

Innate immunity and new adjuvants

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Summary

Vaccination remains the most cost-effective biomedical approach to the control of infectious diseases in livestock. Vaccines based on killed pathogens or subunit antigens are safer but are often ineffective and require coadministration with adjuvants to achieve efficacy. Unfortunately, most conventional adjuvants are poorly defined, complex substances that fail to meet the stringent criteria for safety and efficacy desired in new generation vaccines. A new generation of adjuvants that work by activating innate immunity presents exciting opportunities to develop safer, more potent vaccines. In this review the authors highlight the role of innate immunity in protection against infectious disease and provide some examples of promising new adjuvants that activate innate immunity. They do not review the conventional adjuvants present in many vaccines since they have been reviewed extensively previously.

Keywords

Adjuvant – Infectious disease – Innate immunity – Livestock – Vaccines.

Introduction

Infectious diseases continue to be a major cause of death and economic losses in domestic animals. Today, the most cost-effective strategy for the control of infectious diseases is clearly vaccination. Indeed, vaccination has already greatly improved livestock production and reduced animal suffering. However, there are concerns regarding many of today's vaccines with respect to their safety and efficacy, and therefore there is a need for safer and more efficacious vaccines for livestock. Furthermore, the realisation that the majority of newly emerging diseases not only affect animals but can be transmitted to humans has created an even greater need for effective vaccines for domestic animals. Vaccines based on live and killed pathogens have traditionally been used in the livestock industry, and each of these has its perceived advantages and disadvantages. Live vaccines are often more effective as they tend to stimulate vigorous immune responses, often similar to natural infection, but they can potentially revert to virulence and cause disease especially in

immunocompromised hosts. Killed vaccines or their components are generally regarded as safer, but they often fail to induce protective immunity.

The realisation that certain components in killed vaccines may be harmful to the host has led to the evolution of a vaccine development approach that involves the identification of defined molecules (protective antigens) that are associated with induction of protective immunity. With the recent and rapid progress in molecular biology, genomics, proteomics, and immunology it is now possible to identify a myriad of potential targets for vaccine development. Furthermore, combining the advances in molecular biology with those in immunology and pathogenesis, it is now possible to correlate the immune response induced by specific proteins with different levels of protection. Thus, we now know, in most cases, what components of the infectious agent are critical for preventing infection or aiding in recovery from infection as well as which immune responses are desired. Unfortunately, it is not always easy to induce the correct immune response. This is especially the case where

recombinant proteins or killed vaccines are used as immunogens. These killed antigens are generally poor at inducing immune responses and, more importantly, the quality of the immune response induced may not give optimal protection. This could possibly be improved by developing novel adjuvants and formulation technologies. Conventional adjuvants used in commercially available animal vaccines have been extensively reviewed elsewhere and the reader is referred to these excellent reviews for details (10, 48).

A detailed understanding of the requirements for immune activation has provided an explanation for why recombinant vaccines fail to be effective. These vaccines often lack the components of pathogens that trigger 'danger' signals that activate innate immunity leading to enhanced vaccine efficacy. In this regard, the search for new adjuvants has focused on molecules that activate innate immunity. We will briefly discuss innate immunity and highlight some promising new adjuvants that enhance vaccine efficacy primarily by stimulating innate immunity.

Innate immunity

The immune system has evolved two general strategies to protect the host against infectious diseases: the innate and adaptive immune responses (Table 1). Innate immunity represents a very effective first response against invading pathogens and consists of a set of conserved mechanisms to recognise and counter the constant threat of microbial infections (3, 27). As such, innate immunity is regulated by a network of complex receptor-ligand interactions which eventually lead to the creation of a pro-inflammatory local environment and thereby set the stage for the development of adaptive immune responses. The adaptive immune system, which is relatively slow to respond, forms the second line of immune defence, a 'back-up' strategy called into action to clear any pathogens that survive or evade the innate immune responses. Indeed, in the case of a rapidly

replicating pathogen, this delay affects the success of the naïve host in attacking the invading organism (45). Therefore, the early interplay between innate and adaptive immunity is essential for effective immunity against most invading pathogens (23). By exploiting this link between innate and adaptive immunity, it is possible to develop more potent adjuvants, leading to more effective vaccine formulations.

