HUMAN VACCINES & IMMUNOTHERAPEUTICS
2016, VOL. 12, NO. 5, 1311–1317
http://dx.doi.org/10.1080/21645515.2015.1123829

Taylor & Francis
Taylor & Francis Group

COMMENTARY

Vaccinology: The art of putting together the right ingredients

Alberto Grandi[a], Michele Tomasi[b], and Guido Grandi[b]

[a]Externautics SpA, Siena, Italy; [b]CIBIO, Centre for Integrative Biology, University of Trento, Trento, Italy

ABSTRACT

Historically vaccines were produced using whole attenuated or killed pathogens and still a large proportion of current vaccines utilizes such procedure. However, for safety and quality reasons the development of novel vaccines is preferentially based on the selection and use of specific pathogen components which alone are capable of eliciting protective immune responses against the pathogens they derived from. The big challenge for vaccinologists is how to select the right antigens and to combine them with proper immune stimulatory components (adjuvants) in order to induce protective immunity. This Commentary outlines the authors' view on the current and future strategies for the efficient and rapid identification of the most effective protective antigens and adjuvants. Since efficacious subunit-based vaccines against recalcitrant pathogens are likely to require more than one antigen and/or immune stimulator, this poses the problem of how to make such vaccines economically acceptable. In this regard, the authors also present their view of how bacterial Outer Membrane Vesicles (OMVs) could become a promising platform for the development of future vaccines. The unique properties of OMVs might be exploited in the field of infectious diseases and oncology.

ARTICLE HISTORY
Received 12 October 2015
Revised 4 November 2015
Accepted 19 November 2015

KEYWORDS
bacterial vaccines; cancer vaccines; protective antigens; adjuvants; Outer Membrane Vesicles (OMVs); genomics; proteomics; bioinformatics; high throughput screening

From whole pathogen to subunit vaccines

The benefits of immunization in reducing the morbidity and mortality of infectious diseases have not been surpassed by any other medical intervention so far. Widespread use of vaccines in the past century led to the eradication of smallpox and control of many other pathogen-associated diseases including diphtheria, pertussis, measles and polio.

Vaccinology was born administering killed or attenuated pathogens to healthy people with the aim of protecting them from the deleterious effects of accidental, subsequent exposure to the same, but live and virulent, pathogens. Records of this procedure date to about 1000 A.D. in China and describe how smallpox pustules from infected patients were stored in the air for a month or more (to kill/attenuate the "unknown") and subsequently administered intranasally to healthy individuals together with grinded leaves of *Uvularia grandiflora*, probably the first "adjuvant" ever used in the history of Vaccinology.

At present, vaccines are available against 11 bacterial pathogens and 14 viral pathogens (for simplicity, different serotypes/isolates belonging to the same species are here considered as a single pathogen). Sixty per cent of these vaccines are still constituted by killed or attenuated pathogens.[1] Indeed, one of the best vaccine ever developed, given to hundreds of millions of people and still administered to individuals living in, or traveling to, endemic areas is against the Yellow Fever Virus and makes use of an attenuated viral strain, named 17D strain, which was isolated almost hundred years ago by the Nobel laureate Max Theiler.

Despite their public health triumphs, killed/attenuated pathogens are now rarely considered as a valid option for the development of new vaccines. This is in part due to safety concerns in handling virulent strains during the production processes and to the potential risk of administering something to healthy individuals that could, at least theoretically, be harmful. An anecdotal example is the Oral Polio Vaccine (OPV).[1] This vaccine, developed by Sabin in the middle of last Century, was produced by passages of the 3 main infective viral strains through non-human cells at a sub-physiological temperature. This produced spontaneous mutations resulting in a reduced ability of poliovirus to translate its RNA template within the host cell. The beauty about the attenuated poliovirus strains thus isolated is that they do not cause disease, being unable to replicate efficiently within nervous system tissue, but they can still replicate efficiently in the gut, thus allowing the elicitation of long-lasting protective immunity in vaccinated people. Indeed, OPV is an extremely effective vaccine and has almost succeeded in eliminating polio infections which are now restricted to limited areas in developing countries in which vaccination campaigns are logistically difficult to organize. However, even though at a very low frequency (approximately 1 case per 3 million vaccinees), the attenuated strains can revert to an infective form. The clinical symptoms caused by vaccine-derived poliovirus (VDPV) are indistinguishable from those caused by wild polioviruses. Furthermore, outbreaks of vaccine-associated paralytic poliomyelitis (VAPP) have been reported. This led the USA and other Countries to abandon

CONTACT Guido Grandi guido.grandi@unitn.it PhD, CIBIO, Center for Integrative Biology, University of Trento, Via Sommarive, 9 38123 Povo, Trento, Trento, Italy.

OPV and to replace it with a vaccine based on inactivated poliovirus (IPV).

Switching from whole pathogen to purified subunit(s) creates a different level of complexity to vaccinologists. Those ingredients naturally provided by whole pathogens, namely, the virulent factors that have to be neutralized by the immune system to prevent infection ("*protective antigens*") and the stimulatory components ("*adjuvants*") that activate immune cells and establish adaptive immunity, must be selected and properly combined to elicit the immune responses necessary and sufficient to prevent disease.

