quality included pH, appearance, aggregation by dynamic light scattering, reversed phase HPLC of the vector proteome, and container closure integrity by the microbial ingress method.

Characterization of Vector Stability

Real-time and accelerated stability studies performed to date, on the final vaccine product, demonstrate acceptable stability for periods of 18 months, five months and two months when stored at –20° C, +5° C and +25° C, respectively. Stability studies are currently ongoing to evaluate storage of vaccine product vials beyond 18 months at -20° C.

Clinical Testing of Phase I/II AdHIV Vaccine

In the absence of a clear correlate of immunological protection against HIV, the clinical path for the AdHIV vaccine requires careful design. The initial Phase I clinical trial was a placebo-controlled, double-blinded dose escalation trial examining the safety of 1e9, 1e10 and 1e11 when administered intramuscularly (IM) via needle/syringe in a 1.0 ml volume. Vaccine recipient T-cell responses were assessed *in vitro* using intracellular staining for the production of Interferon-γ (IFN-γ) and Interleukin-2 (IL2) and ELISpot assays for IFN-γ in response to the presence of the *gag* (B), *pol* (B), *env* (A), *env* (B) and *env* (C) antigens. B-cell responses were measured by indirect ELISA titration of serum antibodies and by assessment of neutralizing antibodies to HIV. To date, more than 140 healthy volunteers have received this multi-clade AdHIV vaccine, either alone or in combination with a plasmid DNA prime. Other Phase I/II clinical trials will assess the safety and immunogenicity of the AdHIV vaccine, both alone and as a boost, following immunological priming with multi-clade plasmid DNA vaccine. If successful, these trials will provide the basis for conducting a large "Proof of Concept Efficacy Trial" that would employ AdHIV vaccine manufactured by the comparatively large-scale Phase II/III bioreactor/column chromatography process (described in Table 2).

Conclusions

Replication-deficient adenovirus vectors are a promising platform for delivery of vaccine antigens in humans. The deletions in the E1, E3 and E4 regions of the GV11 vectors provide ample genomic space to accommodate mono-clade HIV antigen transgene cassettes. The 293-ORF6 cell line is

a well-characterized mammalian cell substrate and is suitable for a broad array of vaccine production. A multi-clade adenovector-based vaccine candidate against HIV has been successfully manufactured and progressed into clinical testing in humans.

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