

applications. The murine granulocytic colony-stimulating factor (mGM-CSF) is one of the most promising cytokine candidates for cancer immunotherapy and as adjuvant of DNA vaccines. With the aim of improving the administration and release of cytokines in a very localized area, we have designed vectors expressing the mGM-CSF cDNA with different localization signals. Using this strategy it was shown that: cytokines can be targeted to different subcellular compartments, expressed there as stable active proteins, stored inside the cells and released after cell lysis. Moreover, a plasma membrane targeted form of mGM-CSF displayed substantial amount of biological activity. These vectors could have potential applications in immunotherapy for tumors and DNA vaccination protocols.

755. Use of Dendritic Cells as Cancer Vaccines Targeted against Mutant K-Ras Oncogenes

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The aim of this study is to use this approach to generate new treatment strategies for patients with pancreatic cancer. Codon 12 mutations of the k-ras oncogene occur in over 90% of pancreatic carcinomas. There is evidence that endogenously processed mutant ras peptides in animal models and peptide vaccinations are able to induce CTL reactivity against ras oncogene-expressing tumor cells in mice and in cancer patients. Previous studies in our laboratory using naked DNA vaccination of mutant ras genes have indicated a potential use of these genes as antigens in cancer immunotherapy. Dendritic cells engineered to express tumour antigen represent a powerful method for inducing a more effective anti-tumor response. We are investigating the use of different antigen delivery systems such as bacteria, adenovirus and SV40 viral vectors to transfect dendritic cells *ex vivo* with mutant ras genes. The efficacy of this vaccination approach is being evaluated in animal models to protect against tumor challenge and ultimately explore the possibility of the use of this therapeutic approach in clinical trials with patients affected with malignancies showing a high frequency of k-ras mutations, such as pancreatic and colorectal cancer.

756. Production of Allogeneic Prostate Cancer Vaccine (GVAX®): Characterization and Bioequivalence Studies

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The allogeneic GVAX® cancer vaccine is composed of two prostate cell lines (PC3 and LNCaP) that have been genetically modified by retroviral transduction to secrete GM-CSF. After production, the cells are lethally irradiated and frozen. Manufacture of GVAX® for clinical use was first carried out in 1997 using flat-stock culture vessels and FBS-containing medium. Subsequently, several changes were introduced in the manufacturing process including adaptation of vaccine cells to serum-free suspension culture in the absence of serum. These changes were introduced to make the production of GVAX® a scalable process and to eliminate the risks of introducing adventitious agents with the use of FBS. To assess the effect of these modifications, GVAX® cancer vaccine lots manufactured by different processes were extensively characterized and compared with regard to mRNA and protein expression, viability, GM-CSF production and activity, immunogenicity in mice, and reactivity to serum from GVAX® vaccine recipients. The data collected in this study indicate that, within the limitation of the available technologies, no significant differences are observable between lots of GVAX® produced by the different manufacturing processes. This study represents an approach

towards addressing the characterization of complex biological therapeutics.

DNA VIRUS VECTORS

757. Extending the Packaging of Recombinant Adeno-associated Virus

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Recombinant adeno-associated viruses (rAAV) are among the most promising vectors for gene transfer in humans. However, the parvovirus has restricted packaging capacity that has prevented its wider application in gene therapy. We wished to determine whether recombinant AAV could be similarly over-stuffed to generate vectors with genomes greater than 120% of wild-type. Specifically, we wished to determine whether an expression cassette of ~5.8 kb inclusive of the AAV ITRs can be packaged into an AAV-2 capsid efficiently and without loss of infectivity and transduction titer.

Four viral vectors, AAV/PPE-luc (107% of wild type AAV in length), AAV/PPE-luc-wpre (123% of wild type AAV in length), AAV/GFAP-luc (98% of wild type AAV in length) and AAV/GFAP-luc-wpre (117% of wild type AAV in length), were generated by a helper-free two plasmid transfection method (Grimm et al., 1998). The expression cassette on all constructs was flanked by AAV-2 145 bp ITRs. The two promoters investigated were the neuronal promoter, a 2.7 kb rat preproenkephalin (PPE) fragment, and an astrocytic 2.2 kb human glial fibrillary acidic protein (GFAP) promoter. Each promoter was used to direct expression of a firefly luciferase cDNA, with the 3' component of the expression cassette containing either a 400 bp bovine growth hormone polyadenylation signal (BGH) alone, or a 650 bp Woodchuck Hepatitis Virus Post Regulatory Element (WPRE) immediately 5' to the BGH polyA. For quantitative gene expression analysis, rAAV viral vectors were extracted with 1,2,2-trichlorotrifluoroethane, and then ultracentrifuged in CsCl at 80,000 rpm for 24 hours twice in order to concentrate and remove any empty particles. The rAAV titer was based on a sandwich ELISA technique (kit provided by Progen, Germany). Quantitative transgene expression was determined by a luciferase assay. Luciferase gene expression was further confirmed by immunohistochemistry. For immunohistochemical analysis, viral vectors were purified by a Biocad Sprint high-performance liquid chromatography (HPLC) system with a POROS HE heparin column. The vector titer was determined by an ABI 7700.

Western blotting showed that all four vectors examined contained the viral structural proteins, VP1, VP2 and VP3 in appropriate ratios. Southern blotting revealed that the rAAV genome was present fully intact in each of the vectors respectively, including the 5.75 kb PPE construct. In contrast to previous reports which suggested that rAAV containing >5.2kb genomes lose transductional efficiency, enzyme assays showed significant enhancement of expression from our WPRE plus oversized vectors.

Ongoing studies are attempting to define the absolute limit for efficient packaging as well as determining whether variability in titers obtained from over-sized genomes is sequence dependent. The versatility of rAAV is strongly compromised by its small size. The standard dogma of 110% above wild-type can be challenged, and for a given sequence, packaging can be extended to vector genomes of greater than 120% of wild-type, thus increasing the potential applications of the vector and inclusion of a greater range of cDNAs, promoters and postregulatory elements.

758. WITHDRAWN

759. New Cationic Phosphonolipids with a Cholesteryl Moiety as Lipidic Part

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Decreasing cellular toxicity while maintaining an efficient transgene expression is one of the objectives of search in the field of non-viral vectors.

- Previously, we reported the ability of cationic phosphonolipids ($n=1$, $Z=N$) to transfer DNA into different cell lines and into mouse lungs.

- More recently, we developed a novel class of phosphonolipids

($n=1, 2, 3$; $Z=P, As$) and showed that the replacement of the ammonium by a phosphonium or an arsonium improves transfection activity and decreases cellular toxicity.

- Moreover, the inclusion of cholesterol as co-lipid improves transfection efficiency, especially *in vivo* (mouse). With these results in mind, we designed new cationic phosphonolipids with cholesteryl moiety as lipidic part.

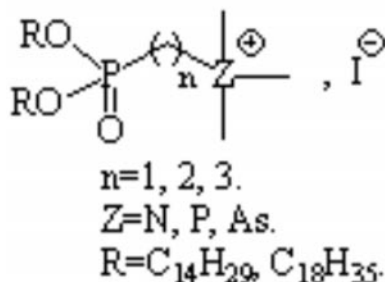


FIG.

760. HSV-1 Viral Vectors for the Peripheral Nervous System: Delivery of Potentially Regenerative Molecules

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We have developed novel herpes simplex virus-1 vectors for gene delivery to the peripheral nervous system. HSV provides a potentially ideal vector for gene delivery to the nervous system as it naturally infects neurons and persists in a latent state in the host cell nucleus for extended and even life long periods of time. In this study, attenuated vectors with different combinations of essential and non-essential gene deletions have been tested *in vivo* for their ability to provide high level, long term delivery of single and multiple genes after peripheral administration. The vectors use latency associated promoter elements to provide long term transgene expression, and after inoculation give highly efficient gene delivery to both sensory neurons of the dorsal root ganglia and motor neurons in the spinal cord. These vectors are being used to deliver molecules such as Reg-2, CNTF, NT-3 and the neuropeptide galanin, in studies aimed at the regeneration of either motor or sensory neurons. A virus containing Cre recombinase has also been generated to allow conditional gene expression in loxP containing transgenic animals.

761. Mutant Adeno-associated Virus Rep Protein with Reduced Cytotoxicity for AAVS1-specific Integration of Foreign DNA

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The adeno-associated virus (AAV) Rep78/68 proteins are required for site-specific integration of the AAV genome to AAVS1 locus (19q13.3-qter) as well as for viral DNA replication. Rep78/68 bind to the GAGC motif on the ITR (inverted terminal repeat) and cut at the trs (terminal resolution site). A similar reaction is believed to occur in AAVS1 harboring an analogous GAGC motif and a trs homologue, followed by integration of the AAV genome. We have reported a gene transfer strategy to integrate foreign DNA to AAVS1, which is achieved by transferring gene of interest flanked by the ITR sequences with simultaneous transient expression of Rep 78/68 proteins. One of obstacles to circumvent in this system is a prominent cytotoxicity of Rep proteins. A mutant Rep protein that lacks cytotoxic effects while retaining the ability to mediate AAVS1-specific integration is an attractive molecule for this system. As the domains responsible for cytotoxicity are thought to exist in the N-terminal half of Rep proteins, we constructed more than 60 mutant Rep 78 proteins in which each charged amino acid in the N-terminal region was mutated to alanine independently. We assessed the cytotoxicity of the mutant Rep proteins by *in vitro* colony-formation assay. We found that H18A (mutation of H18 to A), RD61AA, R122A, E201A, and E226A mutants showed lower cytotoxicity compared to the wild type Rep78 without losing the ability of the site-specific integration. To confirm whether these less-cytotoxic mutants retain the ability to integrate into AAVS1, we expressed these mutants in 293 cells when a plasmid harboring Neo gene flanked by the ITRs was co-transfected. After selection with G418, we isolated clones and expanded. Southern blot analysis revealed that 67-83% of clones obtained by expressing each of the five mutants showed rearrangement of AAVS1, a result comparable to the efficiency with Rep78. We have been screening these mutants in more detail to choose a more suitable mutant Rep for the AAVS1-directed integration of therapeutic genes.

