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Development and use of mucosal vaccines: Potential and limitations

Desenvolvimento e uso de vacinas mucosas: Potencial e limitações

Desarrollo y uso de vacunas mucosas: Potencial y limitaciones

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Abstract
Mucosal surfaces represent a major gateway to microorganisms which may be harmful to health. The humoral immune response has an important action in the defense of these surfaces, as it is able to prevent the entry of pathogens in the body. Vaccines with local application have been evaluated in order to stimulate an efficient immune response in the mucous membranes, since conventional vaccines, for parenteral application, tend to stimulate a mostly systemic response. Vaccines that use the mucosa as an inoculation route are able to generate an immune response directly in the application mucosa and corresponding mucosa, since the mucosal system is integrated, which represents an important advantage in choosing the inoculation route. This paper aims to illustrate some concepts related to mucosal immunity in general, as well as to gather information about what has been studied in relation to mucosal routes of administration of vaccines, immunomodulators and antigen delivery systems.
Keywords: Delivery systems; Immunomodulators; Routes of administration; Vaccines.

Resumo
As superfícies mucosas representam uma importante porta de entrada para microrganismos que podem ser prejudiciais à saúde. A resposta imune humoral tem uma importante ação na defesa dessas superfícies, pois é capaz de prevenir a entrada de patógenos no organismo. Vacinas com aplicação local têm sido utilizadas com o objetivo de estimular uma resposta imune eficiente nas mucosas, uma vez que as vacinas convencionais, para aplicação parenteral, tendem a estimular uma resposta predominantemente sistêmica. Vacinas que utilizam a mucosa como via de inoculação são capazes de gerar uma resposta imune diretamente na mucosa de aplicação e mucosa correspondente, uma vez que o sistema mucoso é integrado, o que representa uma vantagem importante na escolha da via de inoculação. Este artigo tem como objetivo ilustrar alguns conceitos relacionados à imunidade mucosa em geral, bem como reunir

informações sobre o que tem sido estudado em relação às vias de administração de vacinas nas mucosas, imunomoduladores e sistemas de liberação de antígenos**.**
Palavras-chave: Sistemas de entrega; Imunomoduladores; Vias de administração; Vacinas.

Resumen
Las superficies mucosas representan una puerta de entrada importante para los microorganismos que pueden ser perjudiciales para la salud. La respuesta inmune humoral tiene una acción importante en la defensa de estas superficies, ya que es capaz de prevenir la entrada de patógenos en el organismo. Se han utilizado vacunas de aplicación local para estimular una respuesta inmune eficaz en las mucosas, ya que las vacunas convencionales, de aplicación parenteral, tienden a estimular una respuesta predominantemente sistémica. Las vacunas que utilizan la mucosa como vía de inoculación son capaces de generar una respuesta inmune directamente en la mucosa de aplicación y la mucosa correspondiente, ya que el sistema mucoso está integrado, lo que representa una ventaja importante en la elección de la vía de inoculación. Este artículo tiene como objetivo ilustrar algunos conceptos relacionados con la inmunidad mucosa en general, así como recopilar información sobre lo que se ha estudiado en relación con las vías de administración de vacunas mucosas, inmunomoduladores y sistemas de liberación de antígenos.
Palabras clave: Sistemas de entrega; Inmunomoduladores; Rutas de administracion; Vacunas.

1. Introduction

Several microorganisms use mucous surfaces as a gateway and / or initial point of propagation in the body. The mucous membranes of the respiratory, gastrointestinal and urogenital tracts are separated from the outside by delicate barriers of epithelial tissue, which can cause greater fragility to these sites (Brandtzaeg, 2007; Zajac et al., 2016; Chase et al., 2017). Thus, the mucosal immune system represents the initial barrier against these pathogens, using defense mechanisms, both innate and adaptive (Woof & Kerr, 2006).

In comparison to the sterile conditions provided by the immune system in the vast majority of organs, the lymphoid tissue associated with mucous membranes (MALT) have an antigenic microenvironment that is different from the interior of the organism and they also have immunological characteristics that make them unique and independent from the systemic immune system. This tissue is predominantly composed of GALT (Gut-Associated Lymphoid Tissue), BALT (Bronchus-Associated Lymphoid Tissue), NALT (Nasal-Associated Lymphoid Tissue), as well as epithelial tissue and specialized cells (Brandtzaeg et al., 2007; Macpherson et al., 2018). MALT has M cells in its constitution, which vary within different species and according to the degree of stimulatory activity. M cells are important cells specialized in the effective transport of antigens, from the organ lumen to the lymphoid follicle, without causing any damage to them. Upon reaching the follicle, these antigens are captured by antigen presenting cells (APCs), such as dendritic cells (DCs), and presented to auxiliary T (CD4 +) and cytotoxic (CD8 +) lymphocytes, initiating a response (Cruvinel et al., 2010). Several enteropathogenic infectious agents use M cells as a gateway to the body, showing a certain vulnerability in the intestinal epithelial surface (Cruvinel et al., 2010; Cazote et al., 2018).

Mucous surfaces can be termed types I and II (Brandtzaeg, 2007; Cazote et al., 2018). The relevant differences between these two types of mucosa are the presence (type I) or absence (type II) of IgA transport mechanisms, in addition to differences in cellular compositions, considering the presence (type I) or absence (type II) of lymphoid tissues associated with the mucosa (Cazote et al., 2018). Commonly, the type I mucosal submucosa consists of a large number of DCs, macrophages and memory lymphocytes, while the type II mucosal submucosa contains a scarce repertoire of DCs and macrophages, in addition to rare lymphocytes.

