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Review article

Sublingual vaccination and delivery systems

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ABSTRACT

Most infectious agents use mucosal tissues as entry portals, thus, mucosae are frequently defined as a first line of defense against pathogens. Mucosal protection generally operates through antibody-mediated and cytotoxic T-cell responses which can be triggered by mucosal vaccines. Sublingual vaccination provides many advantages such as systemic and mucosal responses (both locally and at remote mucosal sites), besides being a needle-free administration route with high patient compliance and limited adverse effects. Buccal mucosa complexity nonetheless represents a challenge for vaccine administration, hence, many efforts were recently deployed to improve vaccine components, mucoadhesion and/or penetration. Several innovative approaches indeed confirmed that a robust and protective immunity can be achieved by sublingual vaccines. This review will then specify the most recent delivery systems and improvements developed to increase sublingual vaccines efficiency. We will focus our description on the immune mechanisms involved and the requirements for optimal sublingual immunization and mucosal protection.

1. Introduction

1.1. A brief history of vaccination

Historically, first attempts at inducing protective immunity appeared in China during the 10th century with mucosal (nasal) administration of ground-up scab of healing smallpox lesions. This practice spread through Asia and ultimately in Europe where it has been named "variolation". Although relatively effective, this technique was not devoid of risks and could lead to disease spreading, severe symptoms or even be fatal in 2% cases. In 1796, Edward Jenner demonstrated vaccination efficacy against smallpox through cowpox inoculation in the skin, a much safer approach of prophylactic immune protection. In the late 19th century, Louis Pasteur and his collaborators developed preventive vaccines containing attenuated microorganisms and demonstrated their capacity to trigger an immune protection along with decreased virulence [1]. These pioneering vaccinologists established modern vaccines foundations and this had the unfortunate consequence of commonly associating vaccines to injection [2]. Indeed, most vaccines are delivered by parenteral route consisting in intra-muscular injection of a liquid formulation. Yet, the large majority of parenteral immunizations tend to confer protection against infection by generating systemic antibodies and cytotoxic T lymphocytes. However, numerous pathogens use a mucosal port of entry in the host body, and their neutralization requires the induction of a mucosal immune response at their site of entry to be protective. Vaccines administered through mucosae were shown to be effective in inducing serum antibodies as well as secretory IgA (SIgA) antibodies and cytotoxic T cell responses at mucosal sites [3,4]. Besides direct pathogen attachment or translocation prevention, SIgA induction also permits to limit person-to-person transmission and provides indirect (herd) protection [5,6]. Mucosal responses are favoured by the high organization of the mucosal immune tissue which is functionally independent from the systemic immune apparatus [3] stressing the need to carefully choose the type and site of vaccine administration for each pathogen.

1.2. Sublingual vaccination: an alternative for mucosal delivery of vaccines

Immune response dissemination after mucosal administration is defined by its anatomical compartmentalization within a common mucosal immune system [7] which leads to antigen-specific SIgA production at various distant mucosal sites [4]. Several mucosal administration strategies have been explored, and in particular oral (*per os*)

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administration, with several oral vaccines marketed against intestinal infections, namely polio, typhoid fever, cholera and gastroenteritis [8]. However, oral immunization provides an immunity restricted to the digestive tract. An alternative is nasal vaccine administration which is also largely explored, taking advantage of the nasal associated lymphoid tissue (NALT) capacity to induce immune responses at the local oropharyngeal tract but also at distant respiratory, intestinal and genital tract [4,9]. To date, there is only one marketed nasal vaccine, Flumist®, authorized in the US and Canada only. As the nasal administration faced several safety concerns, a new area of research based on the sublingual delivery of vaccines emerged. Even though mucosal vaccine administration is largely investigated on oral or nasal mucosa, specific research had to be performed on the sublingual mucosa due to the variations in cell composition from one mucosa to another, and the expression of different combinations of pattern recognition receptor (PRR) by tissue specific epithelial or innate cells [10,11].