762. Multiply Disabled Herpes Simplex Virus Vectors for Gene Delivery to the CNS

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Replication incompetent HSV1-based vectors designed for the safe and efficient delivery of multiple exogenous genes to the CNS are described. These vectors have been disabled by the deletion or inactivation of genes encoding ICP34.5/ORF P, Vmw65, ICP27, ICP4 and vhs. The combined deletion of ICP27, ICP4 and Vmw65 renders the virus incapable of expressing significant amounts of any of the IE gene products and allows the viral genome to persist in infected cells *in vitro* and *in vivo*, indicating the minimal cytotoxicity of the vector system. Gene expression is maintained in the long term using novel promoter systems containing elements from the HSV latency associated transcript promoter.

The vectors described are non-toxic to cultured neurons at high MOI and are capable of expressing multiple exogenous genes in cell culture and *in vivo*. These vectors have been successfully used in this study to deliver genes to either the striatum and substantia nigra, or the spinal cord, dorsal root ganglia and brain stem of adult rats following inoculation in the striatum or spinal cord respectively. Widespread gene expression results in

each case from retrograde transport of the vector from the site of inoculation to connected sites within the nervous system in a manner not efficiently possible with other vectors. Stable transgene expression then occurs. The expression patterns observed suggest that these vectors have potential applications in therapeutic gene delivery to either the injured spinal cord or in neurodegenerative conditions such as Parkinson's disease. In addition, this system provides an investigative tool for the rapid evaluation of genes and gene combinations implicated in the pathogenesis of several neurological diseases.

763. The E1b 55-kDa and E4orf6 Proteins Cooperate in a p53-independent Manner to Enhance Transduction by Recombinant Adeno-associated Virus Vectors

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Recombinant vectors derived from the single-stranded parvovirus, adeno-associated virus (AAV), show significant promise for gene therapy applications but the biology of rAAV mediated gene transfer is poorly understood. Transduction by rAAV vectors is dependent on both the target cell type and the intracellular environment. Understanding the steps that limit successful rAAV gene transfer will facilitate the utilization of rAAV vectors in gene therapy applications. Both genotoxic agents and adenovirus infection increase rAAV transduction efficiency by enhancing the conversion of the input ssDNA vector genomes into a double-stranded, transcriptionally competent form. The adenovirus-mediated enhancement of transduction has been mapped to a single gene, E4orf6, and expression of E4orf6 alone has been considered necessary and sufficient to mediate this effect. Genotoxic agents are thought to enhance rAAV transduction by stimulating DNA repair synthesis but it is unclear how E4orf6 expression promotes second strand synthesis of input vector genomes, since E4orf6 has no direct role in DNA replication or repair. We were interested to identify cellular proteins and activities that mediate the enhancement of rAAV transduction. Our initial focus was to identify cellular proteins that interacted with E4orf6 and were required for enhancement of rAAV transduction. We generated an E4orf6 expression construct, pCIE4orf6-neo, and examined the rAAV transduction efficiency of four cell lines following transient transfection of pCIE4orf6 or a vector control, pCI-neo. As expected, transient expression of E4orf6 greatly enhanced rAAV transduction in 293 cells (adenovirus transformed human embryonic kidney cells). However neither transient nor stable transfection of the E4orf6 construct was sufficient to enhance rAAV mediated gene transfer in other cell lines (H1299, Saos-2 and HeLa). Transfection studies with a plasmid containing the entire adenovirus genome (pFG140) suggested that another adenovirus gene product, in addition to E4orf6, was required for the enhancement of rAAV transduction. These studies, combined with the observation that E4orf6 expression alone enhances transduction in 293 cells, suggested that a gene from the adenovirus E1 region cooperated with E4orf6 in promoting rAAV transduction. The contribution of the E1b 55-kDa protein was examined since it is known to form a functional complex with E4orf6 during adenovirus infection. A requirement for coexpression of both E1b 55-kDa and E4orf6 to enhance rAAV transduction was demonstrated in experiments with E1b 55-kDa mutant adenoviruses (*dl338* and *dl1520*) and cotransfection studies with E1b 55-kDa and E4orf6 expression plasmids. Cellular p53 function was not required for the enhancement of rAAV transduction by E1b 55-kDa and E4orf6. Although a contribution of the adenovirus E1 region to enhancement of rAAV transduction had been identified, it had been considered to be subsidiary to and dependent on E4orf6 expression. These results do not support this conclusion but suggest that the role of E1b 55-kDa and E4orf6 in the enhancement of rAAV transduction, is best characterised as cooperative and interdependent. Further dissection of the interaction between E1b 55-kDa, E4orf6 and the host cell will lead to

identification of cellular proteins that mediate rAAV transduction and be of value in exploiting rAAV biology for gene therapy applications.

764. Construction and Function of a Recombinant Adeno-associated Virus Encoding Human Interleukin-10

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Interleukin 10 (IL-10) is a potent anti-inflammatory / immune cytokine which has received growing attention for its therapeutic potential. To facilitate therapeutic studies of IL-10 *in vitro* and *in vivo*, a recombinant adeno-associated virus (rAAV) encoding human IL-10 (rAAVhIL10) was constructed and characterized. rAAVhIL10 was produced by triple transfection of 293 T cells in a manner similar to that described by Xiao *et al.* (1998), yielding a titer of 2×10^{11} genomes/ml. COS cells, a monkey kidney epithelial cell line, and HSG cells, a human submandibular gland cell line, were infected by different doses of rAAVhIL10 *in vitro* and IL-10 secretion into the media measured by ELISA. IL-10 production was virus dose-dependent resulting in 110 ng/ml IL-10 when the viral dose was 10^8 genomes/ 3×10^5 cells. rAAVhIL10 administration (5×10^8 - 10^9 genomes/gland) to mouse submandibular glands led to a dose-dependent IL-10 secretion into the bloodstream, i.e., in an endocrine manner as previously described (Baum *et al.*, 1999, Wang *et al.*, 2000). At these doses, little IL-10 was detected in mouse saliva. These findings are consistent with our earlier suggestion that rAAVs may be useful gene transfer vectors in salivary glands (Braddon *et al.*, 1998). We next examined the functional activity of the recombinant IL-10 produced. T helper type 1 (Th1) immune responses are associated with protective immunity to viruses and intracellular bacteria. Heat-killed *Brucella abortus* (*B. abortus*) promotes secretion of Th1-inducing cytokines such as IL-12 and has been used as a carrier to induce Th1 responses to vaccines. IL-10 downmodulates the Th1-like cytokine response to *B. abortus* as previously described (Huang *et al.*, 1999). To explore if the IL-10 secreted from rAAVhIL10-infected cells could downmodulate this biological response, we measured IL-12 secretion from spleen cells of IL-10 knockout mice after the cells were cultured with *B. abortus* +/- the supernatants of COS or HSG cells infected with rAAVhIL10. The results showed that the IL-10 in these supernatants could inhibit the IL-12 response to *B. abortus* and that this inhibition was IL-10 dose-dependent. These data suggest that rAAVhIL10 may be useful for modulating the localized cytokine profile in a diseased tissue and, potentially, limiting disease progression.

765. Infectious Transfer of 150 kb of Human Genomic DNA by Epstein-Barr Virus

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The genomic context of a gene contains the features that control the level and tissue specificity of expression. The use of such native control elements will also evade the silencing mechanisms sometimes observed with the strong viral promoters often used in cDNA cassettes. However, current viral gene therapy vectors have a limited capacity for transgenes, which has prevented the use of most genomic loci. Epstein-Barr virus (EBV) is a 172 kb gamma herpesvirus, which persists asymptotically as an extrachromosomal element in the B-lymphocytes of over 90% of the human population. Work in our laboratory has recently demonstrated that human hypoxanthine phosphoribosyltransferase (HPRT) is functionally expressed from a 115 kb genomic

DNA insert carried by an EBV-based episomal vector. The extrachromosomal nature, large size and long term stability of EBV make it a good candidate for modification into a gene therapy vector for delivering genomic loci. We have constructed a BAC-based vector that contains the elements of EBV essential for vector delivery and maintenance: first, the EBV latent origin of replication (*oriP*), and the EBV nuclear antigen-1 (*EBNA1*) gene, which are both essential for the extrachromosomal persistence of the vector; second, the lytic origin of replication, (*oriLyt*) and the EBV terminal repeats, which are required for the amplification and packaging of the vector. The construct also contains the live cell marker, *GFP*, and the hygromycin resistance gene. A series of genomic DNA inserts from 60 to 150 kb in length was cloned into the vector. A set of clonal packaging cell lines, stably carrying each construct, was created by transfection into the EBV-positive Burkitt's lymphoma cell line P3HR1-HH514. The lytic cycle of the helper virus was induced with TPA and sodium butyrate, and the resultant virions were recovered from the supernatant and characterised. The construct carrying a 60 kb genomic insert was packaged as a dimer, and represented up to 5% of the total viral yield. The virus recovered from 5 ml of supernatant was able to infect Loukes cells (an EBV-negative Burkitt's lymphoma cell line), and EBV-immortalised lymphocyte cell lines (LCLs) with an efficiency of up to 40%. Episomal DNA isolated from three clones grown from the infected Loukes cells was shown to be a dimer of the original plasmid that had recircularised at the terminal repeats. Only one of these clonal lines also carries the helper virus. We have shown that the BAC-based plasmid we have developed can be packaged by an EBV helper virus, and can stably infect both EBV-positive and negative B cell lines, thereby delivering up to 150 kb of genomic DNA. We are now working towards the infectious delivery of the genomic loci of genes involved in DNA repair: the *NBS1* gene (50 kb locus in a 120 kb insert) mutated in Nijmegen breakage syndrome; and the *ATM* gene (146 kb locus in a 150 kb insert) mutated in ataxia telangiectasia.