Stimulation of innate immunity is initiated by the interaction of pathogen components with receptors present on immune cells. These pattern recognition receptors (PRR) recognise highly conserved components of pathogens called pathogen-associated molecular patterns (PAMP) (51, 53). Pattern recognition receptors represent a large group of conserved receptor molecules including toll-like receptors (TLR), complement receptors, C-type lectins, and nucleotide-binding oligomerisation domain (NOD) receptors NOD 1 or NOD 2 (22, 32, 43). Following recognition of pathogen PAMP, signalling through these receptors leads to activation of the nuclear factor- κ B, which in turn increases expression of chemical mediators including cytokines, chemokines (Fig. 1) and co-stimulatory molecules (34). Several of these cytokines induce epithelial cells to express antimicrobial peptides, increasing the antimicrobial capacity of the epithelial barrier (63). In addition, expression of these molecules creates a local pro-inflammatory environment, which helps to recruit and activate phagocytic cells, activate the complement cascade, contain the invading pathogen and chemoattract the effector cells of the adaptive immune response (Fig. 1). However, over-stimulation can also result in septic pro-inflammatory responses such as secretion of tumour necrosis factor, which in severe cases can be detrimental to the animal.

Pattern recognition receptors can be found in large concentrations at the cutaneous and mucosal surfaces of the body and are expressed in various types of immune cells including antigen-presenting cells (APC) and lymphocytes. Of special importance are dendritic cells (DC), highly effective APC that express a wide variety of PRR. These receptors are used by DC as 'sensors' for pathogens and they also sample antigens in their microenvironment. Signalling through PRR leads to activation of APC and expression of several responses. Subsequently, these cells migrate towards the draining lymphoid tissues where the antigen is either directly presented or passed on to resident DC for the induction of an adaptive immune response. Thus, DC represent an important link between innate and specific immunity. Furthermore, the type of initial innate stimulus will impact the ability of DC to link innate and adaptive immune responses with regard to the quality and magnitude of the responses. Thus, DC can 'imprint' the adaptive immune response by shifting the type of effector response to either a T helper (Th)1 type (protects primarily against

Table 1
Innate versus adaptive immunity

Innate immunity	Adaptive immunity
Very early after exposure to infection (hours, days)	Delayed (days, weeks)
Activated by a wide range of pathogens	Specifically activated by certain components of pathogens (antigen)
Confers broad protection	Vaccines stimulate this type of immunity
Primary functions:	Primary functions:
Controls spread of infection	Clearance of infection
Direct development of adaptive immunity	Development of memory response

