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Macromolecular Cytosolic Delivery: Cell Membranes as the Primary Obstacle

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The "evolution" of a thing, a custom, an organ is thus by no means its progressus toward a goal, even less a logical progressus by the shortest route and with the least expenditure of force, but a succession of more or less profound, mutually independent processes of subduing, plus the resistances they encounter, the attempts at transformation for the purpose of defense and reaction, and the results of successful counteractions. The form is fluid, but the "meaning" is even more so (Friedrich W. Nietzsche).

Key words : Cytosolic delivery, Cell membranes, Primary obstacle, Gene, Viral vector, Non-viral vector, Bacterial vector

INTRODUCTION

The two topologically-connected subcellular compartments of cells, the cytosolic space and the nucleus, are important target sites for the action of many new therapeutic agents. Remarkable advances of biomedical research in recent years have identified numerous new target sites and novel ammunitions to combat a variety of diseases, including cancer and AIDS, but we still lack the weapons to deliver these potent agents to their targets at therapeutically effective concentrations *in vivo* (Anonymous, 1998). The next generation of pharmaceuticals, including the majority of polypeptide-based drugs (Brugge, 1993), antisense oligonucleotides (Crooke, 1992; Wagner, 1995), catalytic RNAs (Cech, 1988; Castanotto *et al.*, 1994), and plasmid genes (Felgner and Rhodes, 1991; Mulligan, 1993; Felgner, 1993; Crystal, 1995) are powerful therapeutic agents, but are limited thus far in their efficacy *in vivo* due to the difficulty in delivering them to the cytosolic space of target cells. Their large molecular size or polyanionic nature causes them to be intrinsically membrane impermeant. Such agents cannot cross the membrane barriers of cells, and thus, have limited access to their site of action in the cytosolic space or the nucleus. The full efficacy of these agents is contingent on the development of a delivery strategy capable of specifically targeting and transporting these macromolecules across cell membranes into their appropriate subcellular compartments.

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In general, two major limiting factors influence the effectiveness of a drug at the desired site of its action *in vivo*: (i) the local concentration of the drug that can be achieved in the vicinity of the target, and (ii) the cellular interactions of the drug, which determine what percentage of the local concentration gains access to the intracellular site of action. In order to address the first issue, significant efforts have been directed to develop delivery systems that can target drugs to specific cells or tissues. Examples of delivery systems that can increase this local concentration include prolonged circulation of drugs or delivery vehicles in plasma, controlled release strategies (Poznansky and Juliano, 1984; Langer, 1990), or the combination of increased circulation with a cell-specific targeting motif (Wang *et al.*, 1995; Kato and Sugiyama, 1997).

While targeting at the cellular or tissue level is still important, cytosolic delivery is essential for membrane-impermeant macromolecules to be effective. When interacting with the outside environment, cells endocytose or phagocytose exogenous molecules or fluid into small, membrane-bounded vesicles called endocytic compartments, effectively reducing the extracellular volume that the cell must process. However, membranes act as physical barriers between the outside and the inside of cells, thus the cytosol is topologically remote from both the cell exterior and the lumenal space of the endosomal compartment. The molecules internalized into the lumen of these endocytic compartments are still topologically extracellular although they appear to be within the cell (Budker *et al.*, 1992). Most delivery systems, including ligand-mediated, polymer-based, or liposomal vehicles, can target the drugs to specific cells, at which point most cells internalize

them into the endocytic compartments. Membrane-impermeant macromolecules, however, cannot escape this endocytic pathway and are eventually delivered to the lysosomal compartments where they are degraded by hydrolases.