766. Use of Regulatory Sequences of the Human H19 Gene in Bladder Cancer Gene Therapy

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The H19 gene is expressed in ~75% of bladder tumors of all stages, but not in normal bladder tissue. This observation has pointed to H19 as not only a potentially valuable diagnostic and prognostic marker, but also to the possible use of H19 transcriptional regulatory sequences for gene therapy, for example, for targeting adenovirus-based gene therapy of bladder cancer. The aims of this study were: 1) to define the specificity of H19 promoter and enhancer activity; and 2) to assess the infectivity of different bladder cancer cell lines by adenovirus. Promoter activity and enhancer specificity were determined by transient expression assays using the full or truncated 5' and 3' regulatory sequences of the human H19 gene to regulate the expression of a *luc* reporter gene in different cell lines. The infectivity of bladder cancer cell lines was determined by infecting the cells with CMV: *lacZ* replication-defective adenoviruses and assessing the percentage of X-gal-positive cells 48 hours post infection. We found that: 1) The full length human H19 promoter (-818 to +14) had a 5-10 fold higher activity in five bladder cancer cell lines compared to normal cells (IMR-90); 2) The -85 to +4 promoter region was responsible for 45-70% of the activity of the full length human H19 promoter in all five cell lines tested; 3) Although containing two SP-1 binding boxes, the -61 to +4 region exhibited only 1-5% of the activity of the full length promoter in all five cell lines tested; 4) The -85 to -34 region of the H19 promoter contained the necessary elements to interact with the human H19 4.1 kb enhancer; 5) The human H19 enhancer augmented

the activity of the H19 promoter in both H19-positive (Hep3B) and H19-negative cells (HepG2). Thus inclusion of the H19 4.1 kb enhancer would not augment specificity for H19+ tumor cells; and 6) All the bladder cancer cell lines tested (UM-UC-3, T24P, HT 1197, HT 1376, RT112, 5637 and EJ28) were infected by adenovirus 5 at M.O.I 1. Wt adenovirus infection of UM-UC-3 cell line resulted in only 10% survival 5 days post infection. We conclude that the full length H19 promoter (-818 to +14) or a shorter version of it (-85 to +4) are promising tumor selective promoters for adenovirus-based gene therapy for treating bladder cancer.

767. Ex Vivo Stable Transduce Full-length Dystrophin cDNA into Myoblasts Using HSV-1 Amplicon Vector

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Myoblast transplantation has the potential of restoring dystrophin transcription in dystrophic muscles. Although in animal experiments myoblast transplantation has shown promise, all of the human clinical trials have failed. The preponderance of evidence indicates that the immunoresponse against the transplanted heterologous myoblasts was the main cause of the failure. Transplantation of autologous human myoblasts that have been engineered ex vivo to express dystrophin would overcome this problem. However, transfection and integrating the 14 kb full-length dystrophin cDNA into primary myoblasts remains a challenge. In the present study we examined the efficacy of HSV-1 amplicon vectors, one of only three vectors which are capable of transferring full-length of dystrophin cDNA into dystrophic muscles, to stably transduce mdx myoblasts ex vivo with a full-length dystrophin cDNA driven by the muscle specific MCK promoter and enhancer. PHSVDGN contains a full-length dystrophin cDNA with MCK promoter/enhancer, as well as eGFP and a neomycin resistance cassettes driven by viral promoters. Its total size is 27 kb. The vector was packaged with the helper virus free HSV-1 packaging system with a reasonable titer (2×10^6 tu/ml in unconcentrated stock and 108 after concentration). The expression of dystrophin as well as GFP was confirmed by examining the differentiated mdx myotubes infected with the vector virions. The virions were used to infect mdx myoblasts at MOI 1 resulting in more than 60% of infected myoblasts expressing GFP 24 hours after infection. The GFP positive myoblasts were sorted by FACS then plated both with and without neomycin at 300 ug/ml concentration for 7 days. After 7 days the number of neomycin resistant GFP positive clones was the same with and without neomycin selection. analyzed by colony count of by FACS. In both groups 0.4-2% of the original myoblasts still express GFP. 6 of the GFP positive clones were isolated and 100% of the cells continued to express GFP until the culture was terminated (about two months). Some of these myoblasts were differentiated into GFP positive contracting myotubes. Immunohistochemistry revealed that all of these myotubes express dystrophin and that the variation in dystrophin expression from cell to cell appears to be related to the degrees of differentiation of individual myotubes. Our data challenges the traditional concepts of amplicon vector in at least two aspects. First the size limitation of the vector was believed to be less than 15kb but in our case vector PHSVDGN is 27 kb in size and contains three transcriptional cassettes. Second, HSV virion DNA was presumed to remain as an unstable episome in the nuclei of infected cells which would be diluted out during cell division. Our results show that as much as 2% of vector DNA can either be integrated into cellular genome or exists in some other condition which can be amplified during cell divisions. Also we report here, the first time, a possibility of ex vivo stable transduction of a full-length dystrophin cDNA into myoblasts. Its success has the possibility of changing autologous myoblast transplantation from hypothesis to practicality.

768. Treatment of a Mouse Model of Hemophilia A Using AAV Vectors Expressing Murine Factor VIII

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Hemophilia A is one of the most common coagulation disorders which affects approximately 20,000 males in the U.S. It results from a deficiency of the antihemophilic factor, factor VIII. Current treatment for hemophilia A involves replacement therapy using recombinant or plasma-derived factor VIII. Such therapy is limited by the production of factor VIII and the short half-life of factor VIII *in vivo*. Adeno-associated virus 2 (AAV) has many advantages as a viral vector for human gene therapy. AAV has proven to be an effective mode of factor IX gene therapy for the treatment of hemophilia B and recent work has demonstrated its potential usefulness for factor VIII deficiency (*Nat Gen*, 16:270-276, 1997; *Nat Med*, 5:56-63, 1999; *PNAS*, 96:12725-12730, 1999). The factor VIII cDNA cannot be efficiently packaged into recombinant AAV (rAAV) vector. To investigate the mechanism by which 2 rAAV vectors co-transduce a single cell, we have generated two separate AAV vectors to package the heterodimeric heavy and light chains of murine factor VIII individually. Using 2 rAAV vectors expressing green fluorescent protein (GFP) and yellow fluorescent protein (YFP) respectively, we have determined that the co-transduction rate is 50% of the single transduction rate by fluorescence activated cell sorting (FACS) analysis. By *in vitro* cell mixing experiments, we have shown that heterodimerization of the heavy and light chains of murine factor VIII does not occur extracellularly and that co-transduction of a single cell by both vectors is necessary for functional factor VIII production. *In vivo* intramuscular delivery of 10^9 cytomegalovirus immediate-early promoter (CMV)-driven rAAV constructs expressing murine heavy and light chains, respectively, resulted in subtherapeutic levels of functional factor VIII production, evaluated up to 6 months. Further *in vivo* analysis of AAV-derived murine factor VIII in the mouse model of hemophilia will test the ability of this system to generate therapeutic levels of factor VIII using 2 separate rAAV vectors as well as provide insight in the immunological effects of transgene expression in the mouse model of hemophilia A.

769. Recombinant Adenovirus Expressing AAV CAP and REP Supports Production of High-titer rAAV but Is Unstable

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It has been difficult to produce a chimeric vector containing both Ad and AAV rep and cap, and to grow such chimeric vectors in 293 cells. By recombination in bacterial host, we were able to make recombinant plasmid AdAAV, and then recombinant AdAAV was successfully generated in 293 cells. High titer of recombinant adeno-associated virus (rAAV) stocks were produced by co-infection of cells with two vectors. One vector was with a chimeric AdAAV, which contained the AAV rep and cap genes and all necessary adenovirus genes required for AAV replication. The second vector was an adenovirus in which the green fluorescent protein gene flanked by the terminal repeat ends of adeno-associated virus were cloned into the E1 site of adenovirus. Transfection of 293 cells with these two recombinant adenovirus vector resulted in the large scale, high titer production of recombinant adeno-associated virus expressing GFP. Recombinant AdAAV expressing rep leads to delay adenovirus replication but the titer was not affected eventually. However, a deletion within the AAV rep gene occurs after short term passage. These results indicate that co-transfection of 293 cells with 2 recombinant

AdAAV is a feasible method to produce high titer and pure rAAV, but passage is limited by non-homologous recombination within the AAV rep gene.

770. Correction of Chromosomal Insertion/Deletion Mutations by Adeno-associated Viral Vectors

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The treatment of genetic diseases by gene therapy typically involves the random integration or episomal expression of a functional transgene cassette. Research from this lab has shown that adeno-associated virus (AAV) based vectors can correct homologous human chromosomal sequences by gene targeting, which represents an alternative therapeutic approach. To determine the types of mutations correctable by AAV vectors, several insertion/deletion mutations were analyzed. The retroviral vector LAPIH and its derivatives were used to introduce target loci in human chromosomes. This vector contains an alkaline phosphatase (AP) gene under the control of the Moloney leukemia virus (MLV) LTR promoter and the internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV) which permits the translation of a hygromycin-B phosphotransferase gene. Insertion/deletion mutations (2 to 8 bp), which disrupted AP gene function, were made at the same position (bp 375) of the AP gene. Cells transduced by these vectors were selected by growth in hygromycin-B and the resulting polyclonal populations were derived from greater than 104 independent transduction events. The AAV-5'AP-Bss vector used for gene correction contains 2486 bp of sequence homology to the target loci (AP) and is not functional. For gene correction assays, fibroblasts containing target loci were seeded in 48-well plates on day 1, infected with AAV-5'AP-Bss vector on day 2, and on day 3 cells were treated with trypsin and dilutions were plated in larger dishes. Dishes were stained for AP expression on day 9 and the number of AP positive foci counted. Results indicate that the correction rates of deletions are significantly higher than the correction rates of insertions. In addition, small (2 or 4 bp) deletions have higher correction rates than the larger deletion (8 bp). These results suggest that mismatch repair proteins may be involved in gene targeting by AAV vectors and that they discriminate among different mutations in heteroduplexes. Current research includes evaluating smaller (1 bp) and larger (16 bp) insertion/deletion mutations and the use of mismatch repair mutant cell lines to identify the cellular gene products involved.

