Drug Discovery Today • Volume 13, Numbers 19/20 • October 2008

Virus-sized vaccine delivery systems

Jean-Pierre Y. Scheerlinck and Deanne L.V. Greenwood

Centre for Animal Biotechnology, Faculty of Veterinary Science, The University of Melbourne, Parkville, 3010 Vic., Australia

In a return to the early days of vaccine development during which effective vaccines were produced against viruses, virus-sized vaccine delivery systems have made a comeback. Using modern production technologies these nanoparticles have proved to be very effective at inducing cellular and humoral immune responses. Here, we review a number of vaccine delivery systems based on nanoparticles in the size range of typical viruses. Different strategies for generating these particles, ranging from recombinant virus-like particles to inert nanobeads via ISCOMs and nanoparticle-based DNA vaccine delivery systems, are discussed. In addition, possible mechanisms of immune induction are explored.

Effective vaccines require the induction of specific immune responses with only minimal side effects. To achieve this aim it has been essential to resort to adjuvants. Adjuvants are substances that enhance or modulate the immune response towards an antigen to which they have been added. While immune responses to the adjuvant can sometimes be detected, this should be avoided. There are only a few adjuvants approved for use in humans with alum the only one that has been used in very large populations for an extended period of time. Where alum is recognised as an adjuvant that biases immune responses toward humoral immunity there is an urgent need for alternatives that are able to induce a better cellular immune response. In addition, there is a constant drive to reduce concomitantly the side effects often associated with adjuvants, an area that is important not only for human vaccines but also increasingly for the veterinary field. Alternatives to injected vaccines are continuously sought after, because they are often more readily accepted by the public in general and by young children in particular. These alternatives often have the added advantage that they induce not only systemic but often also mucosal immune responses. This is important since mucosal immunity has the potential to prevent infection at an early stage rather than fight infections that have had the time to get established.

Adjuvants have been referred to by the late Janeway as 'Immunologist's dirty little secret' [1]. Early adjuvants, including complete Freund's adjuvant, were indeed quite 'dirty' in that they contained bacterial products that induced very strong side effects. They were also 'secret' in that the mechanism by which they worked was largely unknown. Now it appears that we should also focus on the word 'little' since the discovery that size, and in particular nanoscale sizes, might play a key part in adjuvanticity. Here, we review the recent development of nanosized particles as adjuvants and vaccine delivery systems.

Virus-like particles

The first, and arguably most successful, vaccines were derived from viruses including smallpox; the smallpox vaccine led to the eradication of the disease in the early 1980s. Initially, these vaccines relied on viral infectivity for induction of protective immune responses. Later, inactivated viruses were used, often without adjuvant. Still, today, some virally derived vaccines, including vaccines against flu, do not use any adjuvant. The use of viruses for vaccination has some serious drawbacks including the possibility of accidental infection before inactivation, reversion of inactivating mutation and, in the case of attenuated viruses, potential to cause harm in immuno-compromised individuals.

To circumvent this problem, virus-like particles (VLPs) have been produced. VLPs are self-assembling particles composed of one or several viral proteins expressed *in vitro* through recombinant technologies. The self-assembly property of these proteins results in the formation of sub-viral particles ranging in size from about 20–100 nm. There are currently two VLP-based vaccines on

Corresponding author: Scheerlinck, J-P.Y. (j.scheerlinck@unimelb.edu.au)

1359-6446/06/$ - see front matter doi:10.1016/j.drudis.2008.06.016

the market. The first one to be commercialised is the Hepatitis B vaccine based on expression of the surface antigen of this virus (HBsAg). More recently, some variations have been produced by the addition of the PreS1 and PreS2 proteins. The second commercially available VLP vaccine is composed of the major capsid protein of the human papilloma virus (HPV) L1. In addition, there is a large list of VLP-based vaccines currently in various stages of development [2].

The requirement for the production of large amounts of recombinant proteins has resulted in the use of a wide variety of expression systems for VLPs. Not surprisingly, the most common systems are these amendable to large-scale *in vitro* cultures including yeast, baculovirus and mammalian cell expression systems. Once produced, the VLPs are purified before administration through injection.

More recently, however, plant-based expression systems have also been used [3]. Interestingly, Santi *et al.* [4] recently demonstrated that VLPs derived from the expression of Norwalk virus proteins in tobacco plants were able to induce immune responses in mice, following oral delivery of the VLPs. There is some debate about the need for purification of the plant-produced VLPs. Purification allows for rigorous quality control steps to be implemented and, in the foreseeable future, is likely to remain a requirement for all human vaccines even if they are produced in edible plants. The attraction of using edible plants to vaccinate and therefore remove the need for purification remains a strong driver for this technology, particularly in the veterinary field where cost constraints are more important.

