have relied on Alexa488 or fluorescein isothiocyanate dye–labeled CPP/PTDs and tended to lack phenotypic analysis of cargo function inside of the cells. Given the results here (and, of course, barring all studies that used fixation techniques), we need to question whether these prior studies may have been biased by the particular combinations of CPP/PTD and hydrophobic dye. Second, not all cells in a given population are susceptible to this type of direct-uptake mechanism. By contrast, cellular uptake of CPP/PTDs by macropinocytosis (endocytosis) results in transduction into the entire population of cells, with essentially the same amount of material present inside each cell. Does this suggest an unknown epigenetic contribution or a specific phase of the cell cycle or a definable amount of metabolism (cell growth)? Finally, is there evidence that it occurs *in vivo* in preclinical models (or in human clinical trials)? Although these will be very difficult experiments to design and control for, they will ultimately tell us whether the cell culture studies are directly related to how these molecules transduce into cells in preclinical animal models as well as in the more than 25 clinical trials using the TAT CPP/PTD.

In summary, the study by Hirose and colleagues brings new and useful information to the CPP/PTD field that illustrates the importance of confirming that the peptide and not the cargo is responsible for the observed mechanism(s) of cellular uptake. Although endocytosis may be responsible for the vast majority of CPP/PTD internalization, accumulating evidence suggests that direct penetration does occur at threshold concentrations. In conclusion, the influence of the cargo must be considered when comparing endocytosis and direct penetration, as the present study highlights, and could explain some of the discrepancies that have existed within the field of CPP/PTD uptake for well over 20 years.

REFERENCES

1. Van den Berg, A and Dowdy, SF (2011). Protein transduction domain delivery of therapeutic macromolecules. *Curr Opin Biotech* **22**: 888–893.
2. Hirose, H, Takeuchi, T, Osakada, H, Pujals, S, Katayama, S, Nakase, I *et al.* (2012). Transient focal membrane deformation induced by arginine-rich peptides leads to their direct penetration into cells. *Mol Ther*, in press.
3. Frankel, AD and Pabo, CO (1988). Cellular uptake of the tat protein from human immunodeficiency virus. *Cell* **55**: 1189–1193.
4. Green, M and Loewenstein, PM (1988). Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein. *Cell* **55**: 1179–1188.
5. Ezhevsky, SA, Nagahara, H, Vocero-Akbani, AM, Gius, DR, Wei, MC and Dowdy, SF (1997). Hypophosphorylation of the retinoblastoma protein (pRb) by cyclin D:Cdk4/6 complexes results in active pRb. *Proc Natl Acad Sci* **94**: 10699–10704.
6. Vives, E, Brodin, P and Lebleu, BA (1997). Truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J Biol Chem* **272**: 16010–16017.
7. Joliot, A, Pernelle, C, Deagostini-Bazin, H and Prochiantz, A (1991). Antennapedia homeobox peptide regulates neural morphogenesis. *Proc Natl Acad Sci USA* **88**: 1864–1868.
8. Derossi, D, Joliot, AH, Chassaing, G and Prochiantz, A (1994). The third helix of the Antennapedia homeodomain translocates through biological membranes. *J Biol Chem* **269**: 10444–10450.
9. Lundberg, M, Wikström, S and Johansson, M (2003). Cell surface adherence and endocytosis of protein transduction domains. *Mol Ther* **8**: 143–150.
10. Wadia, JS, Stan, RV and Dowdy, SF (2004). Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat Med* **10**: 310–315.
11. Nakase, I, Niwa, M, Takeuchi, T, Sonomura, K, Kawabata, N, Koike, Y *et al.* (2004). Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement. *Mol Ther* **10**: 1011–1122.
12. Kaplan, IM, Wadia, JS and Dowdy, SF (2005). Cationic TAT peptide transduction domain enters cells by macropinocytosis. *J Control Release* **102**: 247–253.
13. Charras, G and Paluch, E (2008). Blebs lead the way: how to migrate without lamellipodia. *Nat Rev Mol Cell Biol* **9**: 730–736.
14. Palm-Apergi, C, Lorents, A, Padari, K, Pooga, M and Hällbrink, M (2009). The membrane repair response masks membrane disturbances caused by cell-penetrating peptide uptake. *FASEB J* **23**: 214–223.
15. Fretz, MM, Penning, NA, Al-Taei, S, Futaki, S, Takeuchi, T, Nakase, I *et al.* (2007). Temperature, concentration- and cholesterol-dependent translocation of L- and D-octa-arginine across the plasma and nuclear membrane of CD34+ leukaemia cells. *Biochem J* **403**: 335–342.
16. Kosuge, M, Takeuchi, T, Nakase, I, Jones, AT and Futaki, S (2008). Cellular Internalization and distribution of arginine-rich peptides as a function of extracellular peptide concentration, serum, and plasma membrane associated proteoglycans. *Bioconjug Chem* **19**: 656–664.
17. Duchardt, F, Fotin-Mleczek, M, Schwarz, H, Fischer, R and Brock, R (2007). A comprehensive model for the cellular uptake of cationic cell-penetrating peptides. *Traffic* **8**: 848–866.