The search for the "right ingredients" turned out to be relatively simple for some bacterial and viral pathogens whose infection can be prevented by eliciting functional antibodies against single pathogen components. This is the case for lethal toxins, which can be inactivated by antibodies, and for surface components (for instance, capsular polysaccharides), which are the target of either bactericidal/opsonophagocytic antibodies or antibodies inhibiting pathogen binding to cellular receptors. In these cases, these specific components purified from pathogens or from engineered organisms and eventually combined with Alum as adjuvant have been sufficient to make vaccines that stimulate long lasting antibody-mediated protection. By contrast, for those pathogens which have evolutionarily evolved to escape the immune system by exploiting gene variability and/or redundancy of virulence factors, and for those pathogens which require both humoral and cell-mediated immunity (CMI) to be neutralized, the selection of the right ingredients can be a frustrating challenge.

High throughput technologies and a better understanding of microbial pathogenesis and basic immunology now offer new opportunities to identify protective antigens and to discover novel adjuvants which can better orchestrate the effector functions of our immune system.

Selection of protective antigens

A number of innovative technologies are now in the quiver of vaccinologists to facilitate the identification of pathogen-associated protective antigens. Four of these technologies are briefly described below.

Antigenome technology – The Antigenome approach, proposed by Etz and co-workers,[2] stems from the assumption, experimentally confirmed to be basically true for antibody-mediated immunity, that all protective antigens induce antibodies during natural infection. On the basis of that, the genomic DNA of the pathogen of interest is fragmented and the fragments are cloned to create bacterial expression libraries, each clone expressing random portions of the pathogen proteins. Those clones expressing immunogenic proteins are then identified by immunoassays using sera from convalescent patients. Normally, from a bacterial pathogen, 40 to 50 immunogenic proteins are identified and these proteins are subsequently tested in appropriate surrogate-of-protection assays (SoPAs) to select those to be used as vaccines.

Genome ("Reverse Vaccinology") technologies – Differently from the previous approach, this technology is completely "unbiased" and originates from the indisputable assumption that protective proteins must be encoded by the genetic makeup. Therefore, the genome of the pathogen of interest is sequenced and all annotated proteins are expressed and purified to be finally tested in SoPAs. The approach was conceptualized for the first time by Stephen Johnson and coworkers using DNA immunization,[3] subsequently proposed using protein immunization by L. Lissolo and coworkers (Abstracts WHO Conference 'Utilization of genomic information for tropical disease drug and vaccine discovery', Geneva, 1998;[4]), and finally demonstrated to be effective by Pizza and coworkers.[5] A *Neisseria meningitidis Group B* vaccine based on antigens (and Outer Membrane Vesicles (OMVs), see below) selected by Reverse Vaccinology has recently been registered in Europe.[6] One interesting aspect of this approach is that it can also be applied for the identification of antigens that stimulate Cell-Mediated Immunity (CMI).[7]

Proteomic technologies – The main drawback of genome technologies is that several hundreds of recombinant proteins have to be screened in time-consuming and labor intensive assays usually involving animal models. Since protective antigens of extracellular pathogens are surface-exposed and/or secreted, technologies which selectively identified this category of antigens can substantially accelerate the antigen discovery process. In Gram-positive bacteria surface and secreted proteins can be identified by treating bacteria with proteases under conditions that preserve cellular integrity. Mass spectrometry analysis of the proteolytic fragments released from the cell surface after enzymatic digestion allows the identification of the corresponding surface/secreted proteins.[8] In the case of Gram-negative bacteria, surface and secreted proteins can be characterized by mass spectrometry analysis of Outer membrane Vesicles (OMVs), which are naturally released by all Gram-negative bacteria and are constituted by outer membrane and periplasmic proteins.[9] Since a few tens of proteins are present on the bacterial surface as determined by the 2 mass spectrometry analyses, the number of antigens to be tested in SoPAs is markedly reduced with respect to the genomic approach. Following these strategy several protective antigens have been discovered.[10,11,12]

Recently, a combination of proteomics with an Antigenome-like approach (differently from the original Antigenome approach, immunogenic proteins are identified by screening sera from convalescent patients on protein arrays carrying purified recombinant proteins from the pathogen of interest) has been described.[13] The strategy allows to narrow the antigens to be tested in SoPAs down to less than 10. This is probably one of the most effective approach for vaccine antigen identification ever reported so far. The approach could theoretically allow to move from protective antigen identification directly to humans without the need of "filtering" vaccine candidates through SoPAs. This has a number of advantages. First, the most time consuming step of the antigen discovery process, namely the use of animal models, is basically eliminated. Second, the approach is in line with the new international regulations governing the utilization of animals in research. Third, since SoPAs generally make use of models which not necessarily recapitulate human infections, the approach should avoid the risk that "false positive" antigens enter clinical trials while "false negative" antigens are excluded.