One interesting aspect of the production of VLPs in different expression systems is that in some cases products from the expression system are incorporated into the VLPs, including lipids that make up the lipoprotein envelope and host proteins [2]. Thus, one can expect that expressing the same viral proteins in different expression systems will result in the production of different VLPs. In addition, it has been possible to express several viral proteins simultaneously, so as to produce VLPs containing several antigens within the same particles [5]. It has also been possible to enhance the immunogenicity of VLPs further by adding co-stimulatory molecules such as influenza hemaglutinin or cholera toxin B [6]. The addition of immuno-regulatory molecules such as GM-CSF and CD40L has also significantly enhanced the adjuvanticity of the VLPs [7].

ISCOMs and ISCOM-based vaccine delivery systems

Over 20 years ago viral antigens were mixed with the saponin Quil A, derived from the bark of *Quillaja saponaria*, to form immunostimulating complexes (ISCOMs) [8]. ISCOMs appear as hollow particles of approximately 40 nm in diameter with the antigen embedded into the particles. Typically, these particles are composed of Quil A saponin (or sub-fractions of Quil A), phospholipids and cholesterol. The Quil A components confer immuno-modulatory properties to the adjuvant and variations in the exact composition of these components allows for the induction of different types of immune response [9].

ISCOMs have been shown to be highly immunogenic not only when injected but also when delivered through the oral route [10]. This is intriguing because orally delivered proteins are thought to induce tolerance rather than immunity. One possible explanation for this unexpected result is that ISCOMs target and activate dendritic cells (DCs) in the mesenteric lymph node following

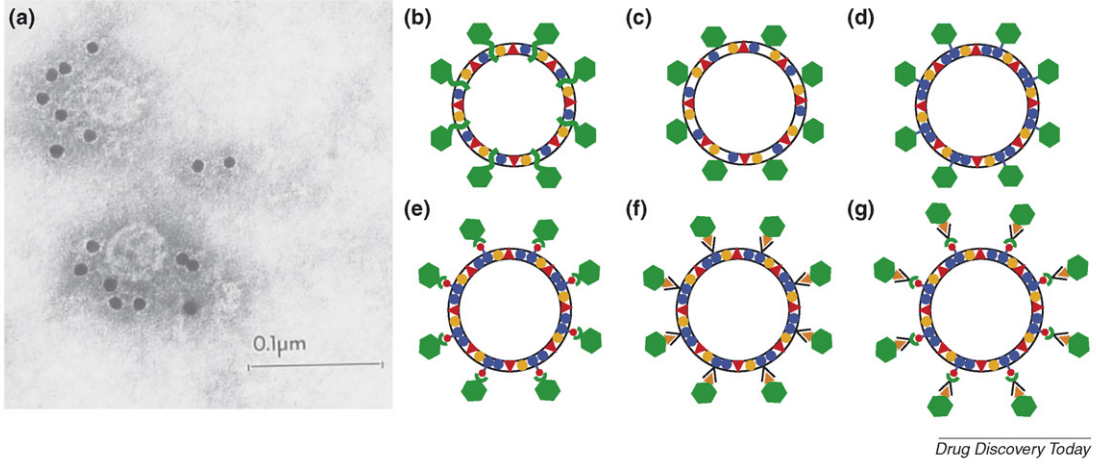

FIGURE 1

Strategies to produce ISCOMs for a wide variety of antigens. (**a**) Immuno-gold electron-microscopic photograph of ISCOMs incorporating the lipid anchored PSA2 antigen of Leishmania major. Antibodies to PSA2 were used to detect the antigen on the surface of the ISCOM and are visualised using an anti-mouse immunoglobulin antibody conjugated to gold particles (black dots on the photograph). The distance between the particles and the gold correspond to the size of the combined antigen, anti-PSA2 antibody and gold conjugated anti-mouse immunoglobulin antibody. (**b**) Classical ISCOMs in which the hydrophobic part of the antigen is incorporated into the particle. (**c**) ISCOMs formed by electrostatic interaction between the negatively charged particles and the positively charged antigen. The charge of the particle can be manipulated to accommodate different antigens. (**d**) Lipid anchors are added to the antigen allowing them to interact with the particles. (**e**) Divalent cations are conjugated to lipids incorporated into the ISCOMs to make chelating ISCOMs. The divalent cations bind to poly-histidine tagged antigen. (**f**) ISCOMs conjugated with biotin can bind a fusion protein linking streptavidin and the antigen (**g**) Chelating ISCOMs are combined with poly-histidine tagged streptavidin and biotinylated antigen. The poly-histidine tagged streptavidin forms a bridge between the biotinylated antigen and the chelating ISCOMs. Electron micrograph used, with permission, from A. Sjolander.

uptake of antigen in the intestinal lumen. The inflammatory signals provided to the DCs are thought to be caused by the presence of the Quil A components of the ISCOMs. In the absence of these inflammatory signals the DCs would also take up and present the antigen but this would lead to immune tolerance instead of immunity [10].