Currently, targeting of delivery vehicles to specific cell types can be achieved via the exploitation of ligand-receptor interactions. For example, after decades of research to develop targetable carriers, liposomes have recently proven their delivery potential with the discovery and development of liposomes with long circulation life in plasma (Papahadjopoulos *et al.*, 1991; Lasic and Papahadjopoulos, 1995; Papahadjopoulos, 1995). Until the advent of these long circulating, sterically stabilized liposomes (SSL), efforts to target to specific cells other than their natural target, macrophages of the reticuloendothelial system, failed. The endocytic uptake of conventional liposomes by macrophages is so rapid (Poznansky and Juliano, 1984) that the targeting motif on the liposome surface has little opportunity to interact with the receptors on target cells, decreasing its effectiveness. Even though the details of targeting with SSL are still under investigation, the current consensus in the liposome field is that these long circulating liposomes can be selectively targeted (Lasic and Martin, 1995). However, if one is to deliver macromolecules using SSL, there still remains the second critical problem of cytosolic delivery, which will be the focus of this article. Targeted SSL are internalized by the endocytic pathway via ligand-receptor interactions and routed to the lysosomes, resulting in rapid degradation of both liposomes and liposomal contents, without the drugs ever gaining cytosolic access. Thus, even if we can specifically deliver 100% of the drugs to the target cells, we can expect little therapeutic value if 99% is routed to the lysosomal compartment and degraded.

Gene therapy, which aims at using genes as the ultimate therapeutic agents, is faced with the particularly daunting task of physically delivering large, negatively charged DNA molecules across the plasma membrane of cells into the cytosol and then to the nucleus. Since plasmid DNA possesses all salient features common to other membrane-impermeant macromolecular drugs, the cytosolic delivery problems of macromolecules may be discussed using gene delivery as a prime example, and can be generalized to the delivery of most nucleic acid or protein based drugs. The breadth of this article is not intended to be thorough in covering different delivery systems for gene therapy, but rather focused on recent studies that exemplify the subcellular targeting strategies which we believe are the most crucial in moving forward the delivery field. Particular emphasis will be placed on delivery using "non-viral, non-bacterial vectors", a non-viral vector class into which mechanisms used by intracellular bacteria for entering the cytosol of host cells are incorporated. Other delivery paradigms will only be discussed in brief for the sake of comparison.

GENE DELIVERY

The implementation of gene therapy in particular can be viewed as the pinnacle of pharmaceutical progress and the most challenging of drug delivery tasks. Gene therapy, which has profited greatly from the explosion of knowledge in areas such as bacterial and human genetics, recombinant DNA technology, cell and molecular biology, gene transfer technologies, and the human genome project, has the potential to revolutionize the way modern diseases are treated by offering a long term, fundamental cure through an administered agent instead of simple, temporary alleviation of the pathological conditions (Wolff and Lederberg, 1994). Replacement of a malfunctioning or absent gene with a functional one presents the possibility to cure by correcting the disease state at the upstream level in the central dogma of genetic information flow, before the faulty information becomes amplified downstream during translation. The effectiveness of such a treatment, however, is currently undermined by the difficulties of effectively delivering a bulky, anionic macromolecule. Bringing the futuristic idea of gene therapy from experimental to clinical settings using an efficient and safe delivery vehicle is being explored through the incorporation of both viral and bacterial transfection strategies which have been proven effective in delivering macromolecular drugs, proteins, small nucleic acids, and plasmid DNA. The goal is to achieve selective expression of genes in the target cells at therapeutic levels by delivering the exogenous DNA in a safe, protected manner to the proper cells without excessive dilution, and then delivering it into the cytosol with high efficiency. While the accessibility of the administered gene to the target cell is still an enormous challenge, once the delivered plasmid gene makes contact with the target cell, the situation can be viewed as more analogous to the *in vitro* situation in which the physical transport of DNA across membranes is the first rate-limiting factor.

Traditionally, the gene delivery effort has been divided broadly into two strategies: the use of vectors either viral or non-viral (Mulligan, 1993; Crystal, 1995). Additionally, new paradigms for delivery include the use of bacterial vectors and non-viral, non-bacterial vectors. Viral vectors are known for their transfection efficiency but have a number of drawbacks including possible pathogenicity, immunogenicity, difficulty in targeting, and duration of expression, despite many recent improvements in their design (Robbins *et al.*, 1998). Non-viral vectors, on the contrary, have advantages such as ease of manufacturing, apparent safety,

stability, low immunogenicity, non-pathogenicity, and potential for targetability; however, relatively low transfection efficiency has been a significant problem with the existing non-viral vector systems.