The mucosal immune response is moderated by the nature of the antigen, the type of antigen presenting cells (APCs) involved and the local microenvironment (Leung et al., 2000; Júnior et al., 2010). After sensitization of B and T lymphocytes at the induction site, these defense cells circulate via lymph and blood vessels, with consequent colonization of the corresponding mucosa, where they differ in effector and memory cells. The differentiation of B lymphocytes activated by

antigen recognition causes a process of proliferation and differentiation that culminates in the humoral immune response, when immunoglobulin-secreting plasma cells are generated, directed to the antigenic epitope that originated the response (Brandtzaeg et al., 2007; Júnior et al., 2010).

Plasma cells derived from antigenic stimulation of inactive or memory B lymphocytes reside in different tissues (Cruz, 2018). Stimulation occurs in the secondary lymphoid organs, such as spleen and lymph nodes, and at the first contact of the B cell receptor with its corresponding antigen, a process of division and differentiation of B cells into memory or secretory cells (plasma cells) begins (Kraan et al., 2017). The initially formed plasmocytes, responsible for the primary humoral response, secrete Immunoglobulin M (IgM), an antibody of low affinity with the antigen (Campbell et al., 2003). After the co-stimulation of active B cells by CD4 + helper T lymphocytes, through the secretion of certain cytokines (Kurashima & Kiyono, 2017), a series of changes occurs, the result of which is evident in the generation of memory B cells and plasmocytes that synthesize high affinity antibodies (Macpherson et al., 2008; Macpherson et al., 2018). Cytokines, for example Interleukin 5 (IL-5) and transforming growth factor β (TGF-β), secreted by helper T lymphocytes CD4 +, lead to the secretion of IgA, the predominant antibody in mucous secretions (Campbell et al., 2003; Macpherson et al., 2008). It is also known that the isotype of the immunoglobulin secreted by the plasma cell is determined by the site of presentation of the antigen by the APCs, as well as the nature of the antigenic stimulation. The site of induction and the expressed isotype will determine the tissue into which the plasma cells will be destined, according to the cytokines found (Macpherson et al., 2008; Macpherson et al., 2018).

Immunoglobulin A (IgA)

The Immunoglobulin A molecule is the most abundant produced by mammals, and is present in serum and mucous secretions, such as tears, saliva, colostrum and also in the secretions of the intestinal, respiratory and genitourinary tracts (Macpherson et al., 2008; Lycke et al., 2012; Macpherson et al., 2018). In all non-ruminant mammals, IgA is the predominant antibody in mucous membranes, in contrast to IgG, dominant in systemic humoral immunity (Neutra & Kozlowski, 2006; Lycke et al., 2012). However, in ruminants, IgG, more specifically IgG1, plays an important role in the defense of mucous surfaces, especially in the mammary gland, where it is found in quantities greater than IgA (Woof & Mestecky, 2005). In the bovine species, there is a predominance of IgA in the vaginal mucosa, while in the uterus the IgG predominates (Leung et al., 2000). Only a fraction of IgG1 found at this location comes from the endometrium, the remainder being derived from the peripheral circulation (Leung et al., 2000; Woof & Kerr, 2006).

IgA monomers have a molecular weight of approximately 150 kDa, but this immunoglobulin is normally secreted as a dimer and, occasionally, as a polymer (Woof & Mestecky, 2005). Like most antibodies, dimeric IgA is secreted by plasma cells, this form occurs by binding two monomeric IgA molecules that are linked by the J chain, this molecule is transported and binds to the polymeric (pIgR) receptor on epithelial cells. The pIgR transports IgA through epithelial cells through a process of transcytosis, which results in the translocation of secretory IgA complexes (sIgA) to the mucosal surface (Lycke et al., 2012). These complexes impart IgA mucophilic characteristics, including resistance to degradation in an environment rich in proteases, as is the case with mucous surfaces (Woof & Kerr, 2006). This resistance to enzymatic degradation is also due to the high degree of glycosylation during its synthesis and is of great importance in the gastrointestinal tract, due to the presence of proteolytic enzymes such as pepsin, trypsin and chymotrypsin in this site (Brandtzaeg et al., 2005).

Human IgA is the most widely studied and characterized, consisting of two subclasses called IgA1 and IgA2 (Pakkanen et al., 2010). It has been reported that 71% of circulating IgA-secreting plasma cells synthesize IgA1, the dominant subtype in human blood serum, tears, nasal fluid and saliva (He et al., 2007). On the other hand, IgA2 is found in greater quantity in the intestinal and genitourinary tracts, routinely colonized by commensal microorganisms and the levels of IgA1 and IgA2 are proportionally equivalent in the vaginal fluid (Murphy, 2014). These subclasses differ structurally in terms of the

size of the hinge region, longer in IgA1, which causes greater susceptibility to bacterial proteases, which target the immunoglobulin hinge region (Murphy, 2014; Tsuruhara et al., 2017). In relation to IgA2, the presence of special carbohydrate chains, in addition to a weak or absent hinge region, gives it protection against proteolytic enzymes (Tsuruhara et al., 2017). The distribution of subclasses in secretions is determined by the origin of the antigen found at the site and the specific stimuli provided to local lymphocytes (Woof & Kerr, 2006; Macpherson et al., 2008).