One of the challenges of producing ISCOMs is that not all antigens are easily incorporated into the particles. To circumvent this issue ISCOMATRIX™ adjuvant has been developed in which the antigen is mixed to pre-formed antigen-free particles. ISCOMs and ISCOMATRIX™ induce strong humoral (i.e. antibody) responses as well as T helper responses. But, while ISCOMs have the ability to induce cytotoxic T cell (CTL) responses, the CTL response induced by ISCOMATRIX™ adjuvanted vaccine is variable from one antigen to the other [11]. It has been speculated that this difference could relate to the association between the antigen and the particles, with a stronger association resulting in better uptake of the antigen into the intracellular compartment of cells involved in the presentation of the antigen in association with MHC class I molecules [12]. Because the association between antigen and particles is important for CTL induction several methods to promote this association have been developed.

Initially, ISCOMs were made only with hydrophobic antigens such as membrane proteins or selected viral proteins, which spontaneously associate with the particles (Figure 1a). Subsequently, soluble proteins were made hydrophobic by the addition of hydrophobic lipid tails to hydrophilic antigens [13] (Figure 1b). Through their composition ISCOMs are naturally negatively charged, and positively charged antigens could associate to these particles through electrostatic interactions. In order to accommodate negatively charged antigen, positively charged particles were generated by substituting cholesterol with cationic derivatives [14]. To facilitate the production of ISCOMs incorporating a wide range of antigens in a standardised manner the poly-histidine tagged antigens were chelated to ISCOMATRIX™ produced in the presence of 1,2-dipalmityl-rac-glycerol-3,8-(3,6-dioxy)octyl-1-amino-*N*,*N*-diacetic acid (DPIDA) and copper ions [15]. This method has the advantage that the antigen can easily be purified using the chelating characteristics of the poly-histidine tag and a chromatography column conjugated with divalent cations. Using the very high affinity of streptavidin for biotin, a streptavidin–antigen fusion protein can be associated to ISCOMATRIX™ that as been conjugated to biotin [16]. More recently, the latter two methods were combined by using fusion protein between a poly-histidine tag and streptavidin as a bridge between biotini-