Bioinformatics technologies – Surface-associated and secreted proteins, the proteins that, as said above, include antigens inducing antibody-mediated protection, carry characteristic signatures, such as secretion signal sequences and transmembrane spanning regions, recognizable at the level of their primary sequence. Algorithms are available that allow the reliable identification of these categories of proteins from genome sequences. Since surface and secreted proteins normally constitute approximately 30% of the whole proteome of a bacterial pathogen, bioinformatics tools have been combined with Reverse Vaccinology to simplify the screening of vaccine candidates.[5,14] More refined algorithms are currently under evaluation in an attempt to better single out protective antigens by *in silico* inspection of bacterial genomes. Among these are algorithms which further dissect the population of surface proteins and predict those that not only are surface-associated but also possess structural and functional domains that well extend out of the bacterial outer membrane/cell wall and therefore are potentially accessible to antibodies.[15] Another approach has been reported where the discrimination of protective antigens versus non-protective antigens was carried out using statistical methods based on amino acid compositional analysis and auto cross-covariance.[16] Along this line, very recently a novel *in silico* prediction strategy has been reported that appears to be particularly promising.[17] In essence, the strategy assumes that protective antigens carry specific signatures ("protective signatures" (PSs)) that discriminate them from the plethora of non-protective proteins. To identify PSs, all known protective antigens described in the literature (the "Protectome Space") are collected and computer-analyzed in search of common structural/functional motifs. In the paper described by Altindis et al.[17] from 241 antigens belonging to 36 bacterial pathogens approximately 35 PSs were identified. PSs are then used to predict protective antigens of the pathogen of interest by scanning the pathogen genome in search of proteins carrying one or more PSs. Although further optimization is needed, the approach paves the way to an "*in silico* only" antigen discovery strategy, which would allow to jump "from genome to clinics" without the need of "wet science."

Selection of adjuvants

Protein antigens alone are usually poorly immunogenic. When a purified protein is injected into experimental animals (and humans) antibody titers are low, isotype switching is inefficient and immunological memory is poorly induced. We now know that this is due to the inability of most of the proteins to properly activate professional Antigens Presenting Cells (APCs) which, in turn, do not activate antigen-specific T helper (T_H) cells. To be activated T_H cells not only have to recognize their specific antigen presented by APCs in the context of the Major Histocompatibility Complex (MHC) II, but also they must receive stimulatory signals. Such signals are released by APCs as long as they sense the danger of a potential intruder through specific receptors (Pathogen Recognition Receptors (PRRs)), which recognize "Pathogen-Associated Molecular Patterns (PAMPs)" and "Danger-Associated Molecular Patterns (DAMPs)." Several PRRs have been identified in mammals, of which the transmembrane Toll-like receptors (TLRs) and the cytoplasmic Oligomerization Domain (NOD)-like Receptors (NLRs) are the best characterized. Together, by recognizing PAMPs and DAMPs they initiate innate immune responses and activate APCs to properly orchestrate adaptive immunity toward the invading pathogen. The chemical nature of PAMPs and DAMPs is now sufficiently well characterized and, a part from some important exceptions, pathogen proteins do not proficiently interact with PRRs. This is why protein-based vaccines must include adjuvants: the proteins are taken up by APCs, degraded into peptides and presented in the context of MHC, while the adjuvants activate APCs so that the T cells which recognize the peptide-MHC complex can differentiate into effector and memory T cells. Ideally, protein antigens and adjuvants should be physically associated so as to guarantee that all APCs presenting the antigen-derived peptides are also activated.

Although the importance of adjuvants was recognized very early on in vaccination (the use of *Uvularia grandiflora* in the thousand year old Chinese smallpox virus vaccine has already been cited) the only adjuvants approved till recently were Aluminum salts and the oil-in-water emulsions MF59 and AS03. Interestingly, these adjuvants were approved in the absence of a minimal understanding of their mechanism of action. Although we still do not know exactly how they work, it is now well documented that both Aluminum salts and the oil-in-water emulsions act as antigen delivery systems. Antigen adsorption to alum increases antigen uptake by DCs[18] and alum injection recruits monocytes which then migrate to the draining lymph nodes to differentiate into inflammatory DCs capable of priming T cells.[19] Similarly to alum, oil-in-water emulsions enhance antigen uptake by DCs, which seem to internalize both antigen and MF59 and then migrate to the draining lymph nodes where they can prime an adaptive response.[20] There is evidence that internalization of Alum and MF59 by immune cells also leads to the activation of specific immune pathways, thus suggesting that, in addition of being good delivery systems, they can also exert some immune potentiating activities.