IgA protects mucous membranes through different mechanisms, such as viral and toxin neutralization (Neutra & Kozlowski, 2006; Murphy, 2014), in addition to having antibacterial activity, through immune / antigenic exclusion. This immune / antigenic exclusion action is based on the neutralization of pathogenic bacteria by IgA, resulting in the consequent decrease in the adhesion of these bacteria to epithelial cells (Sedgmen et al., 2004; Tsuruhara et al., 2017).

Studies on viral neutralization suggest a relationship between the number of IgA molecules per virion (Van Egmond et al., 2001). In addition, it is important to highlight that IgA, during its transport by epithelial cells, is able to interact with intracellular pathogens, such as viruses, blocking its replication and / or budding (Woof & Mestecky, 2005). In this case, IgA-virus complexes are formed inside the infected cell, which are possibly expelled into the organ's lumen (Park et al., 2016). This mechanism has already been demonstrated in vitro in several viruses such as Sendai, influenza viruses and human immunodeficiency virus (HIV) (Van Egmond et al., 2001; Park et al., 2016).

Mucosa immunity and vaccines

Application site and the induced response

The first interaction between the pathogen and the host occurs at the mucosa level, which represents the mechanical barrier between the external and internal media, hindering or preventing the adhesion of microorganisms to the surface of the lining epithelial cells (Brandtzaeg, 2007; Macpherson et al., 2018). These cells express different receptors, which recognize and bind pathogens, initiating the cell activation process, synthesizing and secreting new peptides (Erume & Partidos, 2011). Thus, vaccines that are applied directly to the skin and mucous membranes are promising, and several successful examples have already been reported, such as vaccines for influenza and measles via the nasal route, the use of the vaginal route for immunization against herpes simplex. and the oral route for hepatitis E and polio (Holmgren et al., 2003).

Mucosa vaccines have been a great attraction, as they are a safer alternative, reducing the risk of contamination and not causing damage, as in the case of vaccine applications made with the use of needles, in addition to inducing both an immune response of the mucosa and systemic (Brandtzaeg, 2007; Macpherson et al., 2018). The mucosal immune response is most effectively induced when the inoculation of the antigens of interest occurs directly in this location, whether in the oral, nasal, rectal or vaginal mucosa, since systemic pathways usually induce a weak immune response in these surfaces (Xiong et al., 2012). Although this concept is stabilized, the vast majority of vaccines used are administered parenterally, which is permissible because this route allows greater accuracy in the amount of antigen injected into the body (Bernasconi et al., 2016). Even so, when the purpose is to induce an immune response at the mucosa and systemic level, the best strategy is mucosal immunization (Woof & Kerr, 2006).

When choosing the mucous pathway to be used for vaccine application, it must be considered as an integrated system, where T and B lymphocytes or APCs activated in a given inducing site can migrate through the lymph vessels to another effector site in the mucous membranes, with consequent secretion of specific IgA in a location other than the one where the primary antigenic stimulus occurred (Brandtzaeg, 2007; Júnior et al., 2010). This mechanism is based on the production of tissue-specific adhesion molecules (chemokines, integrins and cytokines) and their receptors, leading these cells back to where the antigenic stimulus occurred and to related effector sites16. As an example, the chemokine receptor CCR10 and its chemokine ligands CCL27 and CCL28, are exclusively involved in epithelial immunity. CCL27 is expressed predominantly in

the skin by keratinocytes, while CCL28 is expressed by the epithelial cells of various mucosal tissues. CCR10 is expressed by several subsets of innate T cells that are programmed to be located in the skin during its development processes in the thymus. Circulating T cells may be attracted to skin-presenting antigen-presenting cells to express CCR10 for recruitment to the skin during the local immune response. For another side, B cells producing IgA antibodies generated in lymphoid tissues associated with the mucosa express CCR10 for their migration and maintenance in mucosal sites (Xiong et al., 2012).

Many pathogens penetrate the host through mucosal surfaces (Woof & Kerr, 2006). In this sense, it is essential to develop vaccines that induce an effective immune response with the production of memory cells in the mucous membranes. Therefore, there are several studies in the scientific literature on the use of mucosal vaccines in different species, and the present study describes the work of several authors and addresses a discussion about the results obtained by our research group.

The ideal mucosa vaccine

When deposited directly on a mucous surface, the antigen can be diluted in local secretions, captured by mucus, attacked by proteases and nucleases, in addition to being excluded by epithelial barriers (Júnior et al., 2010; Macpherson et al., 2018). Thus, it may necessary to inoculate a large volume of vaccine to overcome these challenges. In addition, so far there are no reliable ways to determine exactly the dose needed to cross these barriers and stimulate an immune response (Brandtzaeg et al., 2005; Woof & Mestecky, 2005).

Therefore, an ideal mucosal vaccine formulation should be able to: (1) - protect the vaccine antigen from enzymatic and chemical degradation; (2) - limit its elimination or excessive dilution in the body; (3) - the absorption of the antigen by the specialized M cells of NALT / GALT / BALT in order to enable its capture by APCs; (4) - promote the capture of antigen and adjuvant by APCs, aiming to stimulate, in an appropriate way, an adequate immune response, composed of neutralizing IgA and / or CD4 + and CD8 + T lymphocytes (Aguilar et al., 2007; Pavot et al., 2012; Bernasconi et al., 2016). Unfortunately, this ideal vaccine does not yet exist and, in order to achieve these goals, or at least some of them, it is extremely important to choose the appropriate adjuvant, since inactivated, subunit or non-microbial particles vaccines usually stimulate a response weak or undetectable adaptive immune system when administered directly to mucous membranes (Ansel et al., 2000). Still, it is important that this vaccine has the ability to mimic some key aspects present in pathogens, such as the fact that they are multimeric and / or particulate, have the ability to adhere to mucous surfaces (or directly to M cells), efficiently stimulate an innate response, and incite an adaptive response (Pavot et al., 2012). Thus, for a vaccine with characteristics very close to the ideal to be developed, delivery systems and immunostimulants have been studied with a view to increasing the immune response in the mucous membranes (Bobbala et al., 2018).