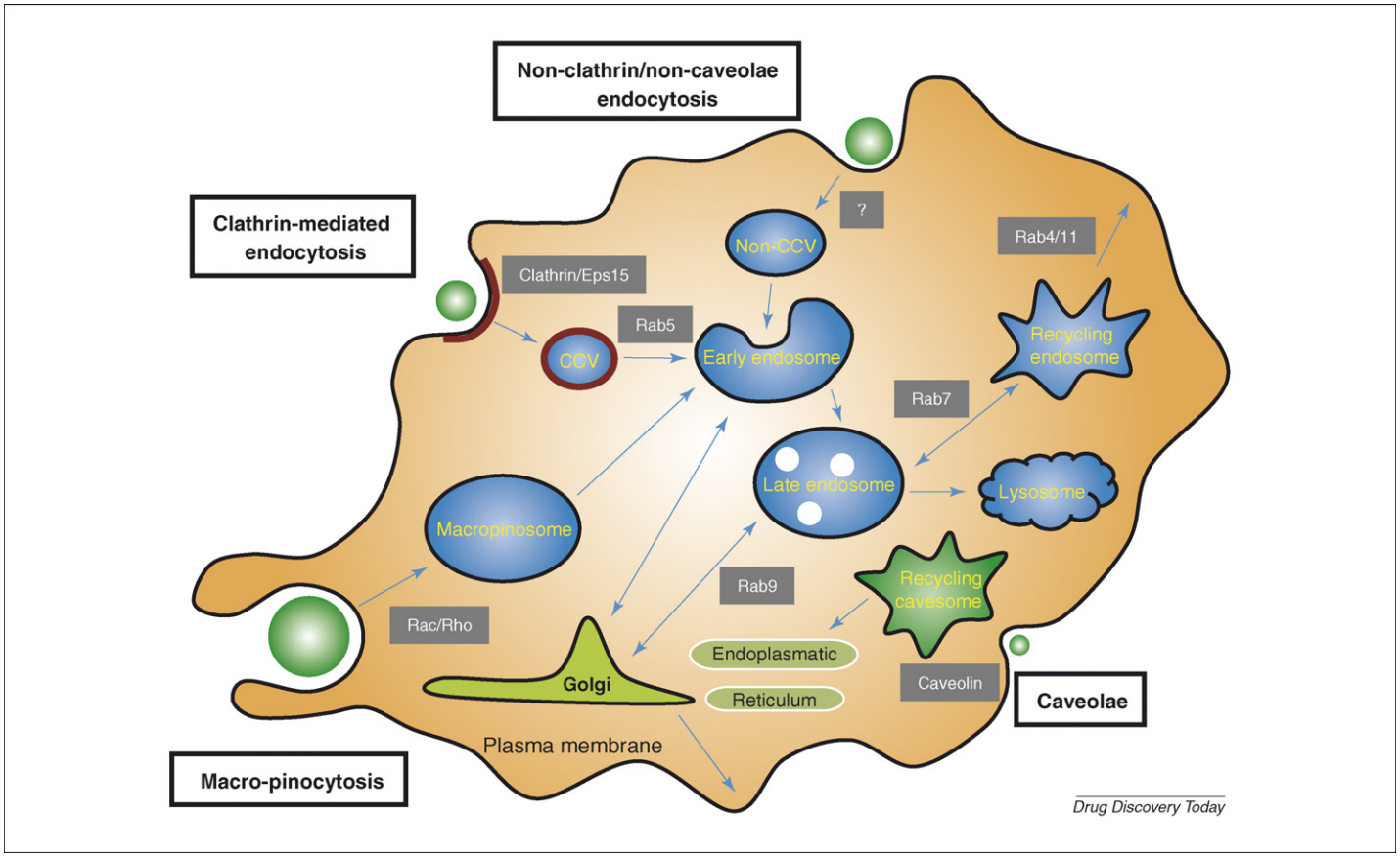

FIGURE 2

Summary of the major routes of endocytosis used by particles of various sizes. The most established route for particles <150 nm is entry via clathrin-coated pits forming clathrin-coated vesicles (CCV), requiring Eps15 among other molecules. CCVs progress to early endosomes via involvement of Rab5. Early endosomes can recycle particles back to the cell surface (controlled by Rab4 and/or Rab11) or progress to the increasingly acidic environment of the late endosome/lysosome (controlled by Rab7). Other possible internalization pathways are non-coated pits (non-CCV) involving particles of a yet-to-be determined size. Macropinocytosis (dependent on the Rac/Rho family of GTPases) typically involves larger particles around 0.5–5.0 μm. By contrast, caveolae (dependent on caveolin) involves particles 50–80 nm in size. The Golgi apparatus is central in membrane trafficking and transport within the secretory and the endocytic pathways.

lated antigen and Ni^{2+}-loaded ISCOMATRIX™ [17]. Immunising mice with recombinant NcSRS2 antigen from intracellular protozoan parasite *Neospora caninum* formulated in this way provided significant protection against cerebral neosporosis [17].

Inert nanobeads

The use of inert 10–100 nm nanobeads has gained notoriety in recent years and their application has been documented in a number of systems [18] providing a promising new approach. The most simplified system utilises antigen covalently conjugated to a solid core nanobead of a defined size. Interestingly, nanobeads of defined size 40–50 nm have been found to be preferentially taken up by DCs [19]. In studies by Fifis *et al.* [20,21], researchers found that particles in this size range can elicit antibody and CD8 T cell immunity in mice, and protect against tumour challenge. Subsequent studies demonstrated that antigen conjugated to nanobeads of this size also induced antibodies and cell-mediated immune responses in sheep [22,23].

The size range of these nanobeads falls within the range of most viruses. Historically it has been suggested that beads between 20 and 200 nm are taken up by clathrin-coated vesicles (pits), caveolae or their independent receptors [24] (Figure 2). It is likely that a number of mechanisms are utilised to allow the efficient uptake of nanobeads into DCs. Antigen delivery to, and activation of, DCs is complex, involving antigen transport, DC binding and antigen uptake, and antigen processing and presentation [25].

Particles of this defined size are able to generate strong CD4 and CD8 Type 1 T cell responses [21], similar to a number of viruses. In this context, it is worthwhile mentioning that particles of various sizes including the size range reviewed here were shown to be able to induce NF-κB mediated release of pro-inflammatory cytokines [26]. But because a heterogeneous mix of particles was used and larger (titanium) particles are also able to activate NF-κB it is not possible to conclude unequivocally that the observed effect is linked to virus-sized particles.