The main reason why very few adjuvants have been approved so far is because adjuvants are "double-edge swords": their ability of potentiating the immune system can theoretically lead to excess inflammation and autoimmunity. Therefore, vaccine companies have been reluctant to develop novel adjuvants which at the end could not pass the strict requirements which regulatory authorities imposed also to respond to the society demand for extremely high level of safety for pharmaceutical products. The realization that Alum and oil-in-water emulsions are not adequate to induce the proper immune responses against a number of pathogens, particularly intracellular pathogens and pathogens causing chronic diseases, is changing this attitude and one new adjuvant, the GSK AS04, has recently been approved and many others have reached the clinics.[21] In general the new generation adjuvants include a delivery system (Alum, oil-in-water emulsions, liposomes, etc.,) and immune potentiators which specifically bind to one particular PRRs. AS04 is constituted by Alum and monophosphoryl lipid A (MPL), a TLR4 agonist purified from *Salmonella Minnesota* lipopolysaccharide (LPS). The adjuvant is used in human licensed vaccines preventing human papillomavirus (HPV) and hepatitis B virus (HBV) infections. Clinical studies

have shown that HPV vaccination with the MPL/alum adjuvant combination enhances both humoral and memory B-cell immunity compared with alum alone. *In vitro* studies have shown that AS04 activates human DCs, resulting in the production of IL-12 and increased expression of MHC class II and costimulatory molecules.

Several other PRR agonists are being tested in humans, including new generation TLR4 agonists, synthetic oligonucleotides targeting TLR9, TLR3 and MDA5, flagellin (TLR5 agonists), and small molecules targeting TLR7. The search for new immune potentiators often starts from the understanding at molecular and structural level of how natural PAMPs interact with their cognate PRRs and subsequently new molecules are designed which optimize the activation signals triggered by PAMP/PRR interactions. For instance, Glucopyranosyl lipid A (GLA) is a new synthetic immune stimulator which has been designed starting from the structure of the TLR4 agonist LPS. GLA combines 6 acyl chains with a single phosphorylation site and when formulated as a stable oil-in water emulsion (GLA-SE) it features excellent safety and immunostimulatory profiles in humans.[22] Recently, the group led by N. Valiante at Novartis Vaccines has demonstrated the power of *in vitro* high throughput screening in the identification of novel adjuvants.[23] Focusing their interest in TLR7 agonists, Valiante and co-workers exploited an engineered TLR7-expressing cell line which fires the expression of a reporter gene anytime a molecule binds to TLR7. The cell line was used in a high throughput modality to single out TLR agonists among a vast chemical library ($>10^6$ compounds). A few molecules were identified among which one, named SMIP.7–10, appeared to be particularly promising. Interestingly, the chemical structure of the molecule turned out to resemble the backbone of RNA, the natural agonist of TLR7. When such an immune potentiator was chemically functionalized to be efficiently adsorbed to aluminum hydroxide, the resulting formulation was shown to have excellent adjuvanticity properties when combined with different antigens, including *Meningococcus B* antigens, *Bacillus anthracis* toxin[23] and *S. aureus* antigens.[24]

Chemical modification of known and natural PRR agonists, high throughput screening of chemical libraries, and computer-assisted design of novel chemical entities are likely to play a relevant role for the development of future adjuvants characterized by low reactogenicity and optimal immune stimulatory capacity. Furthermore, there is an increasing body of evidence suggesting that when combined different immune potentiators can work synergistically.[21] Therefore, combinations of immune potentiators can be exploited to activate proper gene expression profiles which ultimately orchestrate effector functions necessary to combat the target pathogen/disease.

Once protective antigens and effective adjuvants are selected, they have to be produced, purified, and properly formulated to become vaccines. Considering that effective vaccines against recalcitrant pathogens could require a cocktail of several antigens, delivery system and immune potentiators, the production costs could not be compatible with the health care budget of many countries, particularly developing countries. Therefore, the challenge for modern vaccinology is to find strategies which simplify vaccine production processes and abate costs while fulfilling the need of making complex combination of antigens and adjuvants.

Bacterial outer membrane vesicles (OMVs) and vaccines

OMVs are closed spheroid particles of a heterogeneous size, 20–300 nm in diameter, released by all Gram-negative bacteria so far studied. Several experimental evidences indicate that OMVs are generated through a "budding out" of the bacterial outer membrane and, consistently, the majority of their components are represented by LPS, glycerophospholipids, outer membrane proteins and periplasmic proteins.

A multitude of functions have been attributed to OMVs, including inter and intra species cell-to-cell cross-talk, biofilm formation, genetic transformation, defense against host immune responses, and delivery of toxins and virulence factors to host cells.[25] OMV interaction to host cells can occur by endocytosis after binding to host cell receptors or lipid rafts. Alternatively, OMVs have been reported to fuse to cell membrane, leading to the direct release of their content into the cytoplasm of the host cells.[25,26]

