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PLATFORM FOR THE GENERATION OF ORAL VACCINES BASED ON PROTOZOAN SURFACE PROTEINS

MARIANELA DEL CARMEN SERRADELL, LUCÍA L. RUPIL, HUGO D. LUJÁN

Centro de Investigación y Desarrollo en Inmunología y Enfermedades Infecciosas (CIDIE), CONICET-UCCOR, Córdoba, Argentina

Abstract The international spread of infectious diseases is a global problem of health security. Vaccination is one of the most successful and profitable health interventions. Oral immunization has significant advantages over the widely used parental vaccines. Intestinal and free-living protozoa express on their surface a dense layer of proteins that protect them from hostile environmental conditions. The use of variable surface proteins (VSPs), such as those of the intestinal protozoan *Giardia lamblia*, is a feasible mechanism for the generation of oral vaccines, since they are highly immunogenic as well as resistant to changes in pH and proteases. In a recently published article, we showed that these properties of VSPs can be exploited to protect and enhance the immunogenicity of vaccine antigens, thus enabling their oral administration. We recently generated an oral vaccine against influenza virus composed of virus-like particles (VLPs) containing VSPs of *G. lamblia* and the HA antigen (viral hemagglutinin) in its envelope. When administered orally to mice, these coated particles elicit HA-specific humoral (systemic and local) and cellular responses, without the need of any additional adjuvant. Treated mice are protected against viral challenge as well as against the development of tumors expressing the HA vaccine antigen.

Key words: oral vaccine, protozoan membrane proteins, viral hemagglutinin, virus-like particles

Resumen ***Plataforma basada en proteínas de superficie de protozoarios para la generación de vacunas orales***. La propagación internacional de enfermedades infecciosas constituye un problema global de seguridad sanitaria. La vacunación es una de las intervenciones en salud más exitosas y efectivas. La administración por vía oral presenta ventajas significativas sobre la vía parental utilizada comúnmente. Protozoarios intestinales y de vida libre expresan en su superficie una densa capa de proteínas que los protegen de condiciones ambientales hostiles. La utilización de proteínas de superficie variante-específicas o VSPs (del inglés "*Variant-specific Surface Proteins*") tales como las del protozoario intestinal *Giardia lamblia* constituye un enfoque eficiente para la generación de vacunas orales, dada su alta inmunogenicidad y su resistencia a cambios de pH y proteasas. En un trabajo reciente mostramos que estas propiedades pueden ser explotadas para proteger antígenos vacunales y potenciar su inmunogenicidad, facilitando así su administración oral. Como modelo inicial, generamos una vacuna oral contra el virus de la influenza compuesta por partículas similares a virus (VLPs, del inglés "v*irus-like particles*") que contienen en su envoltorio VSPs de *G. lamblia* y el antígeno HA (hemaglutinina del virus de la influenza). La administración oral a ratones de estas partículas recubiertas con VSPs y HA induce una respuesta inmune humoral (sistémica y de mucosa) y celular específica para HA sin la necesidad de adyuvantes externos. La respuesta inmune generada protege frente al desafío con el virus y también frente al desarrollo de tumores que expresan el antígeno vacunal HA.

Palabras clave: vacuna oral, protozoarios, proteínas de membrana, hemaglutinina viral, partículas de tipo viral

Giardia lamblia is a protozoan that colonizes the upper small intestine of many vertebrates, including humans, and is the causal agent of giardiasis. To survive in the gut, the surface of the *Giardia* trophozoites is completely covered with variant-specific surface proteins (VSPs). These are integral membrane proteins that contain a variable extracellular region rich in cysteines (mainly present as CXXC motifs), a single transmembrane domain, and a highly conserved cytoplasmic tail[1]. Approximately 200 VSPs encoded in the genome of *Giardia* are involved in the process of antigenic variation, but only one is expressed in each trophozoite at any given time. Surface proteins similar to VSPs (database of the protein family PF03302) are also present in other protozoan parasites such as *Entamoeba histolytica*, which colonizes the large intestine, and in the free-living ciliates *Paramecium tetraurelia* and *Tetrahymena thermophila*, among others.