Gene transfer technology is often developed *in vitro*, but difficulty can arise when these systems are transferred to *in vivo* situations; direct microinjection of DNA into cells, calcium phosphate precipitates of DNA, or electroporation techniques are examples. Electroporation, which is well known to induce membrane permeabilization, has been reported as a means of improving transfection efficiencies but is limited by tissue accessibility to superficial tissue sites or *ex vivo* procedures (Potts and Chizmadzhev, 1998). A variety of non-viral vectors such as pH-sensitive liposomes, ligand-DNA complexes, and polymer-DNA complexes have been reported (Ledley, 1995), although the most studied non-viral vectors are cationic lipid-DNA complexes (Felgner and Ringold, 1989; Gao and Huang, 1995; Lee and Huang, 1997). Cationic lipid-mediated transfection is moderately efficient, but is relatively cytotoxic and not easy to target; whereas, other non-viral vectors tend to have greater potential for safety and targetability but suffer from the problem of lower efficiency (Ledley, 1995; Gao and Huang, 1995; Lee and Huang, 1997; Felgner *et al.*, 1994).

VIRAL VECTORS

Much of the current research in macromolecular delivery and gene delivery is based on using viral paradigms for delivery. By harnessing the natural infection mechanisms viruses have developed to fuse with target cell membranes and deliver their genetic contents into the cytosol and ultimately to the nucleus of cells, pharmaceutical scientists hope to improve gene delivery efficiencies. Only the viral gene delivery vector systems that are notable will be briefly reviewed with particular emphasis on their weaknesses, as more attention will be paid to the non-viral vector systems or to the hybrid vectors of recent development. For details on viral vectors, review articles by Robbins *et al.* (1998) and Crystal (1995) are referred to.

Currently, research focuses heavily on the use of retroviral vectors and adenoviral vectors, although progress is also being made using herpes-simplex and adeno-associated viruses. Recombinant retroviral vectors, which have been rendered replication deficient through elimination of essential viral genes and replacement with foreign genes to be delivered, are capable of transferring genetic information into the host cell genome (Crystal, 1995; Robbins *et al.*, 1998). Retroviral carriers offer a single-dosage gene transfer system that has a very high transfer efficiency and is stably passed on to progeny cells (Tolstoshev, 1993). Drawbacks associated with this method include the ability to deliver only 9 kb of exogenous genetic information. Genetic information delivered by retroviral vectors integrates at random into the host cell genome, which is an advantage in increasing the duration of gene expression, but also poses certain risks associated with permanent modification of host genome, the possibility to disrupt tumor-suppressor genes through insertional mutagenesis, and potentially toxic overexpression (Crystal, 1995). Furthermore, integration occurs only in proliferating cells, thus limiting the generality of retroviruses as a delivery system. In the case of the most commonly used retroviral system, Murine Leukaemia Virus (MLV), risks associated with recombinant events leading to wild type viral production are minimized by supplying the three viral genes, *gag*, *pol*, and *env*, in *trans* (Robbins *et al.*, 1998). Adenoviral vectors are advantageous in that they can transfer genes into both proliferating and non-proliferating cell types and are epichromosomal, eliminating the concern for insertional mutagenesis. Adenoviruses are produced in high titer, and can carry a large expression cassette (Kochanek *et al.*, 1996). The advantages in safety offered by adenoviral vectors bring with them a number of problems. The lack of chromosomal integration limits the duration of vector expression to a time span of weeks to months, which necessitates repeated dosing in many cases. Nonspecific inflammation as well as virus-specific immune reactions have been reported, which make the repeat treatments difficult and less effective (Crystal, 1995; Robbins *et al.*, 1998). Correlation with oncogenesis has also been seen in adenoviruses retaining the endogenous E4orf6 protein, which interferes with the function of a p53 tumor suppressor protein (Kling, 1996).