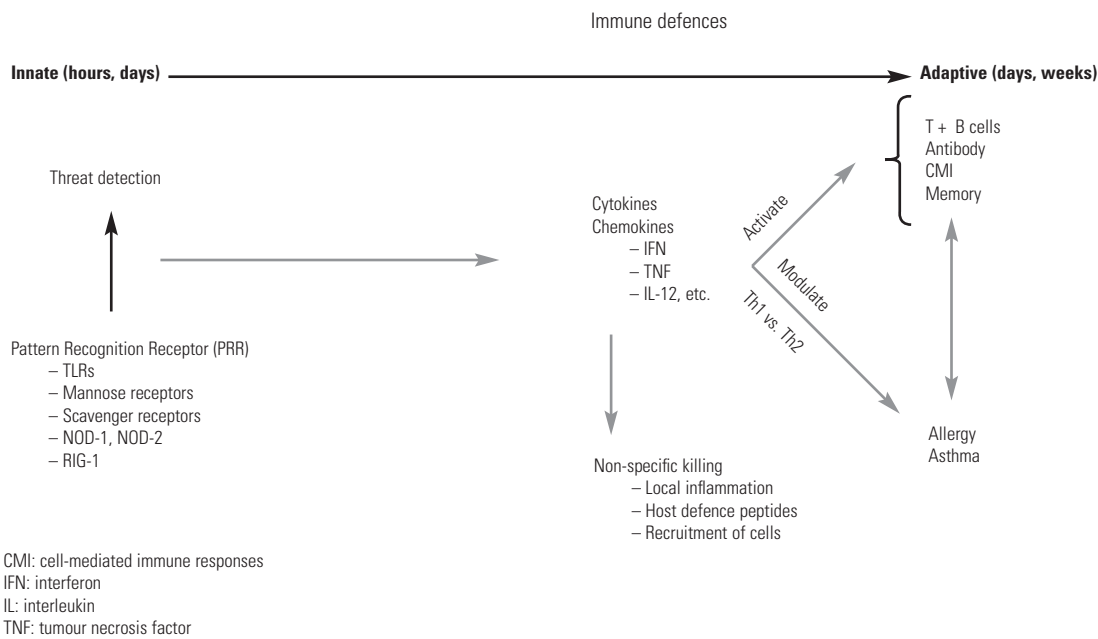


Fig. 1
Activation of innate and adaptive immunity through pattern recognition receptors

The innate immune system uses a network of pattern recognition receptors to detect the presence of infectious agents. These include toll-like receptors (TLR), nucleotide-binding oligomerisation domain receptors (NOD) and retinoic acid inducible genes (RIG). Engagement of these receptors initiates a signalling cascade that results in production of a variety of mediators (cytokines, chemokines), which mediate the effector responses.

These responses serve two primary functions:

- a) to control spread of infection via non-specific killing
- b) to activate and direct the development of the adaptive immune responses (T helper [Th]1 and Th2)

intracellular pathogens) or a Th2 type (protects primarily against extracellular pathogens) of immune response, and by instructing effector cells to selectively home back to certain compartments of the immune system. Thus, stimulation of DC by vaccine adjuvants represents an important strategy for novel vaccination.

Adjuvants

Adjuvants were first described by Ramon (41) as ‘helper’ substances which when added to an antigen produce stronger immune responses than can be induced by the antigen alone. Since then many different natural and synthetic substances have been evaluated, primarily by trial and error, and some have been found to have adjuvant activity.

Adjuvants can be classified into two broad categories:

- a) delivery systems
- b) immunostimulatory adjuvants (48).

Delivery systems include many conventional adjuvants such as alum, liposomes, microparticles and oil/water

emulsions. The mechanisms by which these adjuvants work are not well understood, but many of them form a ‘depot’ at the site of injection, where the antigen is slowly released and stimulates infiltrating cells of the immune system. Furthermore, these are often poorly defined, crude substances that have been associated with severe tissue damage at the site of injection. Ironically, the efficacy of some of these adjuvants is dependent on the degree of tissue damage, i.e. a substance that causes severe tissue damage has more potent adjuvant activity. Therefore, the challenge for vaccinologists is to discover and develop adjuvants that activate protective immunity but do not cause severe tissue damage. This paradigm shift has generated great interest in the second class of adjuvants, the immunostimulatory adjuvants, which tend to stimulate immunity with minimal or no tissue damage. These adjuvants are predominantly microbial components (Table II) and as the name suggests their adjuvant activity is dependent on their ability to stimulate innate immunity. Current understanding of how the body senses infectious threats involves the use of a variety of receptors (see innate immunity, above) which sets the stage for a ‘danger’ signal that triggers a cascade of innate immune responses, subsequently leading to the recruitment and expansion of cells involved in the development of adaptive immunity.