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Postal address: Hugo D. Luján, Av. Armada Argentina 3555, 5016 Córdoba Argentina.

e-mail: hlujan@ucc.edu.ar

VSPs resist the hostile gut environment

We previously demonstrated that antigenic variation is ruled by a mechanism similar to RNA interference (RNAi); by knocking down key enzymes of the RNAi pathway, we generated trophozoites expressing their entire VSP repertoire[2]. Oral immunization with VSPs purified from these cells protected gerbils[3], dogs and cats[4] against *Giardia* infections. This finding confirmed the immunogenic importance of the VSPs and revealed that these proteins are able to survive in the adverse gut environment. In a recent work[5], we postulated that the surface proteins of microorganisms living in the hostile gut environment (characterized by changes in pH, redox potential and presence of proteolytic enzymes) play a key role in their protection. We confirmed this hypothesis by demonstrating that trophozoites of *G. lamblia*, *E. histolytica*, *T. thermophila* and *P. tetraurelia* remain viable after exposure for more than one hour to trypsin in high concentrations. We also showed that three recombinant VSPs of two different *Giardia* isolates were highly resistant to extreme pH and to *in vitro* degradation by many proteolytic enzymes present in the stomach and the small intestine. The underlying biochemical mechanisms would involve the formation of inter- and intra-molecular bonds through the coordination of metal and/or disulfide bridges, since the treatment with reducing agents (2-mercaptoethanol) or with chelating agents (EDTA) markedly decreased VSP resistance to proteolysis.

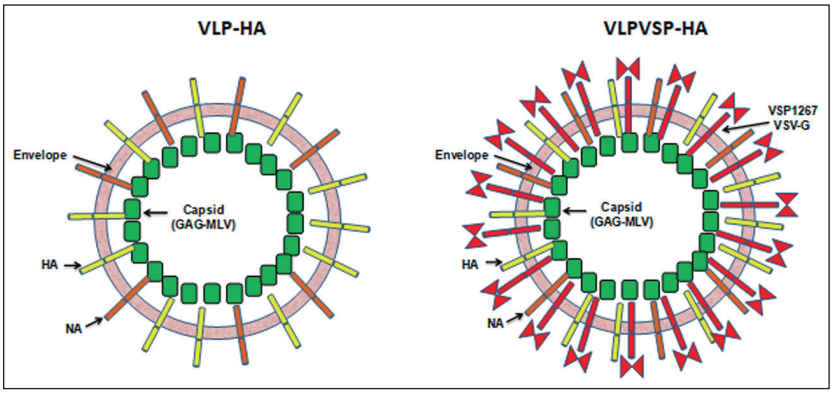
VSPs trigger local and systemic innate immunity

The three recombinant VSPs were able to activate innate immunity signaling through Toll-like receptor-4 (TLR-4), a key player in the immune activation against infectious agents. This interaction with the innate immune system was confirmed by the ability of these proteins to activate dendritic cells, the main professional antigen-presenting cells. These new findings contribute to the understanding of the mechanism by which the oral anti-Giardia vaccine, composed of the entire VSP repertoire, triggers its protective response in experimental and domestic animals[3, 4]. Pathogen-associated molecular patterns (PAMPs) are composed of conserved structures which are widely extended in different pathogens (6). Given the ability of different VSPs to interact with the innate immune system through TLR-4 and the fact that these surface proteins with a high content of CXXC motifs are found in different protozoa, we suggest that they could be considered a new PAMP.

VSPs act as shields on the surface of chimeric vaccines

To test our hypothesis, we designed and produced chimeric virus-like particles (VLPs) that express the main antigens of the influenza virus (HA and NA) along with VSPs on their surface (VLPVSP-HA) as a “shield” (Fig. 1). Virus-like particles (VLPs) are highly organized structures that self-assemble from virus proteins. These non-infectious, stable and versatile subviral particles are susceptible to manipulation to transport heterologous molecules, which retain their native antigenic conformation[10]. In contrast to soluble antigens, VLPs can induce a broad array of immune responses, including T and B cell responses. Therefore, they are not only safe but also potent immunogens, which make them an ideal platform for vaccine development, as has been demonstrated with

Fig. 1.– Schematic representation of VLPs. VLPs pseudotyped with influenza HA and NA, with or without the co-expression of the extracellular region of VSP1267 fused with VSV-G (Vesicular stomatitis virus G protein transmembrane region). The expression in eukaryotic cells of the Gag protein of the murine leukemia virus capsid (MLV) is enough to generate retroviral particles free of genomic material. These particles can be pseudotyped by many types of envelope proteins or by fusing peptides with the transmembrane domain and the cytoplasmic tail of VSV-G.