Progress in the Development of Hepatitis C Virus Vaccines

Hildegund CJ Ertl[1]

doi:10.1038/mt.2012.30

Vaccines save millions of lives each year. Although vaccines are available for many of the viral infections that can be readily prevented by neutralizing antibodies, vaccines for more complex pathogens—including viruses that mutate very rapidly and may require induction of broadly cross-reactive cellular immune responses—remain elusive. Two recent articles[1,2] report on vaccine vectors derived from adenoviruses (Ads) of three different species isolated from chimpanzee feces. Vectors encoding antigens from expression cassettes placed into the deleted E1 domain were found to be highly immunogenic in mice and monkeys.[1] Even more important—because mice can lie and monkeys can exaggerate—vectors expressing the NS3-5B region of hepatitis C virus (HCV) genotype 1B induced potent and sustained transgene product–specific CD8+ T-cell responses in human volunteers.[2]

Traditional vaccines are based on inactivated or attenuated pathogens, purified proteins, or modified toxins. Cellular immunity, especially CD8+ T-cell responses, can best be achieved by gene transfer vehicles that induce *de novo* synthesis of the vaccine antigens, which are in part cleaved by the proteasome in the cytoplasm. Peptides derived from the degraded antigens are actively transported into the endoplasmic reticulum, where they associate with major histocompatibility class I antigen, and then undergo translocation to the cell surface, where they can interact with the T-cell receptors on CD8+ cells.

More than 15 years ago, replication-defective human serotype 5 adenovirus (AdHu5) vectors originally developed by

[1]*The Wistar Institute, Philadelphia, Pennsylvania, USA*

Correspondence: *Hildegund CJ Ertl, The Wistar Institute, 3601 Spruce Street, Philadelphia, Pennsylvania 19104, USA.*
E-mail: ertl@wistar.upenn.edu

gene therapists for correction of genetic defects were shown to be potent inducers of B and $CD8^+$ T cell–mediated immunity.[3] Not only are AdHu5 vectors far more immunogenic than other genetic vaccine carriers such as poxvirus or plasmid vectors, they also induce remarkably more sustained immune responses, most likely because of the vectors' low-level persistence in a transcriptionally active form in T cells.[4] Nevertheless, as had already been appreciated by gene therapists and was then confirmed in vaccine studies, preexisting AdHu5-specific neutralizing antibodies, which are very common in humans, reduce the vectors' transduction rates and hence expression of the transgene products.[5] More than 10 years ago, E1-deleted vectors derived from chimpanzee Ad viruses (S-AdV or AdC) were developed to circumvent preexisting neutralizing antibodies in humans,[5] and since then a steady stream of publications has shown that AdC vectors are highly immunogenic in mice and nonhuman primates (reviewed in refs. 6 and 7).

The recent article by Colloca *et al.*[1] is therefore not highly innovative, as it confirms a wealth of previous studies. Nevertheless, it is praiseworthy in its breadth by isolating and sequencing in excess of 1,000 Ad viruses from monkey feces. Clearly, any Ad vector used as a vaccine carrier for a given pathogen will induce, in addition to the desired vaccine antigen-specific immune response, carrier-specific neutralizing antibody responses, which will render vaccines based on the same Ad virus inefficient for subsequent use in that target population—whether it be for boosting or the induction of responses to other pathogens. Generation of new simian Ad vectors in addition to those already available[8] is thus of value.

However, some of the reported findings should be viewed with caution. Relative immunogenicity of vectors was determined using HIV-1 Gag as the transgene product; in our experience with a more limited number of human and simian serotype Ad vectors,[5,7] immunogenicity of an individual Ad vaccine carrier is in part dictated by the nature of the transgene as well as by characteristics of the expression cassette, such as the presence of introns or enhancers, which may explain discrepancies between Colloca and colleagues' results and those of earlier studies. For example, both AdHu26, a D family member of the family *Adenoviridae*,[9] and SAd-V23 (also called AdC6), an E family member, have previously been reported to be just as immunogenic in mice and rhesus macaques as AdHu5 vectors.[10] Rare human as well as nonhuman serotype Ad vectors were originally developed to overcome preexisting neutralizing antibodies in humans. Prevalence rates of such neutralizing antibodies vary depending on the geographical region; prevalence rates are markedly higher in humans residing in developing countries—especially in sub-Saharan Africa—than in individuals from the United States or Europe.[11,12] In addition, as was shown in animals[5] and then in human volunteers participating in the ill-fated STEP trial,[13] which aimed to test the efficacy of an AdHu5 vector for prevention of chronic HIV-1 infections, titers of Ad-neutralizing antibodies at or above 1:40 or 1:18, respectively, impact the performance of Ad vector vaccines. Colloca *et al.* tested seroprevalence rates in Caucasians residing in the United States or Europe and report frequencies of neutralizing antibodies to the different Ad viruses above 1:200, which may paint an unduly optimistic picture of the true value of the different AdC viruses as vaccine carriers for use in humans.