From a biotechnological standpoint OMVs are emerging as promising vaccine platform. Indeed, OMVs purified from several pathogens, including *Neisseria, Salmonella, Pseudomonas, Vibrio cholerae, Burkholderia*, and *E. coli*,[27] induce potent protective immune responses against the pathogens they derive from, and highly efficacious anti-Neisseria OMV-based vaccines are already available for human use. The attractiveness of OMVs in vaccine applications reside in several key features. First, OMVs are highly immunogenic being readily phagocytosed by professional APCs and carrying many PAMPs, including, among others, LPS, lipoproteins and peptidoglycan.[26] As a result, OMVs elicit potent Th1-skewed immune responses without the need of adding additional adjuvants or delivery systems. For instance, mice immunized with Salmonella OMVs develop robust Salmonella-specific B and T cell responses.[28] Similarly, immunization with Escherichia coli–derived OMVs prevented bacteria-induced lethality and OMV-induced systemic inflammatory response syndrome. As verified by adoptive transfer and gene-knockout studies, the protective effect of OMV immunization was found to be primarily by the stimulation of T cell immunity, especially by IFN-γ and IL-17 from T cells.[29] Second, OMVs are amenable for large scale GMP production. The original processes foresee the treatment of bacterial cells with a mild detergent to obtain portions of the outer membrane which in a hydrophilic environment subsequently collapse to form vesicles. Such vesicles can be purified by ultrafiltration. However, OMVs can be directly purified in large quantities from culture supernatant if specific genes in the OMVs-producing strains are inactivated. It has been shown that such "naturally released" OMVs are not only easier to produce but also immunogenically much superior than their detergent-derived cousins, which are heavily contaminated with cytoplasmic proteins and deprived of important membrane-associated antigens and PAMPs as a consequence of the detergent treatment.[9] At least 100 mg of "naturally derived" OMVs per liter of culture can be purified.[30] Assuming that 10 to 50 μg

per vaccine dose are used, 1 L of culture is sufficient to produce something between 2.000 to 10.000 doses. Third, the protein content of OMVs can be manipulated by applying molecular and synthetic biology strategies to properly alter the genetic makeup of the OMV-producing strain. After the first demonstration by Kesty and Kuhen that heterologous proteins can be compartmentalized in OMVs,[31] an increasing number of publications report the decoration of OMVs with heterologous proteins. Two main strategies for delivering proteins to the OMVs are used. According to one strategy, a leader sequence for secretion (LP) is fused at the N-terminus of the protein of interest. In so doing the protein enters the secretory pathway and reaches the periplasm where can be trapped in the lumen of OMVs during the budding out process.[31,32] Alternatively, the protein of interest is fused to endogenous periplasmic and membrane-associated proteins which chaperone the protein to the vesicular compartment. For instance, a number of heterologous proteins have been successfully exported to the surface of OMVs when fused to the β-barrel forming autotransporter AIDA, the hemolysin ClyA and Hemoglobin protease Hbp, proteins that compartmentalize in *E. coli* OMVs.[33,34,35,36]

The growing number of studies on the immunogenicity properties of engineered OMVs indicate that potent immune responses against the recombinant antigens used to decorate OMVs are elicited in immunized animals. For instance, alkaline phosphatese (PhoA) from *E. coli* has been expressed in *Vibrio cholera* OMVs and mice immunization with such OMVs induced anti-PhoA antibodies.[37] Similarly, OMVs expressing a ClyA-GFP fusion induced anti-GFP antibodies.[35] Furthermore, OMVs carrying the *Chlamydia trachomatis* antigen HtrA induced antibodies capable of neutralizing Chlamydia infection in vitro.[38] Finally, in a recent study, Fantappie' and coworkers extensively analyzed antigen-specific antibodies responses induced by OMVs engineered with 6 different heterologous proteins, including known protective antigens from *Group A Streptococcus* (GAS).[32] With no exceptions, all vesicles induced functional antibodies and, in particular, mice immunized with OMVs decorated with GAS antigens were protected from a subsequent challenge with a lethal dose of GAS virulent strains. Another important information from published studies is that good antigen-specific antibody responses can be induced even if the recombinant antigens are expressed in the lumen of the vesicles. Although the exact mechanism remains to be elucidated (it has been postulated that part of the vesicles are destroyed at the site of injection thus making the antigens accessible to B cell receptors), this observation makes the OMV vaccine platform even more attractive and flexible since delivering of antigens to the OMVs surface is not strictly necessary to induce functional antigen-specific antibody responses.

An increasing body of evidence also indicate that immune responses induced by OMVs are skewed toward a Th1/Th17 profile and include the activation of antigen-specific CD4 and CD8 T cells. For instance, OMVs purified from *E. coli* protected mice from a lethal challenge with a pathogenic *E. coli* strain and protection was largely mediated by cellular immunity.[29] Furthermore, Kuipers and co-workers have demonstrated the intranasal administration of Salmonella OMVs expressing pneumococcal antigens protect mice from pneumococcal colonization and protection was partially mediated by interferon γ and Th17 in the nasal tissue.[39] In addition, Zhang and coworkers exploited MisL autotransporter to present CD4 T cell epitopes on the Salmonella OMVs surface and showed that such engineered OMVs induced epitope-specific T cell responses.[40] Finally, very recently, we have decorated OMVs with several B and T cell epitopes, including CD8 T cell epitopes, and such OMVs induced epitope-specific antibodies and T cell responses in mice (Grandi A., Tomasi M. and Grandi G., unpublished).

In conclusion, built-in adjuvanticity, possibility of manipulation and simplicity of production process make OMVs a promising vaccine platform.

The main drawback of OMVs is represented by their heterogeneity in size and composition which make lot consistency a manufacturing challenge. Rigorous production processes and new analytical procedures need be established to minimize product variability and to assure that each vaccine dose has the expected potency and safety. However, such complications in manufacturing are not unsurmountable as clearly demonstrated by the fact that OMV-based vaccines are already available for human use, including the recently approved vaccine against Meningococcus B.