VLP-based vaccines for hepatitis B or for human papillomavirus, already approved for mass use[11].

VSPs protect the heterologous antigen and enhance its immunogenicity

First, we observed that the presence of VSPs on the surface of VLPs improved their immunogenic properties by promoting the activation of dendritic cells in a TLR-4-dependent manner. In addition, we observed that when VSPs were present on the surface of VLPs, not only they were resistant to proteolysis, but also HA and NA were protected from degradation by intestinal proteases. This is the first time that the property of VSPs to protect a heterologous antigen from degradation is described. Regarding the mechanism of this protection, we are currently able to affirm that VSPs do not act as protease inhibitors, nor do they protect antigens by "shedding" them against proteolytic enzymes, and that both VSPs and antigens must be present in the same particle. As mentioned above, we postulate that intra- and inter-molecular interactions involving disulfide bridges and/or metal coordination play key roles in antigen protection.

VSP chimeric vaccines are effective by the oral route

To test the ability of the VLPVSP-HA as a mucosal vaccine, we administered the particles orally to mice. Indeed, oral immunization of the animals with these chimeric VLPVSPs containing HA induced robust immune responses against this vaccine antigen. In contrast to the VLPs without VSP ("naked" VLPs), the VLPs expressing VSP were efficient in generating an antibody response, which not only was present in serum, but also prevailed in samples from the mucosal compartment (bronchoalveolar lavage and feces). The presence of antibodies in these pathogen entry sites is of great importance, because the mucosal immunity represents the first barrier encountered by infectious agents. It should be noted that the S-IgA response detected in bronchoalveolar lavage and feces was only achieved by oral immunization with the VLPVSP-HA and not by its parenteral administration. S-IgA is key in the mucosal immune system and is produced locally in effector tissues[12]. The presence of HA specific S-IgA in bronchoalveolar lavage, a mucosa effector site distant from the inductive site (digestive tract), where the initial administration of the antigen occurred, is an important and useful characteristic of the mucosal immune system. In addition, because mucosal immunization induces not only mucosal S-IgA but also systemic IgG, this novel oral vaccination platform could be used in the same way as the parenteral vaccines, providing double protection against pathogens that enter the organism through the mucosa membranes, but that are later distributed to other tissues.

VSP chimeric vaccines trigger humoral and cell-mediated responses

Ideally, vaccines should trigger cellular immune responses in addition to antibodies. In fact, our VLPVSP-HA vaccine successfully activated this arm of the immune system, increasing the cytotoxic cellular response and inducing the production of HA-specific IFN-γ-producing T cells. We observed that this cell-mediated response was present not only in the spleen but also in mesenteric lymph nodes, key inductive sites of the immune responses to oral vaccines. It should be noted that IFN-γ and TNF-α secreted by CD8+ T cells in the lamina propria play an important role in allowing phagocytic cells to effectively fight pathogens that have crossed the mucosal barrier[13].

VSP chimeric vaccines protect against tumors

In the next step, we studied the effectiveness of this oral vaccine to generate *in vivo* protection using two strategies: challenge with live virus and with tumor cells. In the intranasal infection model, VLPVSP-HA vaccinated mice were completely protected against infection with the influenza virus, and this protection correlated with the generation of anti-HA neutralizing antibodies. On the other hand, the challenge assay with tumor cells is a useful tool to evaluate the generation of efficient cellular cytotoxic responses. Mice immunized orally with VLPVSP-HA showed almost complete control of tumor growth when inoculated with cells derived from a malignant mesothelioma (cell line AB1) expressing the HA vaccine antigen. Splenocytes isolated from these animals showed greater *in vitro* cytotoxicity against tumor cells, confirming the efficient generation of specific cytotoxic cells against cancer cells expressing HA.