771. Herpes Simplex Virus LAT Intron as a Vehicle for Delivery of Biologically Active RNA Species to Neurons

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HSV can exist in a latent state in neurons for the lifetime of the host. During latency, the viral genome is stable, episomal, non-toxic and transcriptionally silent with the exception of the latency-associated transcript (LAT) loci. These genes, present in two copies, give rise to an unstable, untranslated 8.3kb primary transcript from which a 2.0kb intron is spliced. The intron accumulates as a stable lariat structure of unknown function. We have introduced sequences encoding various biologically active RNA species (BARS) into the 2.0kb LAT, enabling us to test two related hypotheses. First, the bioavailability of BARS might be improved by their incorporation into the stable lariat structure following the splicing event. Second, the LAT may provide a means of achieving long-term delivery of BARS *in vivo* as 'passengers' in the latent infection of neurons by disabled HSV vectors. As a first step towards addressing these questions, we have cloned genes encoding antisense and ribozyme RNA species directed against amyloid precursor protein (APP) into the 2.0kb LAT intron region

of HSV. APP is mutated in some forms of familial Alzheimer's disease and has been implicated in the pathogenesis of the common sporadic form. The LAT/BARS constructs are expressed under the control of a hCMV/LAP2 chimaeric promoter and have been inserted into a recombinant lentivirus to facilitate their manipulation. We have shown that the LAT region retains its unique splicing and stability properties outside the context of the HSV genome, and following insertion of therapeutic transgenes. Thus, a 2.0kb stable LAT intron containing BARS is detectable at high copy number in infected cells. Mutation of the consensus LAT splicing signals abolishes expression of this 2.0kb LAT intron, confirming that the detected species arises by a similar mechanism to that operating in wild-type HSV infection. Cells infected with the recombinant lentiviruses show a specific and marked reduction in the expression of APP protein in response to the presence of anti-APP BARS. Ongoing quantitative analysis aims to establish whether the BARS are more or less effective when incorporated into the LAT intron.

772. A Comparison of Two HSV-Based Gene Therapy Vectors for Immune Therapy of Leukemia

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HSV-based vectors have several biologic features well suited for gene therapy of leukemia/lymphoma. These include high transduction efficiency, ability to infect post-mitotic cells and a large packaging capacity. The recent development of Helper virus-free (hf) packaging methodology using the bacterial artificial chromosome (BAC) system has improved the potential for use of these vectors for clinical gene therapy. We have studied two Herpes Simplex virus (HSV) based amplicon vector systems for transduction of human leukemia. We constructed an HSV amplicon plasmid encoding either b-galactosidase or CD80 (HSVlac and HSVB7.1 respectively), packaged them using either a helper-based (HSVB7.1 or HSVlac) or helper-free system (hf-HSV-B7.1, hf-HSVlac), and compared the two vectors using human B-cell chronic lymphocytic leukemia (B-CLL) and chronic myeloid leukemia (CML) cells. Both vectors were highly efficient at transducing the target population of tumor cells. Using either helper containing or helper free amplicon vector we obtained transduction efficiency exceeding 80% using b-galactosidase as a marker for gene transfer and in the 60-90% range for CD80 (B7.1) detected by flow-cytometry. We studied cells transduced with these two viruses for viability and maintenance of MHC-I expression. We found that cells transduced with helper-containing viral stocks were associated with more significant cytotoxicity and loss of MHC-I expression compared to hf-HSV transduced cells. The cytotoxicity observed for helper containing amplicons is presumably related to the pro-death effects of the immediate early proteins, while loss of MHC-I expression may be due to the effects of the ICP-47 gene product encoded by the helper virus. CLL cells transduced with the CD80 (B7.1) cDNA using either the helper containing or helper-free HSV vectors were then used as stimulator cells in an allogeneic mixed lymphocyte-tumor cell (MLTR) reaction. Although both amplicon preparations were equally efficient at expressing CD80 (B7.1) as detected by flow-cytometry, only cells transduced with the helper-free virus were successful at inducing a T-cell proliferative response as measured by ³[H] thymidine incorporation as well as inducing IL-2 secretion as detected by ELISA. Providing signal one by adding PMA restored both the proliferative T-cell response as well as IL-2 secretion. We hypothesize that either the loss of MHC-I expression, direct cytotoxicity of the helper virus to the T-cells, or possibly perturbation of MHC-II antigen presentation might explain the requirement of PMA for proper T-cell activation using helper containing HSVB7.1 amplicon. We conclude that hf-HSV is a preferable

vector for gene transfer into leukemia cells with excellent potential for use in immune therapy-based translational clinical trials.

773. Metabolic Biotinylation of Recombinant Proteins for Viral Gene Therapy Vector Targeting

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In vitro biotin labeling has been applied to a variety of drug targeting and viral gene therapy vector targeting strategies. While the existing biotinylation technology is certainly robust, it is limited since the *in vitro* labeling procedure is not site-specific, requires purification of the substrate, and can lead to inactivation of the biotinylated protein. To circumvent this limitation, we have demonstrated the ability to metabolically biotinylate tagged proteins in mammalian cells and in mice using the endogenous biotinylation enzymes of the host (*Mol Therapy*1(1): 96-104).

This technology may be particularly useful for cell targeting applications in which proteins or viral gene therapy vectors can be biotinylated at genetically-defined sites for combination with other targeting moieties complexed with avidin. For viral vector targeting strategies, it is possible to biotinylate capsid proteins of both enveloped and non-enveloped viral vectors, which would allow for the incorporation of targeting moieties linked to the capsid proteins via avidin. To test this application of the technology, we have demonstrated the ability to metabolically biotinylate the fiber protein of adenovirus type 5 at various sites with a minimized biotin acceptor tag. The fiber protein is the cellular attachment protein of adenovirus type 5 which is expressed in a trimeric form on the surface of the virus. Some C-terminal additions and insertions into the fiber protein have been shown by us and others to interrupt trimerization and prevent the formation of adenoviral particles containing the fiber protein. By constructing a series of biotinylation tags that were inserted into the C-terminus and HI loop of the adenoviral fiber, we were able to show that a subset are biotinylated and trimeric. Work is underway to produce biotinylated adenovirus with these modified fibers shown to be biotinylated and trimeric in transient transfection of mammalian cells. A similar strategy for the production of biotinylated envelopes for retroviral and biotinylated capsid proteins for adeno-associated virus is also being tested.

To target retroviruses, we have demonstrated the ability to biotinylate proteins targeted for secretion. In this manner, viral envelope proteins such as the VSVG protein into which biotin accepting domains have been incorporated have been shown to be metabolically biotinylated and targeted to the cell surface. On the cell surface, the biotinylated envelopes can be complexed with avidin to allow for the attachment of biotinylated targeting moieties. The biotinylated envelop proteins are now being used to pseudotype retroviral vectors in an attempt to demonstrate the usefulness of this technology to target enveloped viral vectors.

Further development of this project is centered on producing the various types of viral vectors that can be created with this technology and demonstrating their ability to be targeted with various proteins, peptides, and antibodies complexed with avidin.

774. Transduction of Human PBMC-derived Dendritic Cells by Recombinant Adeno-associated Virus Requires High Multiplicities of Infection: Analysis of Mechanism and Implication for Gene Therapy

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Dendritic cells (DC) are among the most potent antigen-presenting cells known and play an important role in the initiation of antigen-specific T-lymphocyte responses. DC therefore

have great relevance to gene therapy, both as potential targets for genetic manipulation and as biological mediators of undesired immune responses. Accordingly, understanding the nature of interactions between specific vector systems and DC is of great practical importance. The capacity of recombinant Adeno-Associated Virus (rAAV) vectors to mediate stable long-term expression of foreign proteins, such as bacterial beta-galactosidase, in the muscle of immunocompetent mice has been hypothesised to reflect inefficient transduction of murine DC by rAAV (Jooss *et al.* J. Virol 1998, 72:4212-4223). We therefore set out to determine whether human myeloid DC might exhibit a similar phenotype and, if so, to investigate the underlying mechanism. Human DC were derived from the adherent fraction of healthy donor PBMC and characterised as previously described (Diao *et al.* Gene Ther. 1999, 6:845-853). Using rAAV vectors encoding either green fluorescent protein or bacterial beta-galactosidase, under the transcriptional control of a CMV immediate early promoter, we were unable to transduce PBMC-derived DC, at a limit of sensitivity of one event per 3000 DC, at multiplicities of infection (MOI) of less than 500 transducing units per cells (titer assigned on 293 cells). At higher MOI however, transduction events became evident, with a 10% transduction rate achieved 4 days after exposure to vector at an MOI of 10,000. Transgene expression was followed for up to 16 days and found to remain relatively stable. Similar results were achieved using DC from three unrelated healthy donors, and on DC matured by TNF- α treatment. As a first step towards analysis of the mechanism(s) underlying this resistance to rAAV transduction, we exposed DC to gamma-irradiation (2500 rads). This treatment markedly enhanced transduction efficiencies, but only at high multiplicities, and there was no downward shift in the minimal MOI required for transduction (500). This result suggested that a threshold MOI must be reached before gamma-irradiation can exert an effect. We next examined the effect of super-infection with the replication competent adenovirus, *dl309*. Again marked enhancement of rAAV transduction efficiency was observed and, in contrast to the effect of gamma-irradiation, there was a 10-fold reduction in the minimal MOI required for transduction (50). Interestingly, the effects of *dl309* infection and gamma-irradiation were additive, even at MOI below the threshold of detection in DC receiving gamma-irradiation alone. Super-infection of DC with a replication-defective adenovirus vector or treatment with chloroquine had only a minor effect on transduction efficiency, suggesting that some rAAV is trapped in the endosomal compartment. Finally, immunohistochemical and FISH analyses were performed to determine whether rAAV capsid and genomes could be detected within vector-exposed but untransduced DC. As previously found in mouse DC, rAAV capsid and genomes were found in abundance in a perinuclear pattern, and after *dl309* treatment intranuclear genomes could be detected in some cells. These data allow the formulation of a model, the implications of which will be discussed.