The charges of the surface of these nanobeads have also been found to influence the level of immune response owing to differences in binding and internalization [27,28]. Interestingly, positively charged carboxylated poly-L-lysine-coated beads have been found to be more efficiently taken up by human derived DCs, when compared to negatively charged surfaces and bovine serum albumin-coated beads [29]. Therefore, the rate of uptake of DCs and macrophages is dependent not only on particles size and surface charge but is also influenced by the coating material on the surface of the beads. While epithelial cells are also capable of phagocytosis only professional antigen-presenting cells (APCs), namely DCs and macrophages, express an array of receptors (including pattern recognition receptors: lipopolysaccharide, mannose and toll-like receptors) on their surface that allow them to recognize and internalize any range of antigens [30]. Consistently, in the literature beads of a defined size 40–100 nm have been found to be more efficiently taken up by Dec205+ cells DCs, when compared to larger 1 μm beads that were preferentially taken up by F4/80+ macrophages *in vivo* [30].

Interestingly, particles in the same size range (i.e. 40 nm) were shown to cross the human skin readily and enter Langerhans cells following cyanacrylate skin surface stripping [31]. Considering that Langerhans cells are specialised APCs able to present antigen effectively to T cells, this observation may lead to novel transcutaneous vaccination strategies. As far as we are aware, however, the potential for a transcutaneous vaccine based around this technology has not yet been tested.

The simplicity and benefits of inert nanobead-based vaccines is apparent when considering the combination of low reactogenicity (i.e. absence of local tissue damage and inflammation) and the high immunogenicity of these inert particles [22,23]. Thus, overall, the flexibility and ease at producing and manipulating nanobeads of this size has applicability in a number of vaccine delivery systems.

Particulate DNA delivery systems

The use of particles for the delivery of DNA vaccines immediately brings to mind ballistic delivery of gold particles coated with DNA using a gene-gun. The particles used in this system (>600 nm) are typically larger than the virus-sized particles considered in this review and are therefore excluded.

Traditionally, DNA vaccines are poorly immunogenic, but when combined with cationic 128 nm sized nanobeads they are able to induce a 250-fold enhancement of antigen-specific IgG titre when compared to naked DNA alone [32]. This enhancement in the immune response, namely IgG levels, was also observed for DNA-coated nanobeads applied to mice through the skin using a jet injection device [32].

Poly-L-Lysine coated beads complexed with plasmid DNA encoding chicken egg ovalbumin (OVA) has also been used successfully to generate OVA-specific responses in mice and inhibit tumour growth in an OVA expressing EG7 tumour cell line [28]. In a study by Yoncheva *et al.* [33], amino-pegylated poly(methyl vinyl ether-co-maleic anhydride) coated nanobeads ranging between 289 and 500 nm associated with DNA have been shown to be tolerated orally, and were able to cross the cellular membrane of the gastrointestinal mucosa still maintaining DNA integrity. These results show promising advances in the difficult area of oral DNA vaccine delivery. Lastly, cationized gelatin nanobeads (~300 nm) were used as carriers to improve delivery of immuno-stimulatory CpG oligonucleotides (CpG ODN). These CpG ODN have strong adjuvant properties through their activation of toll-like receptors. The CpG ODN from classes B and C associated with nanobeads were able to increase production of interferon-alpha in primary human plasmacytoid DCs [34].

An alternative approach to the production of nanospheres containing DNA is to encapsulate the DNA in poly(D,L-lactic-co-glycolic acid) also known as PLGA. Using this method, it was possible to demonstrate that the plasmid DNA is largely protected from degradation [35]. The nanospheres are readily taken up by macrophages, which are able to express the marker protein encoded by the plasmid but were not tested for their ability to induce cytotoxic T cell responses. It should be noted that this polymer is already used for surgical applications [36] and has been used as a plasmid delivery system in humans [37]. Moreover, using larger DNA-PLGA particles (μm range), Sharpe *et al.* [38] were able to induce immune memory responses in macaques that could be effectively boosted by vaccinia viruses encoding the same recombinant HIV antigens.

Historically, virus membrane envelopes have also been used to deliver DNA. Interestingly, in a recent study the production of DNA-virosomes by virus solubilization using dicaproylphosphatidylcholine and subsequent mixing with a cationic lipid successfully protects the incorporated DNA from nuclease degradation [39]. The immunogenic potential of these particles, however, has not been reported [39].