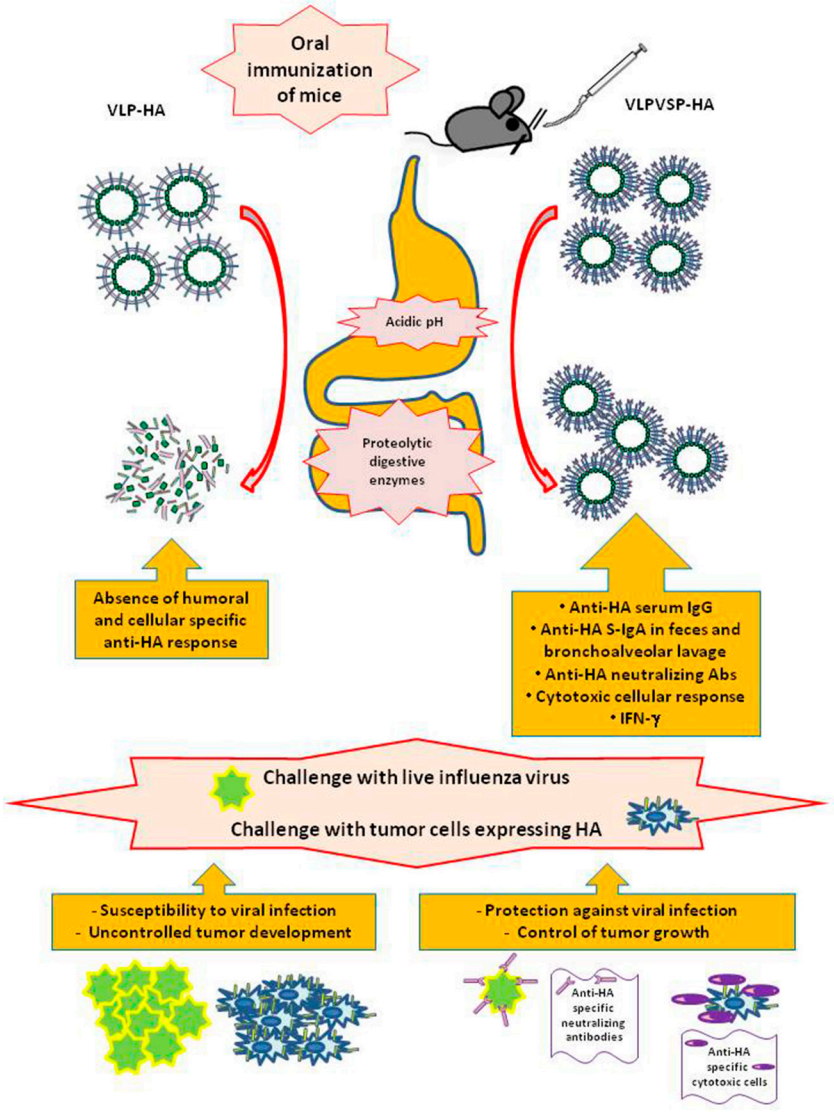
VSP chimeric vaccines are stable

Finally, we proved that the VLPVSP-HA particles were resistant to freeze-thaw cycles, and that they remained stable at different temperatures, without losing their immunogenic properties.

Conclusions

By taking advantage of the high resistance to degradation of protozoan surface molecules and the high immunogenicity and versatility of VLPs, we developed a platform for

Fig. 2.– Resistance and immunogenic properties of the VLPVSP-HA oral vaccine. The VLPVSP-HA particles showed high resistance to exposure to acidic pH and enzymatic proteolysis *in vitro*. The oral administration of the particles covered with VSP1267 to Balb/c mice generated potent humoral and cellular responses. Only the animals vaccinated with VLPVSP-HA and not with VLP-HA developed protective responses both to the intranasal challenge with the influenza virus and to the challenge with tumor cells expressing the HA vaccine antigen. These animal groups exhibited the induction of neutralizing antibodies against the virus and specific cytotoxic cells able to control tumor growth.



the generation of safe, stable and efficient oral vaccines. The oral vaccine against the influenza virus generated using this platform was able to activate the different components of the immune system, generating mucosal antibodies that prevent the binding and invasion of pathogens and neutralize enterotoxins, and serum antibodies that control invasive pathogens at the systemic level, in addition to an effective cellular immunity (Fig. 2).

These results will allow the generation of a wide range of oral vaccines in the near future, which will improve mass vaccination programs, facilitate their delivery in remote areas of the world where refrigerated transport is impractical, and clearly improve the prevention of infectious diseases.

Conflict of interest: None to declare

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