775. WITHDRAWN

776. Towards Integrating Vectors for Gene Therapy: A Study on the Feasibility of Employing the Bacteriophage Mu Integration System for the Insertion of Transgenes in Mammalian Cells

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One of the reasons why retroviruses attracted much interest as vectors for gene therapy is their capacity to insert transgenes

into the genome of the target cells. This ensures persistence of the transgenes in rapidly dividing cells, which is essential for long-term expression. Since the use of retroviral vectors is limited to dividing cells, we sought alternative systems for the integration of transgenes into mammalian genomes. To that end, we adapted the integration machinery of bacteriophage Mu for use in mammalian cells. The integration of the Mu genome in its natural host depends on two phage factors, the MuA and MuB proteins, which recognize the *Att* sites in the viral genome and catalyse integration of the Mu genome. We cloned the MuA and MuB genes in an expression vector already carrying a CMV promoter and extended the coding regions with a sequence encoding a FLAG epitope and an SV40-derived nuclear-location signal. These N-terminal extensions were sufficient to target the MuA and MuB proteins to the nucleus (as demonstrated by immunohistochemistry) and did not impede the function of MuA and MuB, as was demonstrated by their capacity to complement mutations in the MuA and MuB genes of Mu in *E. coli*. As a donor, a miniMu vector was created which, between two *Att* sites, carries between two *Att* sites the neomycin-phosphotransferase gene driven by a SV40 and a TN5 promoter which confer G418-resistance in mammalian cells and kanamycin resistance in *E. coli*, respectively. Transfection of the miniMu-neo plasmid together with the MuA and MuB expression vectors resulted in a slight (40-60%), but significant increase in the number of G418-resistant colonies in human cell lines, when compared to control experiments in which the MuA plasmid was omitted. We cloned 30 integrated miniMu-neo vectors, together with flanking chromosomal DNA. In these clones, the junction between the miniMu vector and the chromosomal DNA is being sequenced and analysed for the target-site duplication that is characteristic for the integration mediated by the bacteriophage Mu machinery. Taken together, our data show that also these tools from the prokaryotic world may be useful for gene therapy.

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777. Baculovirus-mediated Extravascular Gene Transfer into Rabbit Carotid Artery

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Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) has been shown to transduce mammalian cells in vitro. Since baculoviruses offer many advantages over other viruses, we have studied a possibility to use these viruses for in vivo gene transfer by constructing a baculovirus bearing beta-galactosidase (*lacZ*) marker gene under a CMV promoter. With this virus 90.6 $\pm$ 1.6% of rabbit aortic smooth muscle cells (RAASC) and 20.8 $\pm$ 7.7% of human ECV-304 cells were transduced at moi 1000 in the presence of 10mM butyrate. Transfection efficiency was evaluated in vivo by applying 1 X 10⁶ pfu of *lacZ* baculoviruses and adenoviruses in a silastic collar placed around rabbit carotid arteries. It was found that baculoviruses were capable of transducing adventitial cells of rabbit carotid arteries in vivo with efficiency comparable to adenoviruses. The betagalactosidase gene expression was transient staying at high level for at least one week. At 14 days time point only a few positive cell could be detected. The arterial structure and endothelium remained intact in the baculovirus-transfected arteries, but signs of inflammation were detected by macrophage and T-cell specific immunostainings. Baculoviruses can thus mediate an efficient transient gene transfer in vivo and may become a useful tool for gene therapy of human cardiovascular diseases.

778. A Novel and Efficient Method for Large-scale Production and Purification of Recombinant Adeno-associated Virus Vectors

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A simple, high-yield and large-scale method for recombinant adeno-associated virus (rAAV) production and purification is described. To produce the rAAV vectors, a two-component strategy, thus proviral cell line/ helper virus, was designed and shown being able to generate rAAV stocks with high yields (up to 250 transducing units/producer cell). The proviral cell line was established by transducing BHK-21 cells by a recombinant plasmid derived from pSV2neo with AAV two ITRs and in between the exogenous gene expressing cassette inserting at its BamHI site. The helper virus is a recombinant herpes simplex virus expressing Rep and Cap proteins of AAV. To purify the viral stocks, a three-step protocol was developed including chloroform treatment, PEG/NaCl precipitation and chloroform extraction. Using such two-component production and three-step purification method, we can reproducibly obtain, from 2×10^9 of proviral cells cultured by roller bottles, purified rAAV-GFP stocks with titers of $10^{13} \sim 10^{14}$ particles/mL and purity of greater than 95%. The infectious titers of the vector stocks were up to 2×10^{12} TU/mL, thus less than 100 viral particles per transducing unit. The purified rAAV-GFP stocks were successfully used in transducing and expressing GFP gene in tissues including skeleton muscle, retina and liver. Therefore, this new method offers a simple, rapid and cost-effective way for large-scale rAAV production and purification. Further purification approach should be made to remove the residual chloroform left in the stock.

779. Mutational Fine-mapping of the Amino Terminus of Herpesvirus gD Involved in HveA Receptor-dependent Virus Entry

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HSV vectors may be improved by retargeting virus infection to cells where the transgene can be most effective. Retargeting will require modifying the functional components of the virus envelope to alter the virus host-range. As a prelude to accomplishing this long-range goal, we have been dissecting the functional elements of the viral envelope glycoprotein D that are essential to virus attachment and entry. Following virus attachment to the cell surface, the second stage of HSV infection is fusion of the viral envelope with the cell membrane resulting in virus entry. Binding of gD to the cellular receptors HveA or HveC is believed to trigger fusion and is thus required for entry, but it is uncertain whether the involvement of gD extends to the fusion process itself and if it does, whether receptor binding and fusion are separable. To address these questions, we have begun to map the binding and putative fusion domains of gD using a transient assay measuring phenotypic rescue of a gD-null virus for entry into gD-complementing VD60 cells, HSV-resistant CHO cells (negative control), and HveA- or HveC-expressing CHO cells. From a deletion scan of the N-terminal region of gD, the picture is emerging that the first 24 amino acids are essential only in HveA binding but not entry via HveC binding. The next residues to position 32 play a role in HveC binding and increasingly in entry through HveC. This is followed by a highly rigid region including positions 32-40 where mutations abolish all entry activity, and finally a second transition region with evenly diminishing involvement in entry through both HveA and HveC. Individual saturation mutagenesis of positions

28-32 in the first transition region indicates that all five of these positions are important for entry through HveA while only positions 30 and 31 appear to be involved in HveC-dependent entry. Random single amino acid changes at positions 6-9 were found to reduce or abolish HveA-dependent entry without affecting entry through HveC. These results indicate that the N-terminal region of gD has a high density of residues that contribute in some manner to HveA-dependent entry, suggesting that this region assumes a unique tertiary structure that is readily disturbed by sequence changes. Since most of the mutants are functional for HveC-dependent entry, the defects in HveA-dependent entry are most likely due to defective binding. Studies are in progress to determine the end of this binding domain and the role of the neighboring region in entry through either receptor.

780. Directed Evolution of Phage Vectors for Therapeutic Gene Delivery

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We have successfully engineered filamentous bacteriophage for targeted gene delivery to mammalian cells via receptor-mediated endocytosis (1,2). The phage are modified by insertion of a mammalian expression cassette such that when targeted with an appropriate ligand (i.e. FGF2, EGF), they transduce mammalian cells. Like transfection with animal viral vectors, targeted phage gene delivery is concentration, time, and ligand dependent. It also is specific for the ligand's cognate receptor on the target cell. However, phage have distinct advantages over existing gene therapy vectors. They are simple and economical to produce at high titer, have no intrinsic tropism for mammalian cells, and are relatively simple to genetically modify and evolve. Accordingly, we reasoned that genetic selection could be applied to phage particles to direct their evolution towards a more efficient and safe vehicle for gene therapy. To this end, we developed a genetic screen to select for phage that display a functional targeting ligand. In this model system, epidermal growth factor (EGF) targeted phage can be enriched 1 million fold after 3-4 rounds of selection on receptor bearing cells (3). This system termed, LIVE (Ligand Identification Via Expression), has been used successfully to identify peptides that can mediate phage gene delivery to COS cells. Initially, the transduction efficiency of targeted phage was about 1-2%. To improve our ability to genetically select phage directly from complex libraries, we attempted to increase transduction efficiency by modifying both the transfection protocol and vector design. For example, we find that genotoxic treatments more than double the transduction efficiency of targeted phage, presumably by enhancing conversion of single-stranded to double-stranded DNA. In addition, we created new phage-based vectors that are 100 times more potent and achieve transduction efficiencies of up to 20% on some human cell lines. Taken together, these studies establish that we can significantly improve phage efficiency through genetic modification. The ability to use selective pressure to create new vectors for gene therapy represents a significant advance over existing viral and non-viral vector strategies that rely on rational design. We are currently applying this directed evolution approach to overcome some of the common hurdles that impede successful gene therapy such as immunogenicity, toxicity, stability, purity, and target specificity.

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781. Dual Roles for Cre-lox Recombination in a Recombinant AAV Packaging System

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We have developed a new method to produce recombinant AAV on a commercial scale. To date the approach for making recombinant AAV producer cell lines is straight from the retroviral vector handbook — cut the origins and packaging sequences off the viral genes and shove them into cells with a selectable marker. This approach has proven far less successful with AAV than retroviruses. We are presenting a different tactic that uses the ability of AAV to readily integrate into host chromosomes without causing harm to its host cells. In other words, AAV creates packaging cell lines during a normal infection. There are several problems associated with using a replicating virus as the packaging construct. Chief among them is that there cannot be any replicating virus in the product. A less obvious, but equally severe problem is that wild type AAV replicates its own genome much more efficiently than a co-infected rAAV. We have found a way around both of these problems using bacteriophage recombinase. The basis of our system is an AAV with loxP sites inserted between the ITR's and the AAV genes. This floxAHV grows normally but does not preferentially replicate itself over rAAV. We infected HeLa cells with floxAHV and rAAV, quickly generating a producer cell population. When we infected this population with Ad and an Ad vector making Cre recombinase, it made 50 infectious rAAV particles per cell (transduction assay) and no detectable floxAHV (blot). During our analysis, we found a surprising expression switch whose function may help to explain the low efficiency of conventional approaches. We will present data to support our unique mechanism for rAAV production.