Exosomes

In recent years, exosomes have been identified *in vivo*, in association with follicular DCs [40], urine [41], malignant tumour effusions [42], blood [43] and platelets [44]. Exosomes are endogenous particles 50–90 nm in diameter and are thought to be involved in protein sorting and/or the regulation of the immune response [45]. Exosomal production was also suggested to be a mechanism of releasing unnecessary proteins during the maturation of reticulocytes. They are distinct from vesicles produced by apoptotic cells and are only secreted by living cells [46]. Recent work by Caby *et al.* [43], suggests there is a circulating network of exosomes in animals, which is responsible for distal communication between cells. The positive aspect of this network is the enhancement of the immune response.

Aline *et al.* [47] showed that *Toxoplasma gondii*-pulsed DC-derived exosomes were able to prime an antigen-specific cellular and humoral response. This is one of the first studies to demonstrate that exosomes can induce a protective immune response against pathogens. Since then, potential therapeutic use for exosomes in immunotherapy has been demonstrated. The first Phase I clinical trial using autologous DC-derived exosomes was performed in stage II/IV melanoma patients and was completed in 2005 [48] with 50% of patients experiencing enhanced NK cell activity and IFN-γ secretion. On the basis of these results, a Phase I trial in colorectal patients was initiated and the results reported by Dai *et al.* [49], were that exosomes in the presence of granulocyte-macrophage colony stimulating factor (GMCSF) are able to provide a beneficial tumour-specific anti-tumour CTL response. Phase II/III trials in lung cancer patients and a Phase I/II trial in ovarian cancer patients are ongoing. In these studies, exosomes appear as a promising tool for autologous treatments of cancers.

The biological role of exosomes *in vivo* has not been completely elucidated; however, most of what is known about exosomes is derived from *in vitro* produced material and it is therefore unclear as to how far exosomes are produced *in vivo* as a result of external stimuli, how they load antigen (Ag) and the mechanism of uptake by immune cells. Interestingly, in a study by Montecalvo *et al.* [50] delivery of peptides through exosomes to DCs has been found to be significantly more effective at transfer than free peptide. It is interesting to speculate that inert beads of the 40–50 nm size may be tapping into the same mechanistic process utilised by exosomes to enable uptake into DCs. In one of the few *in vivo* studies performed so far, exosomes secreting tumour antigens have been found to induce a more potent antigen-specific anti-tumour immune response, suggesting that the secretion of antigens associated with exosomes has important implications [51].

Concluding remarks

From this review it is clear that nanosized vaccine delivery systems are becoming an important area in vaccine delivery. One common theme to all these developments is that the size of the particles produced is often in the 50 nm range, although particles used for DNA delivery are often larger. Considering that inert particles in this size range are preferentially taken up by DCs one could speculate that the common driving force behind these different antigen delivery systems is the size of the particles themselves and the way they interact with this specialised APC population. Indeed, since DCs are the key cell population for induction of primary immune responses, it makes sense to target these cells using virus-sized particles. One could even speculate that particles of this size might also interact with DCs in such a way that, besides delivering the antigen, they also activate the DCs. Hence, virus-sized particles could, by their size alone, act as a new form of pathogen associated molecular pattern (PAMP).

By virtue of their size, virus-sized vaccine delivery systems described in this review would be able to migrate readily from the site of injection in the skin to the draining lymph node. Indeed, lymphatic capillaries in the skin have a diameter in the 10–80 μm range [52] and would therefore be able to form a conduit for subcutaneously injected particles to reach the draining lymph node. The interaction with the DC could therefore occur at the site of injection as well as within the lymph node.

The fact that virus-sized particles have been shown to induce effective immune responses in different species also suggests that the mechanisms by which they induce immunity are common to these species. Hence, these mechanisms might be fundamental to the defence mechanisms against viral diseases and could be universally applicable across viral antigens and also harnessed to fight other infectious diseases and cancers.

Acknowledgement

The electron micrograph of the ISCOMs was prepared by A. Sjolander in the laboratory of E. Handman at the Walter and Eliza Hall Institute, Australia.