The quite unique properties of OMVs to carry different PAMPs which, by working synergistically, induce potent antibody and cell-mediated immune responses, strongly suggest that their application could not be limited to vaccine against bacterial infections but also, and particularly, against viral diseases and cancer. In this respect we like to close this commentary with an historical note. In 1891 a young New York surgeon, William Coley, began intratumoral injections of live or inactivated bacteria in an effort to reproduce the spontaneous remissions of sarcomas observed in rare cancer patients who had developed erysipelas. Some significant responses were recorded over the ensuing 40 years, and this was attributed to the fact that "Coley's toxins" stimulated antibacterial phagocytes that killed bystander tumor cells. In particular, intravesical injection of live bacillus Calmette-Guérin in superficial bladder cancer patients after surgical resection prolonged patient survival. Such anti-cancer activity, unexplainable at the time W. Coley performed his human trials, can now be attributed to bacterial PAMPs which stimulate immunity by binding to their cognate PRRs expressed on the surface of different cell populations present in tumors. This leads to the optimistic perspective that if properly decorated with tumor-associated and/or tumor specific antigens, OMVs could play an important role in future anti-cancer therapeutic applications.

Disclosure of potential conflict of interest

No potential of conflicts of interest were disclosed.

Funding

Authors' research on Outer Membrane Vesicles(OMVs) is supported by the European Community Advanced ERC Grant "OMVAC" given to Guido Grandi.

References

[1] Sutter R.W., Kew O.M., Cochi S.L. Aylward R.B. Poliovirus vaccine, live. In "Vaccines" 6th Edition. Edited by Stanley A. Plotkin, Walter A. Orenstein, Paul A. Offit. Elsevier, 2013.

[2] Etz H, Minh DB, Henics T, Dryla A, Winkler B, Triska C, Boyd AP, So¨llner J, Schmidt W, von Ahsen U, et al. Identification of in vivo expressed vaccine candidate antigens from Staphylococcus aureus. Proc Natl Acad Sci USA 2002; 99:6573-8; PMID:11997460; http://dx.doi.org/10.1073/pnas.092569199

[3] Barry MA, Lai WC, Johnston SA. Protection against mycoplasma infection using expression-library immunization. Nature 1995; 377:632-5; PMID:7566175; http://dx.doi.org/10.1038/377632a0

[4] Grandi G. Antibacterial vaccine design using genomics and proteomics. Trends Biotechnol 2001; 19:181-8; PMID:11301131; http://dx.doi.org/10.1016/S0167-7799(01)01600-6

[5] Pizza M, Scarlato V, Masignani V, Giuliani MM, Aric∫ B, Comanducci M, Jennings GT, Baldi L, Bartolini E, Capecchi B, et al. Identification of vaccine candidates against serogroup B meningococcus genome sequencing. Science 2000; 287:1816-20; PMID:10710308; http://dx.doi.org/10.1126/science.287.5459.1816

[6] Giuliani MM, Adu-Bobie J, Comanducci M, Aric∫ B, Savino S, Santini L, Brunelli B, Bambini S, Biolchi A, Capecchi B, et al. A universal vaccine for Serogroup B Meningococcus. Proc Natl Acad Sci U S A 2006; 103:10834-9; PMID:16825336; http://dx.doi.org/10.1073/pnas.0603940103

[7] Finco O, Frigimelica E, Buricchi F, Petracca R, Galli G, Faenzi E, Meoni E, Bonci A, Agnusdei M, Nardelli F, et al. Approach to discover T and B cell antigens of intracellular pathogens applied to the design of Chlamydia trachomatis vaccines. Proc Natl Acad Sci USA 2011; 108:9969-74; PMID:21628568; http://dx.doi.org/10.1073/pnas.1101756108

[8] Rodríguez-Ortega MJ, Norais N, Bensi G, Liberatori S, Capo S, Mora M, Scarselli M, Doro F, Ferrari G, Garaguso I, Maggi T, et al. Characterization and identification of vaccine candidate proteins through analysis of the group A Streptococcus surface proteome. Nat Biotechnol 2006; 24:191-7; PMID:16415855; http://dx.doi.org/10.1038/nbt1179

[9] Ferrari G, Garaguso I, Adu-Bobie J, Doro F, Taddei AR, Biolchi A, Brunelli B, Giuliani MM, Pizza M, Norais N, et al. Outer membrane vesicles from group B Neisseria meningitidis Δgna33 mutant: proteomic and immunological comparison with detergent-derived outer membrane vesicles. Proteomics 2006; 6:1856-66; PMID:16456881; http://dx.doi.org/10.1002/pmic.200500164

[10] Doro F, Liberatori S, Rodríguez-Ortega MJ, Rinaudo CD, Rosini R, Mora M, Scarselli M, Altindis E, D'Aurizio R, Stella M, et al. Surfome analysis as a fast track to vaccine discovery: Identification of a novel protective antigen for group B Streptococcus hyper-virulent strain COH1. Mol Cell Proteomics 2009; 8:1728-37; PMID:19401597; http://dx.doi.org/10.1074/mcp.M800486-MCP200