Mucous Adjuvants and Delivery Systems

Currently, studies for the development of new vaccines have sought substances that act as delivery systems, as well as that have intrinsic immunomodulatory activity (Ansel et al., 2000; Bobbala et al., 2018). In this sense, exclusively immunostimulant substances, such as the cholera toxin (CT) and the thermolabile enterotoxin produced by *Escherichia coli* (LTB) are notably important when co-administered to mucus-soluble antigens (Thiam et al., 2015; Ma et al., 2016). These proteins promote a specific response when coadministered or fused to the antigen, including inflammatory Th1, Th2, Th17, cytotoxic T lymphocytes (CTLs) and antibodies. However, more exclusively among classes of adjuvants, they induce specific IgA antibodies to the antigen and long-term memory for coadministered antigens when administered mucosally (Thiam et al., 2015; Ma et al., 2016; John & Elizabeth, 2018).

These CT and LT B subunits (CTB and LTB) were isolated and analysed for their adjuvant action, demonstrating a

mucosal immunostimulatory capacity lower than the complete holotoxin, however significant when administered intranasally. Commonly, the loss of toxic action is reflected in the decrease in adjuvant activity. However, only a few available proteins have significant adjuvant activity in the absence of detectable toxicity. Therefore, an option to exclude subunit A would be a point mutation in domain A1, in order to reduce its toxic potential (Hagiwara et al., 2001).

Among the existing mutants, LTK63 stands out, resulting from the substitution of the amino acid serine for lysine at position 63 of subunit A. This mutant is completely non-toxic and has shown strong immunostimulatory activity in several species, being able to induce high titers of specific antibodies against the co-administered antigen (Palma et al., 2008), in addition to stimulating the production of IL-12 and TNF-α and translocation of NFκβ (nuclear transcription kapa-beta), which is responsible for regulating the expression of the genes of numerous cytokines. In their initial studies, researchers, (Palma et al., 2008), by contrasting the immunostimulant action of the LTK63 mutant and the LT derivative, LTB, emphasize the importance of the A subunit, even if inactive, in the ability to elicit an immune response, not only because it has a greater number of antigenic determinants, present in subunit A, reflecting a greater number of specific B and T cells but also the ability to influence intracellular events, such as antigen processing and presentation (Hagiwara et al., 2001; Palma et al., 2008).

The mucoadhesive delivery systems are quite varied, and their principle is to increase the retention time of antigens in the mucosa, interact with the epithelium of choice and increase the absorption and release of antigens. Delivery systems commonly used in the formulation of mucosal vaccines can be classified into two groups: particulate delivery systems for antigens and solutions (Kim et al., 2019). The first group consists of systems capable of partially protecting the antigen from enzymatic degradation in mucous secretions, involving the antigenic protein, thus making it less vulnerable to attacks by the local innate immune system. Examples are emulsions, liposomes, virosomes, microspheres, immunostimulant compounds (ISCOMs) and pseudo viruses or virus-like particles (VLPs) (Villanova & Oréfice, 2010; Kim et al., 2019). The second group of delivery systems encompasses solutions where the antigen is dissolved or suspended, such as eggs and gels. Eggs, for example, after being inserted into the vagina, have their base softened and dissolve, distributing the antigen on the mucous surface (Villanova & Oréfice, 2010).

Regarding gels with adhering capacity, cellulose derivatives and ploxamers stand out, both very resistant to heat, bacterial enzymes and degradation by exposure to ultraviolet light. In this sense, cellulose derivatives, such as Hydroxyethylcellulose (2-HEC) and Methylcellulose, which are non-ionic ethers, obtained from the processing of cotton cellulose or cellulose pulp, widely used as thickeners (viscosifiers), stabilizers, forming elements of film, gelling agents (gels) in pharmaceutical products, have been evaluated (Zhao et al., 2017). These delivery systems are biodegradable, physiologically inert and have several applications in the pharmaceutical industry. The gel-forming characteristic justifies the use of these cellulose derivatives in the elaboration and development of vaccines that use the mucosa as an inoculation route, since they act as viscosity enhancers and as modulators of drug release, in addition to having mucoadhesiveness properties, increasing the exposure time of the vaccine components with the cells of the immune system (Villanova & Oréfice, 2010; Zhao et al., 2017).

Another delivery system described in the literature are Poloxamer 407 (P407) and 188 (P188), classified by the Food and Drug Administration (USA), as inert for various types of preparations, such as intravenous, inhalation, ophthalmic, topical, oral solutions and suspensions (Roberts, 2004). Poloxamer based formulations have been used as a controlled drug delivery system via routes such as oral (corticosteroids), rectal (anti-inflammatory and analgesic) and intranasal (anti-inflammatory, analgesic, hypertension), due to its gelling property reversible term, high solubilization capacity and prolongation of the pharmaceutical activity of the drug, increasing therapeutic efficacy and decreasing side effects and / or toxicity of the drug (Roberts, 2004).