The sublingual route is already used for transmucosal drug administration [12] and was initially used for allergy treatment and immunotherapy with nowadays several marketed products for sublingual immunotherapy (SLIT). The purpose of SLIT is the hyposensitization towards an allergen and limitation of allergic responses by inducing immune tolerance, an immune mechanism that controls undesired immune reactions against environmental molecules or innocuous molecules. This regulatory response is notably established by the induction of regulatory T-cells (Tregs) and increased production of allergen-specific IgG4 antibodies and IL-10 [13,14]. However, the induction of tolerance is the opposite of anti-infectious vaccination that aims at inducing an immune reaction. Induction of tolerance will thus be a challenge to overcome in order to develop efficient sublingual vaccines.

Interestingly, sublingual vaccine administration, alike nasal administration, induces mucosal immune responses (antibody-mediated and cytotoxic T-cell responses) in the upper and lower respiratory tract, stomach, small intestine, reproductive tract as well as a systemic response [15,16]. This large dissemination of mucosal responses after sublingual immunization is based on two mechanisms: (i) selective migration of IgA antibody-secreting cells (plasmablasts) to distant mucosal sites using CCL28/CCR10 chemokine/chemokine receptor system [17,18] and (ii) recirculation of mucosal antigen-bearing dendritic cells (DCs) to distant secondary lymphoid organs [19] (Fig. 1).

Therefore, besides the obvious advantages of being a needle-free administration route providing high patient compliance and avoiding first pass effect, the sublingual administration of vaccines exhibits several immunological advantages. Indeed, with a proper composition, sublingual vaccines are able to induce both systemic and mucosal immunity at the humoral as well as at the cellular level. Consequently, the large dissemination of the induced mucosal response at distant sites is attracting for the development of vaccines against either respiratory or gastrointestinal pathogens or sexually transmissible infections.

1.3. From preclinical to clinical investigations

Many preclinical studies demonstrated the generation of a potent immune response in several animal models [19–24] and some were pursued by clinical trials, performed to assess prophylactic sublingual vaccines efficiency in humans (Table 1).

In a phase I trial (NCT01488188), nasal vaccine Flumist® was administered under the tongue to overcome difficulties of nasal administration in infant and potential side effects in elderly patients such as rhinitis and sneezing episodes. Sublingual Flumist® administration led to comparable and promising results in terms of immunogenicity and safety when compared to nasal administration. However, to date, no sublingual vaccine is commercially available, except Uromune® (MV140), which is a therapeutic vaccine for recurrent urinary tract infections (RUTI). Safety and efficacy were demonstrated in several prospective studies [25,26], as well as phase 2 and phase 3 clinical trials (NCT04096820, NCT02543827) through daily administration of 2 sprays under the tongue for at least 3 months.

To date, a potential answer proposed for vaccines against challenging infectious agents or problematic unstable compositions would be subunit antigens. However, these formulations, based on peptides or proteins (such as surface proteins) delivery, need to be carried along with appropriate adjuvants to improve their immunogenicity. In addition, as the buccal mucosa is a pro-tolerogenic tissue which evolved to avoid undesired immune responses against innocuous molecules, sublingual vaccine design is particularly challenging and requires clinical trials to assess adjuvants safety and efficacy [27].

The most studied mucosal adjuvants for sublingual administration are ADP-ribosylating enterotoxins including heat-labile enterotoxin of *Escherichia coli* (LT), cholera toxin (CT), and their associated mutants or subunits (reviewed in [28,29]). The safety of sublingual administration of B subunit of CT (CTB), was evaluated in a phase I clinical trial (NCT00820144) (Table 1) and has been successfully used as an antigen carrier (or mucosal vector) in preclinical studies [20]. Another promising adjuvant is double LT mutant (dmLT or R192G/L211A) (reviewed in [28]). Indeed, the moderate immunogenicity of dmLT (even at doses up to 50 μg) impedes its use as an enterotoxigenic *Escherichia coli* (ETEC) sublingual vaccine [30] but confirms its use as a potent mucosal adjuvant for subunit vaccine, such as SHIV gp120-based vaccines in macaques [31]. Its safety and tolerability was demonstrated in a phase I study (NCT02052934) [30] and another phase I is in recruiting status (NCT03548064).