[11] Solis N, Larsen MR, Cordwell SJ. Improved accuracy of cell surface shaving proteomics in Staphylococcus aureus using a false-positive control. Proteomics 2010; 10:2037-49; PMID:20217865; http://dx.doi.org/10.1002/pmic.200900564

[12] Dreisbach A, Hempel K, Buist G, Hecker M, Becher D, van Dijl JM. Profiling the surfacome of Staphylococcus aureus. Proteomics 2010; 10:3082-96; PMID:20662103; http://dx.doi.org/10.1002/pmic.201000062

[13] Bensi G, Mora M, Tuscano G, Biagini M, Chiarot E, Bombaci M, Capo S, Falugi F, Manetti A, Donato P, et al. Multi high-throughput approach for highly selective identification of vaccine candidates: the group A Streptococcus case. Mol Cell Proteomics 2012; 11, 1-12; PMID:22286755; http://dx.doi.org/10.1074/mcp.M111.015693

[14] Maione D, Margarit y Ros I, Rinaudo D, Masignani V, Mora M, Scarselli M, Tettelin H, Brettoni C, Iacobini ET, Rosini R, et al. Identification of a universal group B Streptococcus vaccine by multiple genome screen. Science 2005; 309:148-50; PMID:15994562; http://dx.doi.org/10.1126/science.1109869

[15] Barinov A, Loux V, Hammani A, Nicolas P, Langella P, Ehrlich D, Maguin E, van de Guchte M. Prediction of surface exposed proteins in Streptococcus pyogenes, with a potential application to other Gram-positive bacteria. Proteomics 2009; 9:61-73; PMID:19053137; http://dx.doi.org/10.1002/pmic.200800195

[16] Doytchinova IA, Flower DR. VaxiJen: a server for prediction of protective antigens, tumor antigens, and subunit vaccines. BMC Bioinformatics 2007; 8:4; PMID:17207271; http://dx.doi.org/10.1186/1471-2105-8-4

[17] Altindis E, Cozzi R, Di Palo B, Necchi F, Mishra RP, Fontana MR, Soriani M, Bagnoli F, Maione D, Grandi G, et al. Protectome analysis: a new selective bioinformatics tool for bacterial vaccine candidate discovery. Mol Cell Proteomics 2015; 14, 418-29; PMID:25368410; http://dx.doi.org/10.1074/mcp.M114.039362

[18] Morefield GL, Sokolovska A, Jiang D, HogenEsch H, Robinson JP, Hem SL. Role of aluminum-containing adjuvants in antigen internalization by dendritic cells in vitro. Vaccine 2005; 23:1588-95; PMID:15694511; http://dx.doi.org/10.1016/j.vaccine.2004.07.050

[19] Kool M, Pétrilli V, De Smedt T, Rolaz A, Hammad H, van Nimwegen M, Bergen IM, Castillo R, Lambrecht BN, Tschopp J. Cutting edge: Alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome. J Immunol 2008; 181:3755-9; PMID:18768827; http://dx.doi.org/10.4049/jimmunol.181.6.3755

[20] Calabro S, Tortoli M, Baudner BC, Pacitto A, Cortese M, O'Hagan DT, De Gregorio E, Seubert A, Wack A. Vaccine adjuvants alum and MF59 induce rapid recruitment of neutrophils and monocytes that participate in antigen transport to draining lymph nodes. Vaccine 2011; 29:1812-23; PMID:21215831; http://dx.doi.org/10.1016/j.vaccine.2010.12.090

[21] Maisonneuve C, Bertholet S, Philpott DJ, De Gregorio Ennio. Unleashing the potential of NOD- and Toll-like agonists as vaccine adjuvants. Proc Natl Acad Sci USA 2014; 111:12294-9; PMID:25136133; http://dx.doi.org/10.1073/pnas.1400478111

[22] Behzad H, Huckriede AL, Haynes L, Gentleman B, Coyle K, Wilschut JC, Kollmann TR, Reed SG, McElhaney JE. GLA-SE, a synthetic toll-like receptor 4 agonist, enhances T-cell responses to influenza vaccine in older adults. J Infect Dis 2012; 205:466-73; PMID:22147791; http://dx.doi.org/10.1093/infdis/jir769

[23] Tom Y-H, Wu TY-H, Singh M, Miller AT, De Gregorio E, Doro F, D'Oro U, Skibinski DAG, Mbow L, Bufali S, et al. Rational design of small molecules as vaccine adjuvants. Sci Transl Med 2014; 6:263ra160; PMID:25411473

[24] Bagnoli F, Fontana MR, Soldaini E, Mishra RP, Fiaschi L, Cartocci E, Nardi-Dei V, Ruggiero P, Nosari S, De Falco MG, et al. Vaccine composition formulated with a novel TLR7-dependent adjuvant induces high and broad protection against Staphylococcus aureus. Proc Natl Acad Sci USA 2015; 112:3680-5; PMID:25775551.