Thus, experiments carried out by our research group have involved the evaluation of different types of mucosal vaccine delivery systems. In preliminary studies, with the use of an intravaginal vaccine for cattle, vaginal ova based on high-

grade gelatin were used, and as adjuvants, recombinant proteins and propolis. These presentations were satisfactory, since gelatin demonstrated stability in vaccine production. However, it did not show an increase in the immune response alone, only when in combination with the adjuvant of the recombinant protein and propolis. These findings were fundamental for the improvement of the vaccine preparation process that is under development. We are currently producing mucoadhesive vaginal eggs based on cellulose derivatives and poloxamers, associated with LTB de E. coli. The preliminary results are promising, which leads us to continue developing mucosal vaccines and evaluating different forms and concentrations of delivery systems.

***In vivo* studies using mucosal vaccines**

The development of vaccines administered orally is preferable to the traditional one, since it allows greater security in the administration, avoiding the use of needles and possible injuries and contamination during the application3,4. In addition, the oral, nasal, ocular, rectal and vaginal mucosa pathways allow the stimulation of humoral and cellular responses in the mucosal and systemic pathways, establishing broader and lasting protection (Woof & Mestecky, 2005; Macpherson et al., 2008).

In this sense, the oral polio vaccine (OPV) was the first successful vaccine developed for mucous membranes (Roberts, 2004; Kraan et al., 2017). OPV consists of strains of live attenuated poliovirus, in which three spaced doses are necessary to generate protection via humoral and mucosal immunity (Kraan et al., 2017). After its administration, the stimulation of the immune mechanisms is almost immediate, observing an increase in the level of circulating antibodies in the week of ingestion in primary vaccines, and after 48 (forty-eight) hours in the revaccinated ones (Di Tommaso et al., 1996). In addition, and exclusively for OPV, compared to the inactivated injectable polio vaccine (IPV), OPV produces a local IgA immune response in the intestinal mucosa, which is the primary site of poliovirus entry and replication (Roberts, 2004).

In the study the intravaginal and intranasal mucous pathways were compared regarding the induction of a systemic and vaginal immune response against an ovalbumin (Ova) in female rats, and as a vaccine adjuvant, LTK63 a mutant of the Escherichia coli thermolabile enterotoxin, efficient as a mucosal adjuvant (Tempesta et al., 2007). The result was the generation of a systemic humoral response in both immunization pathways, in addition to high levels of IgG and IgA in the vaginal mucosa. The group of rats immunized by the intranasal route developed a later vaginal IgA response when compared to the group immunized by the intravaginal route, however a higher titer of this antibody was found in the vaginal secretion, after repeated immunizations, in the rats that received the immunogen intranasally. The results indicate that, after immunization, there is recirculation of antibodies or antibody-secreting cells from one compartment of the body to another, confirming the existence of a common mucosa system (Tempesta et al., 2007).

On the other hand, inoculation of goats with an inactivated vaccine against caprine alphaherpesvirus type 1 (CpHV-1) associated with LTK63 intravaginally, induced the animals to develop high levels of IgA and significant protection against the challenge with a virulent strain of CpHV-144, which was proven by the decrease in viral excretion when compared to non-vaccinated animals. Also, when a live attenuated vaccine against bovine alphaherpesvirus type 1 (BoHV-1) was inoculated in goats intranasally, followed by intravaginal challenge with a strain of caprine alphaherpesvirus type 1 (CpHV-1), the antibody titers of CpHV-1, measured by ELISA, were significantly higher than those of the non-immunized group (Parr & Parr, 1999). After the challenge, a significant reduction in the severity of the disease was observed in the vaccinated animals, in addition to a shorter duration and peak of viral excretion, when compared to the control group.

However, when comparing the nasal and vaginal immunization routes in mice, using a live attenuated vaccine against herpes simplex virus 2 (HSV-2), it was observed that the intranasal route did not increase the number of IgA and IgG secreting plasmocytes in the vagina, in addition to not inducing a significant increase in IgA in vaginal secretion, when compared to the intravaginal route (Gomes, 2018). Otherwise, intravaginal immunization generated an increase in the number of IgG-secreting

plasma cells in the vagina and an increase in this antibody, both in vaginal secretion and in serum, indicating local and systemic immunity, since it provided greater protection against the challenge of mice with a strain virulent HSV-2 (Gomes, 2018).

In addition to the choice of the inoculation site, the age of the animals to be immunized must also be considered (McGill et al., 2018) the immune response obtained after intranasal vaccination was compared in calves of 15 and 45 days of age. The study demonstrated that the vaccine response via the intranasal route was not influenced by colostral antibodies and there was also no induction of specific systemic immunity by antibodies. However, in the bronchoalveolar lavage, there was an increase in IgA in the animals vaccinated at 15 days of age and a tendency of increase in animals at 45 days of age. Thus, the author concluded that the response to the vaccine used intranasally in 15-day calves is different from when used at 45 days, and that the vaccine caused changes in clinical examination, leukogram and cytology of bronchoalveolar lavage more intense in animals vaccinated at 15 days of age. In another study, a mucosal nanovaccine, using the intranasal route in calves, against the Bovine Respiratory Syncytial Virus (BRSV) was evaluated (Morrison et al., 1998). The authors used BRSV post-fusion glycoproteins as antigen in polyanhydride nanoparticles. The animals that received the intranasal nanovaccine BRSV-F / G, developed mucosal and systemic antiviral immunity (increased IgA and IgG), exhibited reduced pathology in the lungs and reduced viral load compared to control (unvaccinated) calves.