Indeed, several pathogen-derived components such as bacterial endotoxin (lipopolysaccharide [LPS]), the mycobacterial component of complete Freund's adjuvant, single-stranded ribonucleic acid (ssRNA), and bacterial deoxyribonucleic acid (DNA), including synthetic CpG DNA (sites where cytosine [C] lies next to guanine [G] in the DNA sequence; the p indicates that C and G are connected by a phosphodiester bond), can generate 'danger' signals and thus have adjuvant activity (17, 35, 44, 54). Therefore, molecules that activate innate immunity provide a novel class of adjuvants that not only enhance immune responses but can be selectively used to 'tailor' the quality of the desired response.

Shortly after the discovery that cytokines were critical in inducing immune responses, there was a flurry of activity to use cytokines as adjuvants. Initially, these studies involved interleukin (IL)-2 and interferon gamma (IFN- γ), two potent immune modulators. Some of these studies clearly showed the benefits of incorporating cytokines into vaccines. For example, IL-2 enhanced immune responses to bovine herpesvirus antigens, and other studies have also shown enhanced primary and secondary immune responses in the presence of IFN- γ (24). However, studies also showed that the dose of cytokine was critical and that immune suppression could occur if inappropriate doses were used (24). This is not surprising because the immune system generally is not engineered to respond to a large bolus of a single cytokine. Indeed, a very fine balance between the different cytokines is crucial to ensure appropriate cell signalling. This can be achieved by use of adjuvants that stimulate innate immunity, leading to production of a variety of cytokines and other mediators, resulting in stimulation of well-regulated immune responses.

In addition to their traditional role in preventing infectious diseases, vaccination strategies are also being developed as therapies for other diseases, including cancer and allergies. Development of safe and effective vaccine adjuvants is critical not only for improvement of existing vaccines but also for developing novel vaccines. In the next section the authors discuss some of these new adjuvants.

CpG oligodeoxynucleotides

As early as the 1890s, a surgeon in New York observed that cancer patients injected with crude bacterial preparations had significantly longer remission periods. Subsequently, bacterial DNA was identified as the primary mediator of anti-tumour immunity in mice (50, 58). It has now become clear that bacterial DNA, as well as synthetic oligodeoxynucleotides (ODN) containing CpG motifs (CpG ODN), provides a 'danger' signal that induces vigorous immune responses. To date, numerous investigators have shown that treatment of animals with CpG DNA can protect against a variety of experimental infectious and non-infectious diseases (56). Based on encouraging results from mouse models, human clinical studies are now being undertaken to evaluate the efficacy of CpG ODN therapy against infectious disease, cancer, asthma and allergy (28). In this regard, addition of CpG ODN to a commercial hepatitis B virus (HBV) vaccine resulted in significant increases in HBV surface antigen-specific antibody response in human volunteers (14). Furthermore, immunisation of human immunodeficiency virus (HIV)-infected individuals with an HBV vaccine in the presence of CpG DNA significantly increased the number of seropositive subjects and also increased the HBV-specific lymphocyte proliferative response (15). Thus, CpG DNA is a promising adjuvant for human vaccines.

Synthetic CpG DNA has been evaluated as a vaccine adjuvant in large animals. Unlike conventional oil-based adjuvants, which typically promote Th2 type immune responses that may not be protective against some infections, in these studies, CpG ODN promoted predominantly Th1 type immune responses (13, 28). For example, CpG ODN was shown to be an excellent adjuvant for stimulating immune responses against an experimental vaccine based on a subunit protein (gD antigen) of bovine herpesvirus-1 (BHV-1) in mice, sheep and cattle by producing enhanced serum immunoglobulin 2a levels and IFN- γ in splenocytes or peripheral blood lymphocytes, indicating a more balanced, or Th1 type, response (25, 26). Interestingly, the use of CpG ODN in combination with low levels of mineral oil enhanced the