[25] Schwechheimer C, Kuehn MJ. Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. Nat Rev Microbiol 2015; 13:605-19; PMID:26373371; http://dx.doi.org/10.1038/nrmicro3525

[26] Ellis N, Kuehn MJ. Virulence and immunomodulatory roles of bacterial outer membrane vesicles. Microb Mol Biol Rev 2010; 74:81-94; PMID:20197500; http://dx.doi.org/10.1128/MMBR.00031-09

[27] Collins BS. Gram-negative outer membrane vesicles in vaccine development. Discovery Medicine 2010; 12:7-15.

[28] Alaniz RC, Deatherage BL, Lara GC, Cookson BT. Membrane vesicles are immunogenic facsimiles of salmonella typhimurium that potently activate dendritic cells, prime B and T cell responses, and stimulate protective immunity in vivo. J Immunol 2007; 179:7692-701; PMID:18025215; http://dx.doi.org/10.4049/jimmunol.179.11.7692

[29] Kim OY, Hong BS, Park K-S, Yoon YJ, Choi SJ, Lee WH, Roh T-Y, Lo¨tvall J, Kim Y-K, Gho YS. Immunization with escherichia coli outer membrane vesicles protects bacteria-induced lethality via Th1 and Th17 cell responses. J Immunol 2013; 190:4092-102; PMID:23514742; http://dx.doi.org/10.4049/jimmunol.1200742

[30] Berlanda Scorza F, Colucci AM, Maggiore L, Sanzone S, Rossi O, Ferlenghi I, Pesce I, Caboni M, Norais N, Di Cioccio V, et al. High yield production process for shigella outer membrane particles. Plos One 2012; 7:e35616; PMID:22701551; http://dx.doi.org/10.1371/journal.pone.0035616

[31] Kesty NC, Kuehn MJ. Incorporation of heterologous outer membrane and periplasmic proteins into escherichia coli outer membrane

vesicles. J Biol Chem 2004; 279:2069-76; PMID:14578354; http://dx.doi.org/10.1074/jbc.M307628200

[32] Fantappié L, De Santis M, Chiarot E, Carboni F, Bensi G, Jousson O, Margarit I, Guido G. Antibody-mediated immunity induced by engineered Escherichia coli OMVs carrying heterologous antigens in their lumen. J Extracellular Vesicles 2014; 3:24015.

[33] Schroeder J, Aebischer T. Recombinant outer membrane vesicles to augment antigen-specific live vaccine responses. Vaccine 2009; 27:674854; PMID:19748581; http://dx.doi.org/10.1016/j.vaccine.2009.08.106

[34] Kim J-Y, Doody AM, Chen DJ, Cremona GH, Shuler ML, Putnam D, DeLisa MP. Engineered bacterial outer membrane vesicles with enhanced functionality. J Mol Biol 2008; 380:51-66; PMID:18511069; http://dx.doi.org/10.1016/j.jmb.2008.03.076

[35] Chen DJ, Osterrieder N, Metzger SM, Buckles E, Doody AM, DeLisa MP, Putman D. Delivery of foreign antigens by engineered outer membrane vesicle vaccines. Proc Natl Acad Sci USA 2010; 107:3099-104; PMID:20133740; http://dx.doi.org/10.1073/pnas.0805532107

[36] Daleke-Schermerhorn MH, Felix T, Soprova Z, ten Hagen-Jongman CM, Vikström D, Majlessi L, Beskers J, Follmann F, de Punder K, van der Wel NN, et al. Decoration of outer membrane vesicles with multiple antigens by using an autotransporter approach. Applied Environ Microbiol 2014; 80:5854-65; PMID:25038093; http://dx.doi.org/10.1128/AEM.01941-14

[37] Schild S, Nelson EJ, Bishop AL, Camilli A. Characterization of vibrio cholerae outer membrane vesicles as a candidate vaccine for cholera. Inf Imm 2009; 77:472-84; PMID:19001078; http://dx.doi.org/10.1128/IAI.01139-08

[38] Bartolini E, Ianni E, Frigimelica E, Petracca R, Galli G, Berlanda Scorza F, Norais N, Laera D, Giusti F, Pierleoni A, et al. Recombinant outer membrane vesicles carrying Chlamydia muridarum HtrA induce antibodies that neutralize chlamydial infection in vitro. J Extracellular Vesicles 2013; 2:20181; PMID:24009891; http://dx.doi.org/10.3402/jev.v2i0.20181

[39] Kuipersa K, Daleke-Schermerhornb MH, Jong WSP, ten Hagen-Jongman CM, van Opzeeland F, Simonetti E, Luirink J, de Jongea MI. Salmonella outer membrane vesicles displaying high densities of pneumococcal antigen at the surface offer protection against colonization. Vaccine 2015; 33:2022-9; PMID:25776921; http://dx.doi.org/10.1016/j.vaccine.2015.03.010

[40] Zhang J, De Masi L, John B, Chen W, Schifferli DM. Improved delivery of the OVA-CD4 peptide to T helper cells by polymeric surface display on Salmonella. Microbial Cell Factories 2014; 13:80; PMID:24898796; http://dx.doi.org/10.1186/1475-2859-13-80