The association of two or more inoculation routes often seems to be the solution for inducing a solid and protective mucosal immunity. There are authors (Terauchi et al., 2018) who defend intranasal immunization complementary to parenteral, based on protection against the challenge with HSV-2. These researchers evaluated the immune response conferred by the immunization of mice with a live recombinant vaccine against HSV-2, administered subcutaneously and intranasally. The subcutaneous route generated a strong systemic response, while the intranasal route induced a systemic and mucosal response, evidenced by the increase in IgA antibodies in vaginal secretion. Other researchers in the field (Neutra & Kozlowski, 2006) also defend this theory, pointing out that, in humans who do not have a previous immune response against the vaccine agent, mucosal immunization followed by parenteral inoculation would be the best choice, due to the primary induction of cell receptor expression in mucous and systemic. However, the data obtained by our studies (unpublished data) show that, with the use of the mucosa vaccine only (intravaginal), it is possible to obtain satisfactory and statistically superior results when compared to commercial injectable vaccines, regarding to the increase in the level of antibodies, both in the level of mucosa and systemic. However, we did not evaluate the association with another route of inoculation, which could result in even more promising observations and results.

When evaluating an inactivated vaccine against the influenza virus, through the intranasal route in humans, significant levels of both IgA, present in the nasal mucosa, and circulating IgG in the vaccinated animals were detected. The authors (Farias et al., 2016), obtained a positive correlation between multimeric IgA and neutralizing antibodies against Influenza, indicating that multimeric IgA antibodies play an important role in antiviral activity in the nasal mucosa, corroborating the data obtained by our research group.

Alternative forms of inoculation of vaccines have been evaluated in other species. In a study of vaccine in fish, with intraperitoneal and immersion application, researchers (Reichen et al., 2019), found higher antibody titers for immunization via intraperitoneal, followed by administration via immersion with booster after 30 days and a dose via immersion, respectively. It is worth mentioning that the efficiency of an immersion vaccine depends on the absorptive capacity of the gills and skin. In swine, an increase in monocyte phagocytic activity was observed in animals that received mucosal vaccine through the oral route, through water intake, which may indicate that, in addition to specific immunity, mucosal vaccines also have the potential to stimulate response innate immune (Reichen et al., 2019). Finally, some observations seem to result in important differences in the choice of the inoculation route to be used, such as the species to be inoculated, the nature of the vaccine and the probable

port of entry of the agent in question.

Our group has been conducting research over the years with inactivated intravaginal vaccines against bovine alpha-herpesviruses 1 and 5, and evaluating different adjuvants and vaccine delivery systems. We recently evaluated the use of rLTB as an adjuvant, and the delivery systems, 2-hydroxyethylcellulose and Poloxamer 407 in intravaginal vaccines. As a result, through the ELISA, an increase in IgA and IgG was observed in cattle, both at the level of the vaginal and nasal mucosa, as well as at the systemic level. In addition to the production of neutralizing antibodies, by the Seroneutralization Test. The vaccine for intravaginal application of our research group, provided an increase in the cellular response, observed by the increase in the production of cytokines such as Interleukin 2, Interleukin 13 and Interferon Gamma. Our group has been working with mucous vaccines for years and the results obtained stimulate the continuity of research, aiming at obtaining a commercial product.

2. Conclusion

Vaccines administered directly to mucous membranes are mainly aimed at stimulating an intense local immunity capable of minimizing the infectious load of pathogens that use such surfaces as a gateway into the body. IgA plays an important role in the defense of mucous membranes, through neutralization and antigenic exclusion. For this reason, it is important that the vaccine stimulates a strong humoral response, in addition to stimulating an adequate cellular response.

Several barriers hinder the development of an efficient vaccine, such as choosing the appropriate route of administration and delivery system. Considering the relationship between the mucosa system, it is possible to choose a route that represents greater ease in inoculation, aiming to stimulate an immune response in the corresponding mucosa. This relationship is observed between the respiratory and genital tracts, given that several authors and our research group report that, when administering an antigen in the vaginal mucosa, they observed a specific response in the intranasal mucosa. The vaccine delivery system, in turn, must be able to bypass physical and chemical barriers of the organism and provide a sufficient antigenic load to stimulate an immune response. As described, some substances, in addition to playing this role, have immunostimulant activity, expanding the generated response.

As for exclusively immunostimulatory substances, our research group has been carrying out several works in the area of mucosal vaccines, evaluating different antigens and adjuvants, but further studies are needed in order to clarify the efficiency of these substances and the relationship between mucous administration routes of vaccines in large animals, since several diseases of economic importance, mainly in cattle ranching, are transmitted by contact with these surfaces.

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References

Aguilar, J. C.; Rodríguez, E. G. (2007). Vaccine adjuvants revisited. *Vaccine*, 25, 3752–62.

Ansel, H. C., Popovich, N. G. & Allen, Jr L.V. (2000). *Pharmacotechnics: pharmaceutical forms and drug delivery systems*. Premier.

Bernasconi, V., Norling, K., Bally, M., Höök, F. & Lycke, N. Y. (2016). Mucosal Vaccine Development Based on Liposome Technology. *J Immunol Res*, 1-16.

Bobbala, S., Gibson, B., Gamble, A. B., McDowell, A. & Hook, S. (2018). Poloxamer 407-chitosan grafted thermoresponsive hydrogels achieve synchronous and sustained release of antigen and adjuvant from single shot vaccines. *Immunology and Cell Biology*, 96, 6, 656–665.