Table II
Examples of adjuvants that stimulate innate immunity

Adjuvant	Evidence for adjuvant activity in:	References
CpG deoxyribonucleic acid	Cattle, sheep, pigs, horses, monkeys, humans, laboratory animals	1, 13, 14, 15, 18, 25, 26, 31, 42
Host defence peptides	Laboratory animals	4, 6, 11, 30, 49
Single stranded ribonucleic acid and imidazoquinolines	Monkeys, laboratory animals	54, 55
Polyphosphazenes	Laboratory animals, sheep	33, 38, 40, 57, unpublished observations

immune response and reduced the amount of tissue damage associated with conventional vaccine adjuvants in sheep (25). In addition, CpG ODN in combination with alum demonstrated protection against BHV-1 (42), and CpG ODN in combination with Emulsigen® (a mineral oil adjuvant) was shown to be a potent adjuvant for stimulating a protective immune response against the gD antigen of BHV-1 in cattle (26). Similarly, incorporation of CpG ODN in a commercial equine influenza virus vaccine resulted in significant enhancement of antibody production against influenza virus (31).

Therefore, CpG ODN is compatible with commercially available vaccines, and in some cases CpG synergises with conventional adjuvants present in these vaccines, resulting in even greater enhancement of immune responses. This should expedite the application of CpG in commercial vaccines because there should be less need to perform all the safety trials required for new vaccines as new adjuvants are simply being added to currently licensed vaccines. Indeed, clinical trials are currently in progress to evaluate the benefits of incorporating CpG DNA in commercial livestock vaccines.

Host defence peptides

Cationic host defence peptides (HDP) are endogenous antibiotics found in virtually every life form. Mammalian HDP are very short peptides that can be grouped into defensins and cathelicidins. Typically, HDP are amphipathic positively charged molecules (20, 39).

Host defence peptides are fundamental components of the innate immune response. Their wide spectrum of functions includes direct antimicrobial activities, immunostimulatory functions of both innate and acquired immunity, and involvement in wound healing, cell trafficking and vascular growth (9, 12, 36). While the antimicrobial activities of HDP have been known for a long time, recent evidence suggests that at physiological concentrations mammalian HDP have a number of immunomodulatory functions, including recruitment of immature DC and T-cells, glucocorticoid production, macrophage phagocytosis, mast cell degranulation, complement activation, and IL-8 production by epithelial cells (59, 61, 62). Other HDP have been shown to up-regulate gene expression in epithelial cells and monocytes, and to neutralise pro-inflammatory cytokine induction and lethality in response to LPS/endotoxin (2, 7, 9, 16, 19, 20, 29, 36, 37, 46, 47).

Evidence for the ability of HDP to enhance adaptive immunity (indicative of adjuvant activity) is based on various observations. For example, human neutrophil peptides (HNP) 1 to 3, human beta-defensins 1 and 2, and

murine beta-defensins (mBD) have been described to be chemoattractive for immature DC and lymphocytes (4, 60), and monocytes and macrophages (21). Recognition by immature DC occurs through chemokine receptor 6 (4) and other not yet identified receptors (60). Furthermore, in addition to chemoattraction of immature DC, HDP have also been demonstrated to attract mature DC (4, 6, 16). The immunoenhancing activity of HDP has been demonstrated in several studies. For example, ovalbumin-specific immune responses were enhanced in mice when HNP 1-3 were co-administered intranasally to C57/Bl mice (30). This observation was further supported by other investigators (49) who demonstrated that intraperitoneal injection of HNP 1-3 together with keyhole limpet haemocyanin (KLH) and B-cell lymphoma idiotype antigen into mice enhanced the resistance of immunised mice to subsequent tumour challenge. Brogden *et al.* (11) also confirmed the immunoenhancing activity of various defensins. More evidence for the immunoenhancing activity of HDP is derived from studies using DNA vaccines. When mBD2 and mBD3 were fused with B-cell lymphoma epitope sFv38, strong immune responses and stronger anti-tumour immunity were observed in immunised mice (4, 6). The same researchers also demonstrated that human immunodeficiency virus-1 glycoprotein 120 (HIV gp120) specific mucosal, systemic, and cytotoxic lymphocyte (CTL) immune responses could be achieved after immunisation with a fusion DNA vaccine encoding the murine β -defensin 2 and the HIV gp120 gene (5). Thus, these examples provide evidence that HDP can be used as adjuvants to enhance vaccine-specific immunity.