Adenovirus vectors are endocytosed following binding of the plasma membrane by the fibers projecting from the capsid. Endosomal escape occurs upon acidification of the endosome, which confers a conformational change on the capsid protein which is responsible for disrupting the endosomal membrane (Wu *et al.*, 1994). Unfortunately, the protruding fiber protein which facilitates cell binding interactions is fairly promiscuous, although favoring interactions with epithelial cells (Woo, 1996), and thus must be engineered in order to attain selectivity toward various cell types. This viral entry route has been also tailored to suit the targeting needs of a variety of genes by coupling asialoglycoproteins to the capsid fiber structure in order to targeting receptors that internalize galactose-terminal glycoproteins while blocking recognition of endogenous viral receptor pathways (Curiel, 1994; Wu *et al.*, 1994). This approach of creating mutant retroviral- or adenoviral- vectors with tailored cell surface binding motifs and has become an active area of research in the attempts to render custom-designed tropism and targetability to viral vectors.

Relatively less work in viral delivery has been done using herpes-simplex virus and adeno-associated virus. Herpes-simplex virus has the potential to deliver a large capacity of genes within its 150 kb genome; however, clinical applications of such a vector rely on the development of viral particle deficient in all immediate early genes, which are responsible for regulating replication (Robbins *et al.*, 1998). Adeno-associated virus is a human virus that is not associated with any pathology. Its assets include the fact that the wild type virus integrates in a site specific manner into human chromosome 19; however, current recombinant viruses integrate at random, implying that site specificity lies in an essential replication protein in the recombinant virus (Robbins *et al.*, 1998; Carter, 1996).

NON-VIRAL VECTORS

While non-viral vectors lack the evolutionary development of their viral counterparts, a number of advantages to using non-viral gene delivery are apparent, including the relative ease of preparation of plasmid DNA constructs carrying the necessary control elements, less complex scale-up difficulties in converting to a manufacturing protocol, and elimination of possible side effects of viral vehicles or genomic integration (Felgner and Rhodes, 1991; Ledley, 1995). Non-replicating plasmids have produced expression in cells through direct injection of naked DNA; the use of plasmid DNA encapsulated in liposomes, immunoliposomes, and liposome/red blood cell membrane hybrids; DNA complexed with asialoglycoprotein conjugated to polylysine; or with cationic liposomes. Although the non-viral vectors are continually being improved, the major problem still is the low efficiency of transfection. Some researchers in the field foresee a compromise between viral and non-viral vectors as the two fields converge on a hybrid vector, with viral vectors continuing to de-evolve and become more streamLined while non-viral vectors evolve and become more sophisticated.

The need to improve transfection efficiencies has driven the search for better non-viral delivery systems. Most approaches are based either on the strategies adopted from the viral entry and subsequent delivery of genetic information or on the concept of enhancing the overall gene expression by increasing the cell-associated DNA concentration by incorporating targeting motifs. The latter approach is largely based on our knowledge of ligand-receptor interactions gained through advances in molecular and cell biology, and will not be discussed in detail in this review. Cationic lipid formulations compose a large portion of research on non-viral delivery systems, as they have been shown to spontaneously condense DNA and interact well with anionic cell surfaces (Gao and Huang, 1995; Felgner and Ringold, 1989; Felgner *et al.*, 1994). Studies as to the nature of these interactions indicate that helper lipids such as phosphatidylethanolamine, which is more conducive to membrane fusion because of its tendency to form an inverted hexagonal phase, are necessary for efficient transfection (Felgner *et al.*, 1994). Cholesterol has also been shown to be critical in lipid based delivery systems. In examining the effect of cholesterol in formulations using the cationic lipid DOTAP, an increase in transfection was observed which can be attributed to better positioning of the lipids for attachment to cells and uptake (Crook *et al.*, 1998). Several characteristics have proven desirable in mimicking the transfection ability of viral particles in a non-viral system, including the ability to resist aggregation such that tissue distribution is more uniform and to contain a single copy of the desired DNA. Attempting to copy these traits, lipid preparations were formed in which a single plasmid is condensed using cationic cysteine-based detergent, followed by dimerization of the detergent to form a cystine lipid-based delivery vehicle (Blessing *et al.*, 1998). Cationic liposomal delivery systems offer the possibility of delivering essentially unlimited sized expression cassettes, cannot replicate, and do not contain foreign proteins that are likely to evoke an immune response. Another attribute of cationic liposomes is their ability to complex with DNA by electrostatic interactions. Improvements in the stability of plasmid-liposome preparations by including poly (ethylene glycol)-phospholipid conjugate or pre-condensing the plasmid DNA with polyamines have the potential to make these formulations more easily adaptable to vaccines or standard macromolecular delivery (Hong *et al.*, 1997).