782. Modifications to the Capsid Proteins of Adeno-associated Virus (AAV) for Targeting Gene Therapy *In Vivo*

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One of the challenges in the development of vectors for gene therapy is being able to target a therapeutic gene preferentially to diseased cells. Systemic delivery of recombinant adeno-associated virus (rAAV) results in a broad distribution of the vector throughout the body. This is an undesirable feature, because it may potentially lead to expression of the delivered gene in irrelevant organs. Peptides that recognize specific receptors on the surface of vascular endothelial cells have been previously isolated by *in vivo* selection of phage display libraries. These “homing” peptides have the ability to deliver compounds to specific organs or tissues, by targeting receptors expressed exclusively in their vascular endothelium. In order to target rAAV to specific organs or tissues *in vivo*, we have molecularly engineered the capsid of AAV to include such “homing” peptides. We chose two different sites within the VP3 protein of AAV-2, based on extensive sequence analysis of non-conserved regions between different parvoviruses. The “homing” peptides inserted aim to target rAAV to lung, muscle, or tumor. We have produced the recombinant viruses in 293 cells, and purified them by iodixanol gradient centrifugation. Genomic titers were determined by dot blot hybridization. In some cases, insertion of amino acid sequences in the VP3 capsid protein altered expression levels in the producer cells, as observed by western blot analysis, and therefore yielded lower titers. Moreover, some of the modifications caused an apparent deficiency in DNA packaging. We found that regardless of the inserted amino acid sequence and the site of insertion, most peptide-containing viruses were severely impaired in their ability to transduce susceptible cells, such as 293 and HeLa cells. Therefore, in order to improve the transduction efficiency of these vectors, we have begun to characterize residues in the

capsid proteins, which are important for packaging and uncoating. The role of VP3 sequences in packaging and the use of modified capsid for targeting cells expressing specific receptors will be discussed. Specific targeting of rAAV *in vivo*, will allow for improved safety and efficiency of this viral vector.

783. VSV-G-mediated Rescue of the Attachment and Entry-defective Phenotype of an HSV Vector Deleted for the Essential Envelope Glycoprotein D

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The vesicular stomatitis virus glycoprotein G (VSV-G) has been employed to pseudotype both retroviral and lentiviral vectors in order to increase the stability of these particles and alter the host range of the vectors. The process of attachment and entry of these viruses is normally controlled by the two glycoprotein products of the ENV gene. HSV attachment and entry is a more complex process involving multiple viral glycoproteins and several cellular receptors. HSV glycoproteins B, C, and D (gB, gC, and gD) interact with cell surface receptors prior to a fusion event between the viral envelope and the cell surface membrane. Fusion requires the cooperative action of the essential HSV glycoproteins B, D and H (gB, gD, and gH). gD plays the major role in entry process by binding to either the HveA or HveC receptors, thereby controlling viral tropism. Efforts to re-target HSV vectors thus must either alter the ability of gD to bind to its cognate receptor or delete the molecule entirely and replace it with another molecule capable of binding to a cell-specific receptor and initiating the entry process. Thus, we first set out to determine whether full-length wild-type VSV-G or chimeras with HSV gD or gB were incorporated into virions. Transient transfection-infection analyses showed that the full-length VSV-G molecule was adequately incorporated into mature virions and was able to pseudotype virus deleted for HSV gD. Homologous recombination of VSV-G into an HSV vector deleted for gD yielded a virus that could bind to and enter Vero cells, however an abortive infection ensued following entry of the particles into endosomes with degradation of the particles at the low pH found in lysosomal structures. The use of lysotrophic agents such as chloroquine, protamine sulfate or polybrene resulted in escape of the VSV-G containing HSV virions from endosomes and the production of progeny virions and plaques. In addition, co-infection with replication-defective (E1/E3) adenovirus also resulted in a productive infection in Vero cells. Thus, by raising the pH of the endosomal/lysosomal structures we enabled fusion of the virion with the endosomal membrane. Since the fusion function of VSV-G is only active at low pH, another molecule besides VSV-G must be responsible for fusion, either gB or gH. We are currently evaluating the role of these two HSV glycoproteins in this process using temperature-sensitive mutants and through the use of fusion-deficient VSV-G mutants. This represents the first demonstration that another molecule can substitute for gD in the binding and entry of HSV particles and suggests that other molecules or ligands may be incorporated into the HSV particle to further target virus infection.

784. Simple and Effective Purity Assay for Viral Vectors

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The lack of simple and effective methods to assess the relative purity of viral vectors poses a major obstacle for human gene therapy. A number of traditional biochemical techniques are currently being applied to characterize these complex macromolecules, i.e. SDS-PAGE, ion exchange HPLC, and more recently reverse phase HPLC, and capillary electrophoresis. However, it is often essential to use additional orthogonal separation and/or detection techniques to provide positive sample identification,

and frequently only a partial answer is obtained. We have developed a mass spectrometry-based assay that allows for the direct identification of the individual protein components of a viral particle as well as potential contaminants, in a matter of minutes. The ion data was collected on a matrix assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF) in linear mode. The high sensitivity and good signal-to-noise ratio of these instruments allows for a comprehensive analysis of an adenovirus vector at low particle numbers. The ability to further select ions based on their time-of-flight (timed-ion-selection) allows for direct micro sequencing by post-source-decay (PSD) analysis on small peptides that are only available in very small amounts. Utilizing this assay to compare a number of samples obtained from various purification methods has allowed us to establish a suitable reference standard to which all vectors can be measured against. Furthermore, quantitative results can be obtained by combining this assay with reverse phase HPLC separation of the individual vector proteins.

785. Site-specific Integration by Adeno-associated Virus (AAV): Minimal Requirements for Target Site Selection

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AAV is unique among eukaryotic viruses in its ability to integrate preferentially into a specific locus on human chromosome 19 (the AAVS1 locus). Two elements of AAV are required for targeting integration: the AAV Rep proteins and the viral inverted terminal repeats (ITRs). Rep proteins were shown to bind to a specific Rep recognition sequence (RRS) present in the viral ITR. The same recognition sequence was also found in the AAVS1 locus and we previously showed that Rep can form a bridge between the viral ITRs and the RRS in the pre-integration locus. Rep proteins are also involved in DNA replication and regulation of gene expression during the viral life cycle. Since Rep has been associated with cytostatic phenotypes, the use of the full length protein may not be desirable for targeting integration. However, the application of isolated Rep protein domains in chimeric proteins could bypass this problem. In order to define the minimal requirements for Rep to recognize its target site, we developed one-hybrid assays in which DNA-protein interactions are detected *in vivo*. Chimeric proteins consisting of the N-terminus of Rep fused to different oligomerization motifs and a transcriptional activation domain were analyzed for oligomerization, DNA binding and activation of reporter gene expression. Expression of reporter genes was driven from RRS motifs cloned upstream of minimal promoters and examined in mammalian cells and in *Saccharomyces cerevisiae*. Our results show for the first time that chimeric proteins containing the N-terminus of Rep are able to target the RRS *in vitro* and *in vivo* in the context of an artificial multimer. We are currently examining the minimal DNA sequence requirements necessary for site-specific integration in an *in vivo* integration assay. Together these studies will identify the minimal elements mediating site-specific integration and may allow this unique feature of AAV to be harnessed for gene delivery.

786. Recombinant Human Parvovirus B19 Vectors: Erythrocyte P Antigen is Necessary but not Sufficient for Successful Transduction of Hematopoietic and Non-Hematopoietic Human Cells

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Parvovirus B19 is known to cause impairment of erythropoiesis in humans due to its lytic infection of progenitor cells in the erythroid lineage. The blood group P antigen, known to be abundantly expressed on cells in the erythroid lineage, has been

reported to be the cellular receptor for parvovirus B19. We have described the development of recombinant parvovirus B19 vectors with which high-efficiency, erythroid lineage-restricted transduction can be achieved (J. Virol., 72: 5224-5230, 1998). However, a low-level transduction of non-erythroid cells could also be detected. Since P antigen is expressed in non-erythroid cells, and since additional clinical pathological studies suggest the presence of parvovirus B19 in non-hematopoietic human cells, we re-evaluated the role of P antigen in parvovirus B19 vector binding and entry into cells. A recombinant vector containing the cytomegalovirus immediate-early promoter-driven enhanced green fluorescent protein [EGFP] reporter gene in an adeno-associated virus 2 (AAV) genome was used to infect primary human low-density bone marrow mononuclear (LDBM) cells that had been separated into erythroid and non-erythroid populations using glycophorin A+ conjugated beads. Consistent with our previous studies, ~79% of cells in the erythroid fraction showed transgene expression 48 hrs post-infection, compared with ~13% in the non-erythroid fraction. Cell surface expression revealed that ~75% of glycophorin A+ and ~31% of glycophorin A- cells were P antigen-positive. Two human erythroleukemia cell lines, HEL and K562, and a human promyelocytic leukemia cell line, HL-60, were also examined for P antigen expression and binding and entry of recombinant parvovirus B19 vectors. HEL and K562 cells showed intermediate levels, whereas HL-60 cells demonstrated high levels of expression of P antigen. However, the efficiency of binding of 35S-methionine labeled vector to these cells did not correlate with P antigen expression. Moreover, despite P antigen-positivity and efficient viral binding, HEL, K562, and HL-60 cells could not be transduced with the recombinant vector. These studies were extended to include two primary cell types, human umbilical vein endothelial cells (HUVEC) and normal human lung fibroblasts (NHLF). Low-levels of P antigen expression could be detected in both cell types. In addition, binding of the recombinant vector occurred in both cell types and was reduced by ~17% in HUVEC and ~48% in NHLF, respectively, upon competition with globoside, indicating the involvement of P antigen in virus binding to these cells. In contrast to the established cell lines, these primary cells could be efficiently transduced with the recombinant vector. Taken together, these data suggest that (i) P antigen is expressed on a variety of cell types and is involved in binding of parvovirus B19 to human cells, (ii) The level of P antigen expression does not correlate with the efficiency of viral binding, (iii) P antigen is necessary but not sufficient for parvovirus B19 entry into cells, and (iv) Parvovirus B19 vectors can be used to efficiently transduce HUVEC and NHLF in addition to erythroid cells in LDBM cells. These studies further suggest the existence of a putative cellular co-receptor for efficient entry of parvovirus B19 into human cells.