Brandtzaeg, P., Johansen, F. E. (2005). Mucosal B cells: phenotypic characteristics, transcriptional regulation, and homing properties. *Immunol Rev*. 206, 32–63.

Brandtzaeg, P. (2007). Induction of secretory immunity and memory at mucosal surfaces. *Vaccine*, 25, 5467-84.

Campbell, D. J., Debes, G. F., Johnston, B., Wilson, E. & Butcher, E. C. (2003). Targeting T cell responses by selective chemokine receptor expression. *Semin Immunol*, 15, 277-86.

Cazote, A. S. (2018). *Characterization of human lymphocytes in the HIV / TB association: result in the immunopathogenesis of extrapulmonary tuberculosis in its ganglionic form.* [Dissertation]. Programa de Pós-Graduação em Medicina Tropical: Fundação Oswaldo Cruz.

Cruvinel, W. M., Mesquita, Jr D., Araújo, J. A. R., Catelan, T. T. T., Souza, A. W. S. & Silva, N. P. (2010). Innate immunity fundamentals with emphasis on the molecular and cellular mechanisms of the inflammatory response. *Rev Bras Reumatol*, 50, 434-61.

Cruz, R. H. (2018). *Interaction between specific antibodies and dendritic cells in asthmatic patients.* [Dissertation]. Programa de Pós Graduação em Imunologia: Universidade de São Paulo.

Di Tommaso, A., Pizza, M., Rappuoli, R., Abrignani, S., Douce, G. & De Magistris, M. T. (1996). Induction of antigen-specific antibodies in vaginal secretions by using a nontoxic mutant of heat-labile enterotoxin as a mucosal adjuvant. *Infect Immun*, 64, 974–79.

Erume, J. & Partidos, C. D. (2011). Evaluation of the LTK63 adjuvant effect on cellular immune responses to measles virus nucleoprotein. *Afr Health Sci*, 11, 151-57.

Gomes, R. C. (2018). *Age influence on the immune response of calves to intranasal vaccination*. [Tesis]. Universidade de São Paulo.

Hagiwara, Y., Iwasaki, T., Asanuma, H., Sato, Y., Sata, T. & Aizawa, C. (2001). Effects of intranasal administration of cholera toxin (or *Escherichia coli* heat-labile enterotoxin) B subunits supplemented with a trace amount of the holotoxin on the brain. *Vaccine*, 19, 1652–60.

Holmgren, J., Czerkinsky, C., Eriksson, K. & Mharandi, A. (2003). Mucosal immunisation and adjuvants: a brief overview of recent advances and challenges. *Vaccine*, 21, 89-95.

Farias, T. V., Pala, G., De Moraes, A. C., Prado, E. J. R., Kotzent, S. & Da Costa, J. C. (2016). Immune response of the cutaneous mucosa of Pacus (*Piaractus mesopotamicus*) vaccinated intraperitoneally and by immersion against aeromoniosis. *Rev Ciên Vet Saúde Públ*, 3, 286-88.

He, B., Xu, W., Santini, P. A, Polydorides, A. D., Chiu, A. & Estrella, J. (2007). Intestinal bacteria trigger T cell independent immunoglobulin A2 class switching by inducing epithelial-cell secretion of the cytokine *Immunity*, 26, 812–26.

John, D. C. & Elizabeth, B. N. (2018). The Mucosal Vaccine Adjuvant LT(R192G/L211A) or dmLT. *mSphere*, 25: 215-18.

Júnior, D. M., Araújo, J. A. R., Catelan, T. T. T., Souza, A. W. S., Cruvinel, W. M. & Andrade L.E.C. (2010). Immune System - Part II Fundamentals of T and B lymphocyte-mediated immune response. *Rev Bras Reumatol*, 50, 552-80.

Kraan, H., Peter, S., Amorij, J. P. & Kersten, G. (2017). Intranasal and sublingual delivery of inactivated polio vaccine. *Vaccine*, 35, 2647-53.

Kurashima, Y. & Kiyono, H. (2017). Mucosal Ecological Network of Epithelium and Immune Cells for Gut Homeostasis and Tissue Healing. *Annu Rev Immunol*, 35, 119–47.

Kim, M., Yi, E., Kim, Y., Kim, S. H., Jung, Y. S. & Kim, S. R. (2019). ERdj5 in innate immune cells is a crucial factor for the mucosal adjuvanticity of cholera toxin. *Front Immunol*, 10, 1-11.

Leung, S. T, Derecka, K., Mann, G. E., Flint, A. P. F. & Wathes, D. C. (2000). Uterine lymphocyte distribution and interleukin expression during early pregnancy in cows. *J Reprod Fertil*, 119, 25-33.

Lycke, N. (2012). Recent progress in mucosal vaccine development: potential and limitations. *Nat Rev Immunol*, 12, 592-605.

Ma, Y. (2016). Recent Advances in Nontoxic *Escherichia coli* Heatlabile Toxin and Its Derivative Adjuvants. *Expert Rev Vaccines*, 15, 1361-71.

Macpherson, A. J, Mckoy, K. D, Johansen, F. E. & Brandtzaeg, P. (2008). The immune geography of IgA induction and function. *Mucosal Immunol*, 1, 11–22.

Macpherson, J. A., Yilmaz, B., Limenitakis, J. P. & Ganal-Vonarburg, S. G. (2018). IgA Function in Relation to the Intestinal Microbiota. *Annu Rev Immunol*, 36, 359–81.