References

1 Sant'Angelo, D.B. (2003) Recollections of Charlie: Dr. Charles Alderson Janeway, Jr. (1943–2003). *J. Exp. Med.* 197, 1231–1232
2 Grgacic, E.V. and Anderson, D.A. (2006) Virus-like particles: passport to immune recognition. *Methods* 40, 60–65
3 Santi, L. *et al.* (2006) Virus-like particles production in green plants. *Methods* 40, 66–76
4 Santi, L. *et al.* (2008) An efficient plant viral expression system generating orally immunogenic Norwalk virus-like particles. *Vaccine* 26, 1846–1854
5 Vieira, H.L. *et al.* (2005) Triple layered rotavirus VLP production: kinetics of vector replication, mRNA stability and recombinant protein production. *J. Biotechnol.* 120, 72–82
6 Ludwig, C. and Wagner, R. (2007) Virus-like particles-universal molecular toolboxes. *Curr. Opin. Biotechnol.* 18, 537–545
7 Skountzou, I. *et al.* (2007) Incorporation of glycosylphosphatidylinositol-anchored granulocyte- macrophage colony-stimulating factor or CD40 ligand enhances immunogenicity of chimeric simian immunodeficiency virus-like particles. *J. Virol.* 81, 1083–1094
8 Morein, B. *et al.* (1984) Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses. *Nature* 308, 457–460
9 Hu, K.F. *et al.* (2005) The immunomodulating properties of human respiratory syncytial virus and immunostimulating complexes containing *Quillaja saponin*