787. Site-specific Integration Mediated by an Adeno-associated Virus/Adenovirus Hybrid Vector System

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Effective gene therapy requires a vector system that will deliver a transgene at high efficiency and enable prolonged gene expression, without eliciting an immune response. Our aim is to create a hybrid vector system that combines the high transduction efficiency of adenovirus, with the ability of adeno-associated virus (AAV) to site-specifically integrate a transgene, thus avoiding the necessity for readministration. AAV integrates into a specific site (AAVS1) on the short arm of chromosome 19. This integration event is mediated by AAV Rep gene products, Rep 68 or Rep 78. The Rep proteins function by binding and nicking DNA at recognition sites within both the AAV genome (at inverted terminal repeats) and at the AAVS1, and inducing a recombination event. Two adenoviral vectors expressing Rep from a T7 promoter were constructed and used to characterise site

specific integration. The first vector encodes the wt Rep gene and expresses all four Rep proteins: Rep 78, 68, 52 and 40. The second virus contains a mutant Rep gene and only expresses Rep proteins 78 and 68. HeLa cells were infected with these vectors and subsequent Western blotting experiments showed that in the absence of T7 polymerase, Rep was not only detected, but its level of expression was proportional to the intensity of adenoviral infection. To study the biofunction of Rep proteins, HeLa cells were infected with the Rep-expressing adenoviral vectors and then transfected with plasmid containing a GFP transgene flanked by AAV inverted terminal repeats (ITRs). Clonal isolates were picked and cultured for 5 weeks. PCR analyses of the clonal genomic DNA samples indicate that Rep expression is required for the persistence of GFP transgene expression. Southern blot analyses suggest that GFP persistence is due to the ability of Rep to induce the site specific integration of the GFP transgene. In addition, the integration event may be more efficient in the presence of the mutant Rep adenovirus vector (expressing only Rep 68 and 78) than with adenovirus expressing all four Rep proteins. Current studies are being undertaken to characterise the rescue of an ITR-flanked GFP transgene from adenoviral vectors. Subsequently, the aim is to compare the integration efficiency of ITR-flanked GFP with the integration of a GFP gene which is flanked by modified ITR sequences.

788. Helper Virus-free HSV/AAV Hybrid Amplicon Vector-mediated Transgene Integration into the Genome of Mouse Fibroblasts

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Helper virus-free HSV amplicon vectors have large transgene capacities, high infectibility, low pathogenicity, and the propensity to infect both mitotic and postmitotic cells. HSV/AAV hybrid amplicons were designed to mediate transgene integration into the mammalian genome. Previous studies have shown extended transgene expression in dividing cells with this hybrid vector as compared to the HSV amplicon (Johnston et al., 1997). The HSV/AAV hybrid vector used in this study contains a transgene cassette with a GFP gene driven by the CMV promoter and a neomycin resistant gene driven by the SV40 promoter, both flanked by partial AAV ITRs (inverted terminal repeats; Costantini et al., 1999). The aim of this study was to examine whether the HSV/AAV vector can facilitate the integration of transgenes into the genome of immortalized mouse fibroblasts (generation time 24 hours). Fibroblasts were infected with helper virus-free HSV or HSV/AAV vectors at MOI 2, and 24 hours after infection the cells were cloned under G418 selection. After two weeks the number of resistant colonies were three times greater in cells transduced with the HSV/AAV vector as compared to HSV vector.

After expansion of clones in continued selection for 3 months the number of surviving clones was 35-fold greater in cells transduced with HSV/AAV (7% of transduced cells) than with the HSV vector (0.2% of transduced cells). Southern blot analysis was used to determine whether the transgene cassette had integrated into mouse genome. Genomic DNA was extracted from the stable clones, digested with Hind III (which cuts only once in the HSV/AAV amplicon), resolved by agarose gel electrophoresis, blotted into the nylon membrane, and hybridized to 32P-GFP probe. In all clones examined, hybridization with the GFP probe showed at 12.5 kb band (the size of the hybrid amplicon) of variable intensity and many clones showed bands of varying sizes indicating junctions of transgene integration into the genome. To confirm the integrated rather than episomal state of the transgene cassette in the mouse genome, genomic DNA was either not digested with any enzyme or digested with Asc I (which does not cut the hybrid amplicon DNA). Pulse field gel electrophoresis was used to separate large fragments of genomic

DNA, and membranes were hybridized to a 32P-GFP labeled probe. The undigested genomic DNA from clones infected with HSV/AAV showed a single band retained in the wells with no band in the ~150kb range- the size of packaged amplicon DNA. In contrast, genomic DNA cut with Asc I showed a smeared band in the range of 200-300 kb (with 100 kb being the average frequency of Asc I sites in mouse genome). These data provide evidence that the HSV/AAV amplicon vector can facilitate genomic integration into the mouse genome.

789. Scale-up of Recombinant Adeno-associated Virus (rAAV) Gene Therapy Vector Production and Purification

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Recombinant adeno-associated virus (rAAV), as a gene delivery vector, has demonstrated considerable promise for human gene therapy. Many studies have indicated that rAAV mediates efficient gene delivery to tissues as well as long-term gene expression. However, efficient technology for large-scale production and purification of rAAV remains a major obstacle for clinical applications. We previously described a method to generate rAAV using transfection of cells with plasmids expressing required helper functions, in the absence of both adenovirus and replication competent AAV (rcAAV). One of our current goals is development of clinical gene therapy for hemophilia. In efforts to move rAAV gene therapy to large-scale clinical applications, we have developed a new strategy for rAAV production and purification. Using a fully scaleable adherence cell culture system, we have developed a process resulting in vector production at the level of 10^4 - 10^5 viral genomes per cell. In support of this high yield rAAV vector production process, we have developed an efficient, fully scaleable column chromatographic purification process resulting in highly purified vectors. Our data indicated that this novel process will enable the production of rAAV in quantities sufficient to support our planned clinical applications.

790. Quantitation of Residual Host-cell DNA in Viral Gene Therapy Vectors

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For reasons of safety and tolerance, the manufacturing and purification of recombinant viral vectors must include assays to quantitatively measure amounts of residual host-cell contaminants including host-cell proteins and nucleic acids. Typically, these assays may be used as lot release assays on the final product; they may also be used during the purification process to monitor the clearance of residual contaminants through the various steps.

With regard to contaminating host cell DNA derived from the cell substrate used for vector production, acceptable limits are not specified by the current FDA "Guidance for Human Somatic Cell Therapy and Gene Therapy". One hundred pg of DNA per dose, which is the suggested acceptable limit in the manufacturing of monoclonal antibodies, is often used as a reference for the manufacturing and testing of viral vectors for gene therapy.

We have developed a rapid and sensitive assay to address the need to accurately detect and quantitate residual host cell DNA during downstream purification of recombinant adenoviral vectors and in final formulated products. The assay is based on real-time quantitative polymerase chain reaction (TaqMan PCR) amplification of a sequence in the human endogenous retrovirus (HERV-H) family. Each diploid human cell contains approximately 2,000 copies of the HERV-H sequence, dispersed over several chromosomes. The presence of numerous copies of HERV-H in each genome confers increased sensitivity to the assay. Additionally, since HERV-H sequences are present on most chromosomes, the assay is applicable to human cell lines with different karyotypes. The assay is compatible with several final

formulations, which allows for direct testing of final product without manipulations as a lot release assay. In the case of in-process samples, standard molecular biology techniques are used to extract and purify DNA from aliquots taken at specific steps during the purification scheme, which consists of a series of filtrations and column chromatography with final elution in a formulation buffer. Using genomic human DNA as a standard, we have determined that the assay can reliably detect 40 pg of human genomic DNA in one ml of Adenovirus formulation buffer. This assay may also be used to measure host cell DNA contaminants in the production of recombinant lenti and adeno-associated viral vector lots.

791. Contribution of Rep and Cap Gene Levels to AAV Vector Production

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Adeno-Associated Virus (AAV) has attracted significant attention as a gene therapy vector. Its advantages include: stable integration, long-term gene expression, evasion of host immunity, inability to replicate autonomously, and a lack of any known pathogenicity. However, a major obstacle in the development of AAV vectors is the inability to produce recombinant virus in high titer. We have used various methods to alter levels of AAV replication (Rep) and capsid (Cap) proteins to investigate whether rAAV titers are limited by the copy number of these genes. First, AAV elements, including Rep, Cap, and an *itr* flanked transgene, were cloned into Adenovirus gene expression vectors in order to generate an all-infection system for AAV vector production. To combat Rep induced toxicity during the Adenovirus production process, the T7-phage inducible promoter system was used to generate a Rep expressing Ad vector. The Cap expression vector contains the CMV promoter, and the *itr* flanked transgene vector contains GFP as a reporter gene. Additionally, we generated rAAV with either a standard helper plasmid (pAAV/Ad) or in the presence of replication competent wtAAV. We found similar levels of recombinant virus replication and encapsidation in all cases. Furthermore, increased copy number of Rep and Cap did not significantly change the levels of any replication intermediates, including monomer and dimer replication intermediates. Analysis of DNase resistant virus particles indicated that Rep/Cap copy number contributes minimally to the total number of DNA containing particles present. These studies have led us to investigate the possibility that defective particles may interfere with rAAV vector production.