Encapsulation of DNA inside anionic liposomes utilizes the strategy of destabilizing the lipid bilayer upon pH drop in the early endocytic compartment en route to lysosomes. These studies with so called "pH-sensitive or acid labile" liposomes have shown that DNA encapsulated inside such liposomes can be used for transfection (Wang and Huang, 1987; Wang and Huang, 1989; Legendre and Szoka, 1992); however, the transfection efficiency is 1~2 orders of magnitude lower than that by cationic lipid-DNA formulations.

Interest in polymer-based formulations has also risen. Polycations such as polylysine or protamine condense plasmid DNA, and these polymers are continually being developed or modified to better optimize delivery characteristics. Polylysine is most commonly used as a polymeric base, but it requires the presence of additional agents for improved cellular uptake (Tang and Szoka, 1997). Recently, other polymers have been examined as pharmaceutical scientists attempt to exploit the individual characteristics of diverse polymer systems. For example, polyamidoamine cascade polymers, also

known as "dendrimers", condense and complex with DNA and additionally act as a weak base in buffering the endosomal compartment, thus reducing lysosomal degradation (Haensler and Szoka, 1993), while stearyl-poly(L-lysine) complexed with DNA and low density lipoproteins shows improved size, charge, and compactness characteristics (Kim *et al.*, 1998).

As an alternative to high molecular weight polymer complexes, studies are being conducted using peptides capable of mediating nucleic acid delivery such as the amphipathic peptide, KALA, which uses hydrophobic interactions to permeabilize membranes in a pH dependent manner (Wyman *et al.*, 1997). Many of these cationic peptides are designed to possess the ability to induce membrane fusion or destabilization. Increased transfection efficiency has been reported upon incorporation of the so called "fusogenic peptide" derived from a viral entry mechanism (Wagner *et al.*, 1992; Bron *et al.*, 1994; Plank *et al.*, 1994), which led to non-viral vectors acquiring names like "artificial viruses" or "virosomes". DNA complexed with gramicidin S, an amphipathic cyclic decapeptide which complexes strongly with DNA and destabilizes membranes, was also designed to mimic the function of fusogenic peptides and thus improve transfection efficiency (Legendre and Szoka, 1993). The increased transfection efficiency via endosome disruption has been clearly demonstrated by adenovirus-conjugated to non-viral vector (Wu *et al.*, 1994; Curiel *et al.*, 1991), although using the whole virus in non-viral systems eliminates the advantages of non-viral vectors in practicality. These non-viral vector systems comprise the main effort to increase transfection efficiency by overcoming the plasma or endocytic compartment membranes.

BACTERIAL VECTORS

A recent and different approach to macromolecular delivery and specifically gene therapy relies on the use of bacterial vectors to transfer exogenous genes (Higgins and Portnoy, 1998). Particularly interesting is the exploitation of facultative intracellular bacteria such as *Shigella* and *Listeria* (Ikonomidis *et al.*, 1994; Sizemore *et al.*, 1995; Dietrich *et al.*, 1998). Attenuated mutant *Listeria monocytogenes* containing foreign plasmid DNA were introduced into a macrophage cell line and showed expression of reporter proteins and subsequent antigen presentation (Dietrich *et al.*, 1998). While a direct comparison with any viral vector has not been done, and the frequency of transfection of these systems is still somewhat low, advantages include the potential for *in vivo* delivery and the ability to use a single vector construct for foreign gene delivery (Higgins and Portnoy, 1998). Similar research has shown that attenuated *Shigella flexneri* which is deficient in cell wall synthesis is another candidate for DNA delivery (Higgins and Portnoy, 1998; Sizemore *et al.*, 1995).