components QH-A, QH-C and ISCOPREP703. *FEMS Immunol. Med. Microbiol.* 43, 269–276
10 Mowat, A.M. (2005) Dendritic cells and immune responses to orally administered antigens. *Vaccine* 23, 1797–1799
11 Pearse, M.J. and Drane, D. (2004) ISCOMATRIX adjuvant: a potent inducer of humoral and cellular immune responses. *Vaccine* 22, 2391–2395
12 Skene, C.D. and Sutton, P. (2006) Saponin-adjuvanted particulate vaccines for clinical use. *Methods* 40, 53–59
13 Lenarczyk, A. *et al.* (2004) ISCOM based vaccines for cancer immunotherapy. *Vaccine* 22, 963–974
14 Lendemans, D.G. *et al.* (2005) Cationic cage-like complexes formed by DC-cholesterol, Quil-A, and phospholipid. *J. Pharm. Sci.* 94, 1794–1807
15 Malliaros, J. *et al.* (2004) Association of antigens to ISCOMATRIX adjuvant using metal chelation leads to improved CTL responses. *Vaccine* 22, 3968–3975
16 Wikman, M. *et al.* (2006) Achieving directed immunostimulating complexes incorporation. *Expert. Rev. Vaccines* 5, 395–403
17 Pinitkiatisakul, S. *et al.* (2007) Immunogenicity and protective effect against murine cerebral neosporosis of recombinant NcSRS2 in different ISCOM formulations. *Vaccine* 25, 3658–3668
18 Spickler, A.R. and Roth, J.A. (2003) Adjuvants in veterinary vaccines: modes of action and adverse effects. *J. Vet. Int. Med./Am. College Vet. Int. Med.* 17, 273–281
19 Mottram, P.L. *et al.* (2007) Type 1 and 2 immunity following vaccination is influenced by nanoparticle size: formulation of a model vaccine for respiratory syncytial virus. *Mol. Pharm.* 4, 73–84
20 Fifis, T. *et al.* (2004) Short peptide sequences containing MHC class I and/or class II epitopes linked to nano-beads induce strong immunity and inhibition of growth of antigen-specific tumour challenge in mice. *Vaccine* 23, 258–266
21 Fifis, T. *et al.* (2004) Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. *J. Immunol.* 173, 3148–3154
22 Scheerlinck, J.P. *et al.* (2006) Systemic immune responses in sheep, induced by a novel nano-bead adjuvant. *Vaccine* 24, 1124–1131
23 Greenwood, D.L. *et al.* (2008) Vaccination against foot-and-mouth disease virus using peptides conjugated to nano-beads. *Vaccine* 26, 2706–2713
24 Pelkmans, L. (2005) Secrets of caveolae- and lipid raft-mediated endocytosis revealed by mammalian viruses. *Biochim. Biophys. Acta* 1746, 295–304
25 Reddy, S.T. *et al.* (2006) Targeting dendritic cells with biomaterials: developing the next generation of vaccines. *Trends Immunol.* 27, 573–579
26 Zhou, Y.M. *et al.* (2003) Oxidative stress and NFkappaB activation in the lungs of rats: a synergistic interaction between soot and iron particles. *Toxicol. Appl. Pharmacol.* 190, 157–169
27 Josephson, L. *et al.* (1999) High-efficiency intracellular magnetic labeling with novel superparamagnetic-Tat peptide conjugates. *Bioconjug. Chem.* 10, 186–191
28 Minigo, G. *et al.* (2007) Poly-L-lysine-coated nanoparticles: a potent delivery system to enhance DNA vaccine efficacy. *Vaccine* 25, 1316–1327
29 Thiele, L. *et al.* (2001) Evaluation of particle uptake in human blood monocyte-derived cells *in vitro*. Does phagocytosis activity of dendritic cells measure up with macrophages? *J. Control Release* 76, 59–71
30 Xiang, S.D. *et al.* (2006) Pathogen recognition and development of particulate vaccines: does size matter? *Methods* 40, 1–9
31 Vogt, A. *et al.* (2006) 40 nm, but not 750 or 1,500 nm, nanoparticles enter epidermal CD1a+ cells after transcutaneous application on human skin. *J. Invest. Dermatol.* 126, 1316–1322
32 Cui, Z. and Mumper, R.J. (2003) The effect of co-administration of adjuvants with a nanoparticle-based genetic vaccine delivery system on the resulting immune responses. *Eur. J. Pharm. Biopharm.* 55, 11–18
33 Yoncheva, K. *et al.* (2008) Development of bioadhesive amino-pegylated poly(anhydride) nanoparticles designed for oral DNA delivery. *J. Microencapsul.* 25, 82–89
34 Zwiorek, K. *et al.* (2008) Delivery by cationic gelatin nanoparticles strongly increases the immunostimulatory effects of CpG oligonucleotides. *Pharm. Res.* 25, 551–562
35 Mok, H. and Park, T.G. (2008) Direct plasmid DNA encapsulation within PLGA nanospheres by single oil-in-water emulsion method. *Eur. J. Pharm. Biopharm.* 68, 105–111
36 Greenland, J.R. and Letvin, N.L. (2007) Chemical adjuvants for plasmid DNA vaccines. *Vaccine* 25, 3731–3741
37 Klencke, B. *et al.* (2002) Encapsulated plasmid DNA treatment for human papillomavirus 16-associated anal dysplasia: a Phase I study of ZYC101. *Clin. Cancer Res.* 8, 1028–1037
38 Sharpe, S. *et al.* (2003) Mucosal immunization with PLGA-microencapsulated DNA primes a SIV-specific CTL response revealed by boosting with cognate recombinant modified vaccinia virus Ankara. *Virology* 313, 13–21
39 de Jonge, J. *et al.* (2007) Cellular gene transfer mediated by influenza virosomes with encapsulated plasmid DNA. *Biochem. J.* 405, 41–49
40 Denzer, K. *et al.* (2000) Follicular dendritic cells carry MHC class II-expressing microvesicles at their surface. *J. Immunol.* 165, 1259–1265
41 Pisitkun, T. *et al.* (2004) Identification and proteomic profiling of exosomes in human urine. *Proc. Natl. Acad. Sci. U.S.A.* 101, 13368–13373
42 Andre, F. *et al.* (2002) Malignant effusions and immunogenic tumour-derived exosomes. *Lancet* 360, 295–305
43 Caby, M.P. *et al.* (2005) Exosomal-like vesicles are present in human blood plasma. *Int. Immunol.* 17, 879–887
44 Robertson, C. *et al.* (2006) Cellular prion protein is released on exosomes from activated platelets. *Blood* 107, 3907–3911
45 de Gassart, A. *et al.* (2003) Lipid raft-associated protein sorting in exosomes. *Blood* 102, 4336–4344
46 Thery, C. *et al.* (2001) Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. *J. Immunol.* 166, 7309–7318
47 Aline, F. *et al.* (2004) Toxoplasma gondii antigen-pulsed-dendritic cell-derived exosomes induce a protective immune response against *T. gondii* infection. *Infect. Immunol.* 72, 4127–4137
48 Escudier, B. *et al.* (2005) Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of the first phase I clinical trial. *J. Transl. Med.* 3, 10
49 Dai, S. *et al.* (2008) Phase I clinical trial of autologous ascites-derived exosomes combined with GM-CSF for colorectal cancer. *Mol. Ther.* 16, 782–790
50 Montecalvo, A. *et al.* (2008) Exosomes as a short-range mechanism to spread alloantigen between dendritic cells during T cell allorecognition. *J. Immunol.* 180, 3081–3090
51 Zeelenberg, I.S. *et al.* (2008) Targeting tumor antigens to secreted membrane vesicles *in vivo* induces efficient antitumor immune responses. *Cancer Res.* 68, 1228–1235
52 Swartz, M.A. *et al.* (2008) Lymphatic drainage function and its immunological implications: from dendritic cell homing to vaccine design. *Semin. Immunol.* 20, 147–156