792. AAV-mediated Transduction of Murine Hematopoietic Progenitor Cells *In Vitro* and *In Vivo*

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Although adeno-associated virus 2 (AAV) has gained attention as a potentially useful alternative to the more commonly used retrovirus- and adenovirus-based vectors for human gene therapy, we have accumulated evidence to suggest that efficient gene transfer and transgene expression by AAV vectors require that the following two obstacles be overcome. First, the target cell must express the receptor and the co-receptor for AAV infection (J. Gen. Virol., 77: 1111-1122, 1996; Nature Med., 5: 71-77, 1999), and second, the cell must allow for viral second-strand DNA synthesis (Proc. Natl. Acad. Sci. USA, 94: 10879-10884, 1997; J. Virol., 72: 1593-1599, 1998; J. Virol., 72: 9835-9843, 1998). We have also identified a third obstacle that the cell must allow for efficient intracellular trafficking of AAV to the nucleus (J. Virol., 74: 992-996, 2000). Since those studies were carried out

with established cell lines, we examined primary murine hematopoietic cells to evaluate whether impaired intracellular trafficking of AAV vectors might account for the observed low-efficiency of transduction of these cells. Purified murine Sca1+, lin- hematopoietic cells from C57Bl6 donor mice, with and without treatment with hydroxyurea (HU) [1 mg/g/mouse; intra-peritoneal (i.p.); 24 hrs prior to harvesting bone marrow cells], were either mock-infected or infected with a recombinant AAV vector containing the CMV promoter-driven enhanced green fluorescent protein (EGFP) reporter gene and transplanted into lethally-irradiated congenic recipient mice. Twelve days post-transplantation, spleen cell colonies (CFU-S) were enumerated and analyzed for the presence of the transduced gene in single cell colonies. Both low and high Mr DNA samples were isolated from three CFU-S colonies each and analyzed on Southern blots using EGFP DNA as a probe. A significant hybridization of the probe to the low Mr DNA was detected in each of the colonies derived from vector-transduced cells. Since linear, monomeric duplex forms of the viral genomes were not readily detectable, even in cells obtained from animals with prior treatment with HU, in the next set of experiments, CFU-S colonies from each recipient animal were pooled individually and nuclear and cytoplasmic fractions were prepared. Low Mr DNA samples isolated from these fractions were analyzed on Southern blots. A significant increase in the hybridization intensity to the probe in the nuclear fraction as well as augmentation in monomeric duplex forms of the viral genome observed in cells in CFU-S colonies derived from cells obtained from HU-treated animals suggested that HU-treatment increases the efficiency of AAV trafficking into the nucleus. This hypothesis was tested by using NIH3T3 cells known to be impaired in efficient intracellular trafficking of AAV. Cells were either mock-treated or treated with HU alone, tyrphostin 1 (Tyr1; known to promote the viral second-strand DNA synthesis) alone, or HU+Tyr1, followed by AAV-mediated transgene expression analyses. HU-treatment led to ~11-fold increase in the transgene expression whereas Tyr1-treatment caused <1-fold increase. HU+Tyr1-treatment led to ~20-fold increase. Southern blot analyses of nuclear and cytoplasmic fractions obtained following these treatments further corroborated the conclusion that HU-treatment leads to a significant increase in intracellular trafficking of AAV vectors.

793. Fluorodeoxyuridine Enhances Oncolytic Potency of the Multimutated Herpes Simplex Virus Type-1 G207 by Cell Cycle Alterations

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Introduction: The thymidilate synthetase inhibitor fluorodeoxyuridine (FUDR) induces an imbalance of intracellular deoxynucleotide triphosphate pools with subsequent DNA double strand breaks. An event involved in the ensuing DNA repair is up-regulation of ribonucleotide reductase. The oncolytic Herpes simplex virus type-1 G207 which has viral ribonucleotide reductase inactivated requires dividing cells for its replication. G207, therefore, may have enhanced replication under certain conditions like DNA repair. The present in vitro study was designed to investigate whether FUDR can enhance the oncolytic effect of G207.

Methods: The human colorectal cell line HCT8 and two of its chemoresistant clones (HCT8/4hR; HCT8/7dR) were used for this study. The cytotoxic effects of G207 and FUDR alone and in combination were evaluated at different MOIs (multiplicity of infection) and FUDR concentrations (10 nM; 100 nM). Additive effects of combined treatments were calculated as product of surviving fractions from each single treatment. Viral replication (standard plaque assay) and infectivity rate (β -galactosidase activity) were evaluated for different treatment groups. In order to

determine fractions of dividing and non-dividing cells, cell cycle analysis was performed on FUDR-treated as well as untreated cells by measuring DNA content with flow cytometry.

Results: Cytotoxicity of combined treatments was higher than the calculated additive effects of either G207 or FUDR. Even at low MOIs (0.01), addition of 10 nM FUDR resulted in complete HCT8 cell kill at day 6 post-infection. Cells treated with 10 nM FUDR showed 3.5-5.5 fold higher overall production of G207 and had 2-10 fold higher β -galactosidase activity at day 3 compared to G207 treatment alone (MOI 0.01). These effects were more pronounced for the chemosensitive cell line HCT8. Cell cycle analysis of FUDR-treated cells demonstrated an increase in S-phase fractions with a corresponding decrease of G0/G1-fractions. S-phase fractions increased from 28% to 73% 24 hours after low (10 nM) or high FUDR exposure (100 nM) in HCT8 cells, while chemoresistant cells showed an S-phase shift ranging from 28-84% with only high dose FUDR.

Conclusion: The present study demonstrates enhanced viral infection and replication of G207 during FUDR exposure resulting in synergy between both agents. Since G207 requires dividing cells for its replication, the ability of FUDR to induce DNA damage and increase the S-phase fraction may be responsible for the observed synergy. This knowledge may be helpful in planning therapy by combining these agents to enhance the effect of regional viral therapy and current chemotherapy in colorectal liver metastases.

794. WITHDRAWN

795. Aggregation of AAV Vectors, its Impact on Liver-directed Gene Transfer and Development of Vector Formulations to Prevent and Dissolve Aggregation and Enhance Gene Transfer Efficiency

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AAV vectors lead to efficient gene transfer in the liver. AAV vectors are usually delivered to the liver through portal vein or via peripheral vein. To achieve high level of gene transfer and ensure the safety of vector administration it is desirable to deliver high doses of vector in small volumes. However, we have recently found that, at high concentrations, AAV virions form aggregates of different sizes in a range of different buffer systems and storage conditions. The size of aggregates appears to be concentration dependent. In both in vitro and in vivo studies using human A1AT as a reporter gene, it was found that, when the same vector prep was concentrated to different concentrations, the higher the concentration, the larger the aggregates and less efficient the gene transfer. Particularly, when the vector titer reached $5-10 \times 10^{13}$ GCs/ml, gene transfer efficiency was 10-100 folds lower at the same dose as compared to the vector whose titer was $1-5 \times 10^{12}$ GCs/ml. To track vector uptake and pharmacokinetics AAVAlbA1AT was labeled with Cy3, concentrated to different levels and delivered to mouse liver through both portal and tail veins. The trafficking of AAV was monitored microscopically and gene transfer was measured by serum levels of human A1AT. Our preliminary data suggest that large AAV aggregates are sequestered in macrophages and quickly cleared from the liver. A series of formulation studies were performed to prevent and dissolve AAV aggregation. Our preliminary finding indicated that some of our formulations could lead to a 30-50% reduction in the size of aggregates at high vector concentrations. Furthermore, PEGylation of AAV efficiently dissolves AAV aggregates. Comparative studies of the performance of PEGylated and native AAV in vivo is in process. (The first two authors contributed equally to this work.)

796. REP/CAP Gene Amplification Is Crucial for High-yield of rAAV Production in the REP/CAP Cell Line, B50, and is Strongly Inhibited by rAAV-ITRs in a Dose-dependent Fashion

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We recently reported the production of recombinant AAV vectors that are free of replication competent AAV (rcAAV) by using a Rep/Cap cell line, B50, and an Ad-AAV hybrid shuttle vector. It was found that temporal relationship of infection with adenovirus helper and AdAAV hybrid was critical to the AAV productivity. A 24-hour pre-infection with Ad helper was essential. More recently, in an attempt to eliminate the Ad helper virus, replication competent AdAAV hybrids (rcAdAAV) were created in which AAV vector genomes were cloned into E3 region of a replication competent Ad genome. Efforts to optimize rcAdAAV for AAV vector production failed. A systematic study was carried out to understand gene regulation of rep/cap gene expression in B50 cells and develop novel strategies for high yield AAV vector production. Our studies reveal the following. 1) The rep/cap minigene in B50 cells is amplified 10-20 times between 32-48 hrs post adenovirus infection. 2) Such an amplification process can be strongly inhibited by AAV-ITRs delivered by either AdAAV hybrids and AAV vector infection, or plasmid ITR fragment transfection in a dose dependent manner. 3) The inhibitory effect of ITRs leads to significant reduction of Rep proteins, particularly, large Reps and poor yield of AAV vector. 4) In the experiments using plasmid construct pP5Luciferase as a reporter gene, it was found that ITRs could also directly or indirectly inhibit P5 promoter activity in both B50 and 293 cells. Based on our findings, we developed a co-infection method for high yield rAAV productions in B50 cells, which is much simpler and easier to be scaled up. At this point, we speculate that the rep/cap gene amplification is Rep-mediated and the inhibitory effect of ITRs is through at least two levels of regulations: either direct binding/sequestering of Reps and/or other cellular factors such as YY1 and/or direct interaction with the P5 promoter. Experiments are under the way to further understand the cellular mechanism of our observation and to shed light on developing novel strategies for high throughput AAV vector production.

797. AdAAV support High-titer Production of rAAV but not Stable

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It has been difficult to produce a chimeric vector containing both Ad and AAV rep and cap, and to grow such chimeric vectors in 293 cells. By recombination in bacterial host, we were able to make recombinant plasmid AdAAV, and then recombinant AdAAV was successfully generated in 293 cells. High titer of recombinant adeno-associated virus (rAAV) stocks were produced by co-infection of cells with two vectors. One vector was with a chimeric AdAAV, which contained the AAV rep and cap genes and all necessary adenovirus genes required for AAV replication. The second vector was an adenovirus in which the green fluorescent protein gene flanked by the terminal repeat ends of adeno-associated virus were cloned into the E1 site of adenovirus. Transfection of 293 cells with these two recombinant adenovirus vector resulted in the large scale, high titer production of recombinant adeno-associated virus expressing GFP with little to no contaminating adenovirus. Recombinant AdAAV expressing rep leads to delay adenovirus replication but the titer was not affected eventually. However, a deletion within the AAV rep gene occurs after short term passage. These results indicate that co-transfection of 293 cells with 2 recombinant AdAAV is a feasible method to produce high titer and pure rAAV, but passage is limited by non-homologous recombination within the AAV rep gene.