1.4. Limits of sublingual vaccines

1.4.1. Sublingual mucosa anatomy and associated immune system

The sublingual mucosa, in addition to being a tolerogenic environment, has a specific organization when compared to the extensively studied intestinal or nasal mucosae. It is indeed devoid of M cells (microfold cells), able to sample antigens at the lumen of the epithelium to deliver them to antigen presenting cells (APCs). Additionally, the sublingual tissue does not present any specialized epithelium-associated follicles nor organized lymphoid structures (no follicular DCs) [29,32–34] stressing the central role of DCs in the induction of an immune response at this site [35] (Fig. 1).

A sublingual vaccine is defined as a formulation deposited under the tongue. In liquid form, it can reach the ventral part of the tongue (*per lingual*), gingiva and the floor of the mouth. The sublingual mucosa is a thin stratified tissue consisting in two major layers: the epithelium and the submucosa (connective tissue). The human epithelium is non-keratinized and consists in 8–12 epithelial cell layers of 100 to 200 μm thickness [16]. Pigs, minipigs or monkeys have common histological features with humans and possess a non-keratinized epithelium as opposed to rodents which have a sublingual mucosa lined by a keratinized layer. Rodent models and especially mice which present 6 to 8 epithelial layers in their mucosa can nonetheless be chosen as the most pertinent animal model for evaluation of drug biodistribution, safety or efficacy [36] and still represent a largely used model for sublingual vaccination.

Due to its thinness, the sublingual mucosa is considered as a privileged site for rapid drug adsorption. This prompt adsorption is an essential asset since protein antigens can cross the oral mucosa within 15 to 20 min to accumulate at the mucosal/submucosal junction [37]. For vaccines, antigens can either follow a paracellular or a pericellular pathway before being taken up by immune cells or reaching blood vessels in the submucosa, thus avoiding hepatic first-pass effect [16,38]. Then, once detected by immune sentinels (from 30 min to 2 h), pro-inflammatory cytokines and chemokines are released, attracting immune cells at the site of administration (especially MHC-II$^+$, CD11b$^+$, CD11c$^+$ and Langerin$^+$ cells, attracted by the CCL20 chemokine through their CCR6 receptor) [39] and inducing their maturation (notably through CCR7 expression) [33]. The intensity of the immune response can depend on the number of recruited DCs, the efficiency of antigen