Exploring another avenue of bacterial delivery, genetically engineered *E. coli* or *Salmonella* have been rendered capable of delivering themselves into the cytosol of target cells through the intracellular bacteria's mechanism to overcome the cell membrane barrier and move into the cytosol of target cells. By incorporating listeriolysin O or truncated forms of ActA, proteins which are involved in *Listeria* entry into the cells and motility in the cytoplasm, into the genome of orally delivered, attenuated *Salmonella,* the expression plasmid is transferred to the nucleus of the host cells (Courvalin *et al.*, 1995; Darji *et al.*, 1997; Higgins and Portnoy, 1998). An *E. coli*-based bacterial carrier represents a promising delivery route, due to the improved safety of utilizing avirulent *E. coli* as well as the ability to easily manipulate its genome and attain high plasmid copy number, but is still subject to the limitations of host immunity. These bacterial vectors possess the high efficiency to mediate therapeutically significant delivery of genes, but have many of the weaknesses shared by viral vectors including pathogenicity, immunogenicity, and difficulty in targeting.

Yet another variation to bacterial vector-mediated delivery includes the utilization of purified bacterial proteins which have been selected for their endosomolytic properties in delivering antigenic proteins. A family of sulfhydryl-activated, pore-forming bacterial proteins including listeriolysin O (LLO), streptolysin O (SLO), perfringolysin O (PFO), and pneumolysin (PLY) is known to form pores of a diameter of 25 to 30 nm in cholesterol-containing membranes, dimensions large enough to plasmid DNA or most macromolecules (Portnoy *et al.*, 1988; Cossart *et al.*, 1989; Bielecki *et al.*, 1990; Gottschalk *et al.*, 1995). The mechanism of LLO, for example, which mediates the passage of a whole bacterium from the endosome to the cytosol, is fundamentally different from other endosomolytic agents derived from viral entry mechanisms, as the *Listeria* entry mechanism delivers the entire micron-sized bacterial particle to the cytosolic space (Portnoy and Jones, 1994).

Utilizing isolated endosomolytic bacterial protein mimics the tactic utilized by a facultative intracellular bacteria to enter into the cytosol of cells, which has been demonstrated to be mediated by a single protein (Portnoy *et al.*, 1988; Cossart *et al.*, 1989; Bielecki *et al.*, 1990). Conjugation of PFO to DNA using a biotin-streptavidin bridge also gave high levels of gene expression independent of the presence of a receptor-specific ligand (Gottschalk *et al.*, 1995). LLO administered together or co-encapsulated inside liposomes with a passenger antigen, activated a MHC class I-mediated immune response thus indicating cytosolic

antigen delivery (Lee *et al.*, 1996, Darji *et al.*, 1997). The LLO protein seems to have several inherent regulatory mechanisms, including optimal activity at the pH of endosomes, to ensure high efficiency and low cytotoxicity (Portnoy *et al.*, 1992; Jones and Portnoy, 1994), making it an ideal endosomolytic agent. Additionally, LLO should be better suited for endosome disruption for efficient and safe cytosolic delivery than SLO or PFO as Listeria has evolved to be intracellular while *Streptococcus pyogenes* or *Clostridium perfringens* are extracellular bacteria. The report of genetically engineered *Salmonella* carrying LLO gene as an efficient bacterial vector adds credence to the viability of this hybrid bacterial vehicle (Dietrich *et al.*, 1998).