McGill, J. L., Kelly, S. M., Kumar, P., Speckhart, S., Haughney, S. L. & Henningson, J. (2018). Efficacy of mucosal polyanhydride nanovaccine against respiratory syncytial virus infection in the neonatal calf. *Scientific Reports*, 3021, 1-15.

Morrison, L. A., Da Costa, X. J. & Knipe, D. M. (1998). Influence of mucosal and parenteral immunization with a replication-defective mutant of HSV-2 on immune responses and protection from genital challenge. *Virology*, 2430, 178–87.

Murphy, K. (2014). *Immunology of Janeway*. (8a ed.) Artmed.

Neutra, M. R. & Kozlowski, P. A. (2006). Mucosal vaccines: the promise and the challenge. *Nat Rev Immunol*, (6), 148–58.

Palma, C., Iona, E., Giannoni, F., Pardini, M., Brunori, L. & Farttorini, L. (2008). The LTK63 adjuvant improves protection conferred by Ag85B DNA-protein prime-boosting vaccination against *Mycobacterium tuberculosis* infection by dampening IFN-γ response. *Vaccine*, 26, 4237-4243.

Pakkanen, S. H., Kantele, J. M., Moldoveanu, Z., Hedges, S., Häkkinen, M. & Mestecky, J. (2010). Expression of homing receptors on IgA1 and IgA2 plasma blasts in blood reflects differential distribution of IgA1 and IgA2 in various body fluids. *Clin Vaccine Immunol*, 17, 393–401.

Parr, E. L., Parr, M. B. (1999). Immune responses and protection against vaginal infection after nasal or vaginal immunization with attenuated Herpes simplex virus type-2. *Immunology*, 98, 639–45.

Park, A., Hong, P., Won, S. T., Thibault, P. A., Vigant, F. & Oguntuyo, K. Y. (2016). Sendai virus, an RNA virus with no risk of genomic integration, delivers CRISPR/Cas9 for efficient gene editing. *Mol Ther Methods Clin Dev*, 3.

Pavot, V., Rochereau, N., Genin, C., Verrier, B. & Paul, S. (2012). New insights in mucosal vaccine development. *Vaccine*, 30, 142– 154.

Reichen, C., Dezen, D., Meneguzzi, M. & Kich, J. D. (2019), Use of flow cytometry for the evaluation of phagocytosis produced by a mucosal vaccine against *Salmonella* sp. in swine. *Rev Acad Ciênc Anim*, 17, 244-245.

Roberts, L. (2004). Polio: The Final Assault? *Science*, 303, 1960-1968.

Sedgmen, B. J., Meeusen, E. N. T. & Lofthouse, S. A. (2004). Alternative routes of mucosal immunization in large animals. *Immunol Cell Biol*, 82, 10–16.

Tempesta, M., Camero, M., Bellacicco, A. L., Tarsitano, E., Lorusso, A. & Martella, V. (2007). Caprine herpesvirus 1 vaccine with the LTK63 mutant as a mucosal adjuvant induces strong protection against genital infection in goats. *Vaccine*, 25, 7927–7930.

Terauchi, Y., Sano, K., Ainai, A., Saito, S., Taga, Y. & Ogawa-Goto, K. (2018). IgA polymerization contributes to efficient virus neutralization on human upper respiratory mucosa after intranasal inactivated influenza vaccine administration. *Hum Vaccines Immunother*, 14, 1351-1361.

Thiam, F., Charpilienne, A., Poncet, D., Kohli, E. & Basset, C. B. (2015). Subunits of cholera toxin and thermolabile enterotoxin of *Escherichia coli* have similar adjuvant effect as whole molecules on rotavirus 2/6- VLP specific antibody responses and induce a Th17-like response after intrarectal immunization. *Microb Pathog*, 89, 27-34.

Tsuruhara, A., Aso, K., Tokuhara, D., Ohori, J., Kawabata, M. & Kurono, Y. (2017). Rejuvenation of mucosal immunosenescence by adipose tissue-derived mesenchymal stem cells. *Int Immunol*, 29, 5–10.

Van Egmond, M., Damen, C. A., Van Spriel, A. B., Vidarsson, G., Van Garderen, E. & Van de Winkel, J. G. (2001). IgA and the IgA Fc receptor. *Trends Immunol*, 22, 205–211.

Villanova, J. C. O. & Oréfice, R. L. (2010). Pharmaceutical Applications of Polymers. *Polymers: Science and Technology*, 20, 51-64.

Woof, J. M. & Mestecky, J. (2005). Mucosal immunoglobulins. *Immunol Rev*, 206, 64–82.

Woof, J. M. & Kerr, M. A. (2006). The function of immunoglobulin A in immunity. *J Pathol*, 208, 270–82.

Xiong, N., Yaoyao, F., Shaomin, H., Mingcan, X. & Jie, Y. (2012). CCR10 and Its Ligands in Regulation of Epithelial Immunity and Diseases. *Protein Cell*, 3 (8), 571–580.

Zhao, X., Coulman, S. A., Hanna, S. J., Wong, F. S, Dayan, C. M. & Birchall, J. C. (2017). Formulation of hydrophobic peptides for skin delivery via coated microneedles. *J Control Release*, 10, 2-13.

Zajac, M. P. D. M, Zanetti, F. A., Esusy, M. S., Federico, C. R., Zabal, O. & Valera, A. R. (2017). Induction of Both Local Immune Response in Mice and Protection in a Rabbit Model by Intranasal Immunization with Modified Vaccinia Ankara Virus Expressing a Secreted Form of Bovine Herpesvirus 1 Glycoprotein D. *Viral Immunol*, 1-7.