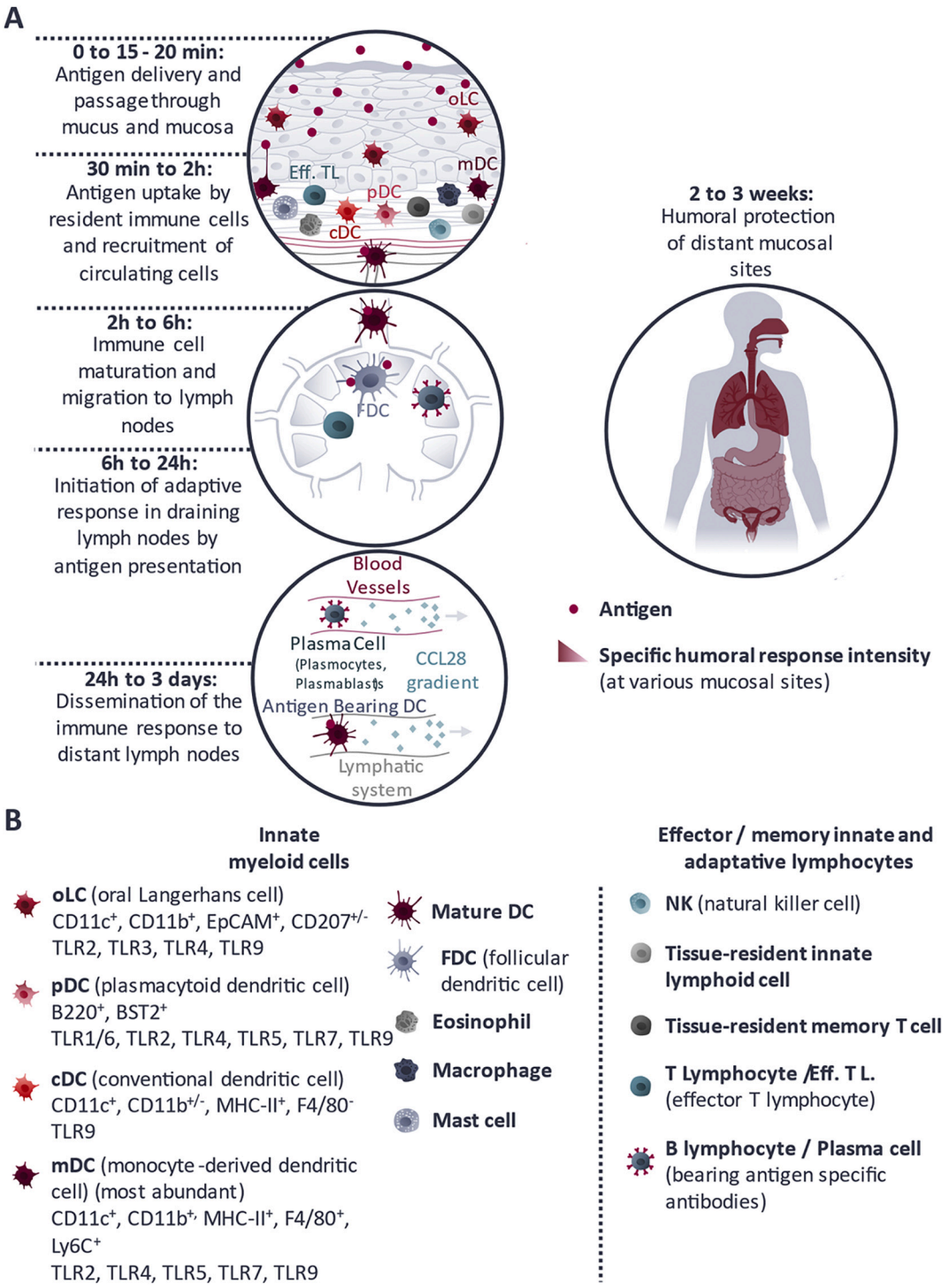


Fig. 1. *Insight on the main phases of the immune response after sublingual vaccination: from antigen delivery to distant mucosal site protection.* (A) Uptake of antigen and dynamics of induction of B and T lymphocytes in the sublingual mucosa with indicative times for each major phase of the immune response: protein antigens usually need 15 to 20 min to cross mucus and sublingual mucosa and reach tissue-resident immune cells. Antigen-presenting cells (APCs) are then able to uptake antigens as well as recruit other immune cells from systemic circulation through cytokine release. While they enter a maturation process, migrating APCs will reach draining LNs within 2 to 6 h. Once in lymphoid organs, the adaptive immune response can be initiated through antigen presentation to T and B lymphocytes, with the help of resident follicular DCs. After a day, the antigen-specific immune response can be disseminated to other LNs and to distant mucosae either through the lymphatic system as antigen-bearing DCs or through blood vessels by plasma cells. First signs of specific mucosal humoral protection can then be detected within 2 to 3 weeks at distant mucosal sites indicated in red. (B) Main immune cell subsets involved in sublingual immune response with common murine phenotypic markers used to identify DCs subtypes described in the sublingual mucosa. Some of their TLR receptors are listed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Clinical trials on sublingually administered prophylactic vaccines in presence or absence of adjuvants.