NON-VIRAL, NON-BACTERIAL VECTORS

The design of a non-viral, non-bacterial vector which achieves a high transfection efficiency without the limitations of viral and bacterial carriers poses a difficult challenge. Clearly, the low efficiencies of the existing non-viral vector systems in delivering therapeutically useful amounts of DNA are due in part to their inability to overcome the membrane barriers of endocytic compartments that limit the accessibility of exogenous DNA to the cytosol. A notable paradigm in the effort to find an agent or a technology that can induce the release of DNA from the endocytic compartments into the cytosolic space of cells has been the import of viral mechanism into non-viral vectors, including conjugation of endosome disrupting adenovirus to non-viral delivery systems (Curiel *et al.*, 1991; Wagner *et al.*, 1992; Legendre and Szoka, 1993; Plank *et al.*, 1994; Wu *et al.*, 1994; Wyman *et al.*, 1997). However, the levels of enhancement seen in these viral/non-viral hybrid systems have thus far fallen short of the potential originally anticipated.

An alternative cytosolic delivery strategy is to temporarily breach the membrane barriers of the endocytic compartment by exploiting the mechanisms evolved by intracellular bacteria as discussed previously. A multidisciplinary approach is being taken to develop these "non-viral, non-bacterial" cytosolic delivery systems by combining cell biological, biophysical, and pharmaceutical views to exploit the mechanism of bacteria while limiting the disadvantages associated with bacterial delivery. Examples of this approach include the delivery of nucleic acids using streptolysin O or perfringolysin O (Barry *et al.*, 1993; Gottschalk *et al.*, 1995). More recently, in an effort to deliver proteins and antisense oligonucleotides, a strategy adopted by our laboratory combines the endosomolytic activity of *Listeria* hemolysin, LLO, with pH-sensitive (*i.e.*, acid labile) liposomes (Lee *et al.*, 1996). Microbiological and cell biological studies of *Listeria* and LLO, as described in the previous section, led to the feasibility and the design of the listeriolysin O-containing liposomes, or "*listeriosomes*," that behave like *Listeria* in their escape from the endosome into the cytosol. Both the LLO molecules and the "cargo" molecules encapsulated inside the *listeriosomes* are thought to be released from the pH-sensitive liposomes upon acidification in endosomes, and then LLO induces a brief breach of the endosomal membrane and the subsequent delivery of the "cargo" into the cytosol. *Listeriosomes* have thus far been tested to deliver molecules of molecular mass up to 50 kD and shown to dramatically enhance the release of fluorescent dyes, proteins (Lee *et al.*, 1996), and 20-mer oligonucleotides from endosomes into the cytosol (unpublished data). If as efficient as have been demonstrated to-date, *listeriosomes* can be utilized as a general delivery vehicle for many membrane-impermeant macromolecular drugs provided they can be encapsulated inside liposomes with a reasonable efficiency. The unique ability of *listeriosomes* to put antigenic proteins into the cytosol make them an optimal vaccine carrier which can deliver antigens into the MHC class I- as well as class II-dependent pathways of antigen presentation and T cell activation. In addition, listeriosomes have the potential to serve as carriers of proteinacious toxins for their use in chemotherapy. The versatility of such a non-viral, non-bacterial system allows for incorporation of a variety of cargos, from proteins to genes, without compromising its delivery characteristics.

EPILOGUE

In reviewing the recent efforts to design better delivery vectors for bulky and charged macromolecular therapeutic agents, it is obvious that paradigms of this field originate from observations as to how nature performs this sophisticated task. Lipid membranes serve as a barrier and gateway for information-conveying molecules, as well as a defense against pathogens which attempt to penetrate the cytosolic space of the cell. However, viruses and bacteria have evolved to understand the masterplans of eukaryotic cells and thus can utilize the cell biology for their own survival. Numerous types of viruses have been delivering their genomic contents with relatively high efficiency, and in some instances with preferential targeting, for millions of years. So have some intracellular bacteria. Researchers using viral or bacterial vectors have been trying to take these evolved endproducts of nature and exploit them in therapeutic applications; however, success in this endeavor is hindered by the defenses which have evolved in humans in response to these pathogens. With this in mind, proponents of non-viral vectors propose to start from scratch, but we are constantly seeking inspiration from the mechanisms that have been already tested and selected by nature.

As the search for the optimal delivery system continues, particularly as gene therapy progresses, the critical turning point arrives when we have enough knowledge to harness the tools that nature has already set before us.

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