To co-formulate HDPs into novel vaccines several issues need to be addressed, including reduction of the cost of producing the peptide, co-formulation and possible interaction with the antigen, and the stability and safety of the vaccine formulation. Recent research has already demonstrated that short peptide derivatives of only 7 to 12 amino acids, which include only specific motifs for certain functions, can behave very similarly to the parental HDP (8). These derivatives are much cheaper to produce and potentially have less interaction with other vaccine components. More research is required to better understand the peptide motifs that are responsible for immunomodulatory and antimicrobial functions. In addition, although a large variety of HDP has been described in domestic animals (11) very little information is currently available about the immunomodulatory functions of these peptides. Thus, future research needs to address the immunoenhancing activities of these HDP in their respective host species, analyse their potential cross-species activity and investigate the prophylactic potential for preventing infectious disease in domestic animals. However, preliminary results provide a degree of hope that these molecules will be able to improve vaccine responses with minimal adverse reactions.

Ribonucleic acid oligonucleotides and imidazoquinolines

Synthetic ssRNA and small anti-viral compounds (imidazoquinolines) activate a class of receptors similar to those stimulated by CpG ODN. Imidazoquinolines have adjuvant activity and appear to promote Th1 rather than Th2 immune responses (52). Studies in mice have revealed that appropriately formulated ssRNA is a potent adjuvant and modulator of vaccine-associated immune responses (54). Furthermore, conjugation of imidazoquinoline derivatives to an HIV experimental vaccine dramatically enhanced the magnitude and altered the quality of Th1 immune responses in monkeys (55). Although they have not yet been tested for adjuvant activity in humans and livestock, based on the results from mice and monkeys it is a reasonable expectation that these molecules will have adjuvant activity in livestock. Evidence in support of this notion comes from numerous studies in the authors' laboratory, which have confirmed that ssRNA and imidazoquinolines are highly stimulatory when tested in immune cells from cattle, pigs and sheep (Mutwiri *et al.*, unpublished observations), strongly suggesting that studies testing these molecules as adjuvants in livestock are warranted.

Polyphosphazenes

Polyphosphazenes are synthetic, water-soluble and biodegradable polymers that are inexpensive to produce. One of the most interesting properties of polyphosphazenes is that they are stable at room temperature and can be stored on the bench for several months without loss of activity, eliminating the need for refrigeration. The prototype member of this class of polymers is poly[di(sodium carboxylatophenoxy) phosphazene] (PCPP) which has previously been shown to have adjuvant activity with a variety of viral and bacterial antigens in mice (33, 40, 57). Despite the compelling evidence for adjuvant activity of these polymers in mice, they have not been tested in large animals. In this regard, the authors have shown that PCPP is also a potent adjuvant in sheep when used at only double the dose used in mice (Mutwiri *et al.*, unpublished data). A new polyphosphazene polyelectrolyte, poly[di(sodium carboxylatoethylphenoxy)phosphazene] (PCEP) seems to have even more potent adjuvant activity (38). Evidence from numerous studies in mice demonstrates that PCEP is a potent enhancer of antigen-specific immune responses, and its adjuvant activity is far superior to that of PCPP and the conventional adjuvant alum (38). PCEP not only enhanced the magnitude but modulated the quality of immune responses, resulting in more balanced immunity

(38). Even more interesting was the observation that the combination of PCEP with CpG showed strong synergy, resulting in dramatic increase in immune responses. The authors hypothesise that because PCEP induces immune responses that have similarities with those stimulated by CpG DNA, PCEP achieves its adjuvant effects by activating innate immunity. Indeed, they have obtained evidence that PCEP activates immune cells to secrete cytokines that have been associated with the development of Th1 type immune responses (Mutwiri *et al.*, manuscript submitted). Thus, activation of innate immunity may be at least one of the mechanisms by which PCEP mediates its potent adjuvant activity. PCEP has not yet been tested in livestock. Given its success in mice, studies in large animals are certainly warranted.