Name	Study design	Date	Participants	Studied disease	Antigen/vaccine	Adjuvant/ immunomodulator	N°
Virus							
Measuring Responses to Sublingual Antigens	Open, non-randomized	2009–2011	18	Sexually Transmitted Disease	Human Papillomavirus 6,11,16,18 Vaccine Recombinant (Gardasil)	Alum	NCT00949572
Immunogenicity and Safety of Live Attenuated Influenza Vaccine (Flumist) Administered by Nasal and Sublingual Route	Phase I, open, randomized	2011–2013	40	Flu	2011–2012 flu vaccine (Flumist)	–	NCT01488188
Evaluation of the Safety and Immunogenicity of a Sublingual Influenza Vaccine NSV0001 in Healthy Male Volunteers	Phase I, quadruple-blind, randomized	2016–2017	90	Flu	NSV0001	ND002 adjuvant	NCT02955030
Bacteria							
Reactogenicity, Safety and Immunogenicity of a TB/FLU-01 L Tuberculosis Vaccine	Phase I, open, randomized	2013–2015	36	Tuberculosis	TB/FLU-01 L	–	NCT03017378
Safety of Sublingual dmLT for ETEC	Phase I, double blind, randomized	2014–2016	80	Gastroenteritis (Enterotoxigenic *E. coli,* ETEC)	Recombinant Double Mutant Heat-Labile Toxin LT (dmLT)	–	NCT02052934
A Double-Blind Placebo-Control Dose Escalating Study to Evaluate the Safety and Immunogenicity of dmLT by Oral, Sublingual and Intradermal Vaccination in Adults Residing in an Endemic Area	Phase I, double-blind, randomized	2018–2020	135	Gastroenteritis (Enterotoxigenic *E. Coli,* ETEC)	Recombinant Double Mutant Heat-Labile Toxin LT (dmLT)	–	NCT03548064
Mucosal adjuvants							
Effect of interferon-α Citizen by Sub-Lingual Way on the Humoral Immunizing Answer (GP-INFA)	Phase III, double-blind, randomized	2005–2008	140	Flu	2005 flu vaccine (i.m. injection of InfluvacTM)	IFNα 2b (Intron ATM)	NCT00647465
Cholera Toxin B Subunit (CTB) Administered by Mucosal Way in Healthy Adult Volunteer	Phase I, open, randomized	2006–2009	40	–	–	Recombinant Cholera Toxin subunit B (CTB)	NCT00820144

uptake and their level of maturation induced by the adjuvant. The number of tissue-resident MHC-II$^+$ cells then decreases until 4 h to 6 h due to their migration to lymph nodes (LNs) (through a CCL19/CCL21 gradient) where the adaptive immune response is triggered due to the education of naive B and T lymphocytes by matured antigen-bearing migratory DCs [29]. By day 3 after antigen administration, antigen-specific B and T effector cells start to disseminate to other LNs [29] and a humoral immune response can be expected 2 weeks after the last immunization in blood and mucosal secretions.

The approximate 27 cm^2 of human sublingual tissue [16] are covered by a mucus layer which is a protective gel composed mainly of water and proteins, such as antibodies, defensins or mucins [40]. Mucins are large glycoproteins decorated with oligosaccharides and represent the main feature of the glycocalyx of all mucosal epithelia [40]. The main mucus function is epithelial surface protection against dehydration, maintenance of lubrication and prevention from infections by restraining pathogens from reaching mucosal epithelium. Interestingly, for protein antigen delivery purpose, buccal mucosa has a low enzymatic activity compared to gastrointestinal mucosa [41]. Sublingual saliva is composed of 99% of water and mainly comes from sublingual and submandibular salivary glands which secrete around 600 mL of saliva per day [42]. The dilution of vaccine components is then a major issue in effective control of the dose that is transported through the sublingual mucosa.