Barnes *et al.*[2] described the immunogenicity of AdHu6 (Ad6) and AdC3 (ChAd3 or SAd-V3) expressing NS3-5B of HCV in a phase I clinical trial. Vectors were used at different doses individually or in a prime-boost regimen. Both vectors induced measurable T-cell responses; at low vector doses, frequencies of vaccine-induced T cells were higher upon AdHu6 immunization. Responses were, as expected from animal studies,[10] dominated by T cells producing interferon-γ alone or in combination with tumor necrosis factor-α. They showed broad cross-reactivity between different genotypes of HCV and remained largely activated, as indicated by sustained levels of PD-1 and granzyme, limited expression of CD127, and marginal *in vivo* expansion upon booster immunization with a heterologous Ad vector.

Ad vectors, initially hailed as perfect gene delivery vehicles for correction of inherited diseases, in the end failed to achieve sustained gene therapy because of their high immunogenicity. Their subsequent claim to fame as vaccine delivery vehicles was called into question in the aftermath of the STEP trial. The studies by Colloca and Barnes and their colleagues reconfirm that Ad vectors, especially those to which humans lack neutralizing antibodies, should continue to be explored as vaccine carriers for complex pathogens that cannot be thwarted by traditional vaccines.

REFERENCES

1. Colloca, S, Barnes, E, Folgori, A, Ammendola, V, Capone, S, Cirillo, A *et al.* (2012). Vaccine vectors derived from a large collection of simian adenoviruses induce potent cellular immunity across multiple species. *Sci Transl Med* **4**: 115ra2.
2. Barnes, E, Folgori, A, Capone, S, Swadling, L, Aston, S, Kurioka, A *et al.* (2012). Novel adenovirus-based vaccines induce broad and sustained T cell responses to HCV in man. *Sci Transl Med* **4**: 115ra1.
3. Xiang, ZQ, Yang, Y, Wilson, JM and Ertl, HC (1996). A replication-defective human adenovirus recombinant serves as a highly efficacious vaccine carrier. *Virology* **219**: 220–227.
4. Tatsis, N, Fitzgerald, JC, Reyes-Sandoval, A, Harris-McCoy, KC, Hensley, SE, Zhou, D *et al.* (2007). Adenoviral vectors persist *in vivo* and maintain activated $CD8^+$ T cells: implications for their use as vaccines. *Blood* **110**: 1916–1923.
5. Zhi, Y, Figueredo, J, Kobinger, GP, Hagan, H, Calcedo, R, Miller, JR *et al.* (2006). Efficacy of severe acute respiratory syndrome vaccine based on a nonhuman primate adenovirus in the presence of immunity against human adenovirus. *Hum Gene Ther* **17**: 500–506.
6. Tatsis, N, Tesema, L, Robinson, ER, Giles-Davis, W, McCoy, K, Gao, GP *et al.* (2006). Chimpanzee-origin adenovirus vectors as vaccine carriers. *Gene Ther* **13**: 421–429.
7. Lasaro, MO and Ertl, HC (2009). New insights on adenovirus as vaccine vectors. *Mol Ther* **17**: 1333–1339.
8. Roy, S, Medina-Jaszek, A, Wilson, MJ, Sandhu, A, Calcedo, R, Lin, J *et al.* (2011). Creation of a panel of vectors based on ape adenovirus isolates. *J Gene Med* **13**: 17–25.
9. Liu, J, Ewald, BA, Lynch, DM, Denholtz, M, Abbink, P, Lemckert, AA *et al.* (2008). Magnitude and phenotype of cellular immune responses elicited by recombinant adenovirus vectors and heterologous prime-boost regimens in rhesus monkeys. *J Virol* **82**: 4844–4852.
10. Tatsis, N, Lasaro, MO, Lin, SW, Haut, LH, Xiang, ZQ, Zhou, D *et al.* (2009). Adenovirus vector-induced immune responses in nonhuman primates: responses to prime boost regimens. *J Immunol* **182**: 6587–6599.
11. Xiang, Z, Li, Y, Cun, A, Yang, W, Ellenberg, S, Switzer, WM *et al.* (2006). Chimpanzee adenovirus antibodies in humans, sub-Saharan Africa. *Emerg Infect Dis* **12**: 1596–1599.
12. Chen, H, Xiang, ZQ, Li, Y, Kurupati, RK, Jia, B, Bian, A *et al.* (2010). Adenovirus-based vaccines: comparison of vectors from three species of adenoviridae. *J Virol* **84**: 10522–10532.
13. Buchbinder, SP, Mehrotra, DV, Duerr, A, Fitzgerald, DW, Mogg, R, Li, D *et al.* (2008). Step Study Protocol Team. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet* **372**: 1881–1893.