Conclusion

Looking to the future, many new generation vaccines will consist of purified antigens and well-defined adjuvants, and these vaccines will be expected to meet more stringent safety and efficacy requirements. A few examples of directions in which the field of adjuvant development may be headed in the future have been provided here. The authors anticipate that, in future, adjuvants will be used as high precision tools to activate the desired immune responses. In this regard, the selection of adjuvants will be much more focused on stimulating specific immune responses, and not just enhancing antibody responses. Thus, there will be more emphasis on the quality of the immune response with fewer adverse reactions. The use of stimulators of innate immunity such as CpG or other selective modulators of the innate immune response, combined with better formulations, should dramatically improve vaccine efficacy and reduce economic losses to the livestock industry. Furthermore, these more defined vaccine formulations, together with the understanding of their mode of action, should provide the regulatory agencies with a greater level of confidence in the new vaccines. Those vaccines currently being developed will be safer for use in livestock, which is particularly important for food-producing animals that will eventually be consumed by humans.

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L'immunité innée et les nouveaux adjuvants

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Résumé

De toutes les approches biomédicales visant à contrôler les maladies du bétail, la vaccination est la plus rentable. Les vaccins dits inactivés (à agent pathogène mort) ou les vaccins sous-unitaires (utilisant uniquement les fractions immunogènes du microorganisme) présentent une meilleure innocuité mais leur efficacité laisse à désirer et nécessite souvent la présence d'adjuvant. Malheureusement, la plupart des adjuvants classiques sont des substances complexes et mal définies qui ne répondent pas aux critères rigoureux d'innocuité et d'efficacité exigés pour les vaccins de nouvelle génération. Une nouvelle génération d'adjuvants qui agissent en stimulant l'immunité innée offre de nouvelles perspectives pour la mise au point de vaccins plus sûrs et plus efficaces. Les auteurs soulignent le rôle de l'immunité naturelle pour se protéger contre les maladies infectieuses et citent quelques exemples prometteurs d'adjuvants capables de stimuler l'immunité innée. Les adjuvants classiques ont déjà fait l'objet de revues détaillées et ne sont pas examinés dans cet article.

Mots-clés

Adjuvant – Bétail – Immunité innée – Maladie infectieuse – Vaccin.



Inmunidad innata y nuevos adyuvantes

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Resumen

La vacunación sigue siendo el procedimiento biomédico más rentable para luchar contra las enfermedades infecciosas del ganado. Las vacunas basadas en patógenos muertos o subunidades antigénicas presentan menos riesgos, pero con frecuencia es preciso administrarlas con adyuvantes para que sean eficaces. Lamentablemente, la mayoría de los adyuvantes convencionales son sustancias complejas, mal definidas, que no satisfacen los criterios estrictos en materia de inocuidad y eficacia que las vacunas de nueva generación deben cumplir. La nueva generación de adyuvantes que potencia la inmunidad innata representa una oportunidad muy interesante para obtener vacunas más seguras y potentes. En este artículo los autores destacan el papel de la inmunidad innata en la protección contra enfermedades infecciosas y presentan algunos ejemplos de nuevos adyuvantes prometedores que la potencian. No examinan los adyuvantes convencionales utilizados en muchas vacunas puesto que ya fueron analizados exhaustivamente en el pasado.

Palabras clave

Adyuvante – Enfermedad infecciosa – Ganado – Inmunidad innata – Vacuna.



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