1.4.2. Dilution of liquid vaccines by saliva and administration protocols

Every sublingually administered liquid formulation is facing the challenge of avoiding as much as possible the wash out of antigen by saliva flow. In order to avoid swallowing, different methods or protocols are applied to animal models. In mouse model, sublingual administration is performed under injected anaesthesia (ketamine/xylazine or analogues) or gaseous anaesthesia, and a concentrated drop of vaccine (volume limited up to 10 μL) is deposited with a pipet directed towards the floor of the mouth [19,21,23,24,43]. In addition, animals could be maintained with heads in anteflexion for 15 to 30 min to avoid swallowing [22,43,44] and by this method, no antigen was found in other buccal mucosae, oesophagus nor small intestine [43]. In non-human primate immunization protocols, up to 800 μL of vaccine are deposited under the tongue of sedated animals (ketamine) [20,45,46] followed by heads bending forward during 15 min and wash of any vaccine excess to avoid oral immunization [20]. However, despite all these precautions, high heterogeneity in induced immune responses can be found due to the lack of control of the dose that is absorbed at the sublingual site [20]. In vaccine clinical trials, participants can only be asked to avoid swallowing during 1 to 2 min after sublingual deposition of the liquid formulation (either as drop or spray), to allow vaccine penetration into mucosa [25]. Yet, in new-born or infant vaccination, where cooperation cannot be expected, alternatives must be developed [47].

To overcome these limitations in the development of efficient sublingual vaccines, one of the main challenges is to increase antigen to mucosa contact time using innovative delivery systems. These advanced devices can have -or combine- mucoadhesive and enhanced permeabilization properties in the form of colloidal or semi-solid formulations [20,29,30].

2. Mucosal devices and formulations enhancing sublingual vaccination

Beyond antigen type, adjuvant choice and vaccine dosage forms, delivery systems are crucial parameters that can highly influence the quality and the intensity of the induced immune response (Fig. 2). Formulations are now fully considered as main actors of a vaccine efficacy, stressing the need to choose them thoroughly to improve

Antigens		Formulations				
Recombinant or subunit proteins	Pathogen mimicking antigens	No formulation	Colloidal formulation	Antigen linked to adjuvant	Studied disease	Refs
No delivery device						
Liquid						
+ Easy formulation and administration						
- High risk of dilution in saliva						
	x	x			Influenza	**[21 , 91]**
	x	x			UTIs*	**[92]**
	x	x			TB†	**[93]**
	x	x			RSV‡	**[50]**
	x	x			HIV§	**[53]**
	x	x			JEV‖	**[51]**
	x	x			HPV¶	**[23]**
x		x	x	x	Influenza	**[94]**
x		x			PIs#	**[58]**
x				x	-	**[19]**
x				x	HIV§	**[20]**
x				x	ETEC**	**[56]**
x				x	Influenza	**[57]**
x			x		-	**[95]**
x			x	x	-	**[96]**
	x		x		Influenza	**[97]**
Transmucosal delivery device						
Needle-free injector						
+ Enhanced antigen delivery through mucosa						
- Evaluation of local inflammation after delivery needed						
x		x			-	**[63]**
	x	x			HIV§	**[31]**
Microneedle array						
+ Enhanced antigen delivery through mucosa						
+ Versatile platform for various antigen formulations						
- Does not avoid partial dilution in saliva						
- Evaluation of local inflammation and tissue damage						
	x	x			Influenza	**[65]**
x			x		-	**[66]**
	x		x		HIV§	**[67]**
Mucoadhesive delivery device						
Solid or semi-solid form						
+ Avoids dilution in saliva						
+ Adapted fabrication process to preserve antigen and adjuvant						
+ Enhanced thermostability						
+ Lengthen contact time between antigens and mucosa						
- Has to be produced with biodegradable components						
x		x			-	**[59]**
	x	x			Influenza	**[70 , 72]**
	x	x			Poliomyelitis	**[47]**
	x		x		GAS††	**[55]**
x			x		-	**[71]**
	x		x	x	HIV§	**[73]**

Fig. 2. Sublingual vaccines formulations and delivery devices. Antigen formulations and delivery devices used for sublingual vaccination studies over the past five years, with a description of their main advantages (+) and drawbacks (−). (*Urinary Tract Infections, †Tuberculosis, ‡Human Respiratory Synctial Virus, §Human Immunodeficiency Virus, ‖Japanese Encephalitis Virus, ¶Human Papillomavirus, #Pneumoccocal Infections (*Streptococcus pneumoniae* or Haemophilius Influenzae), **Enterotoxigenic *Escherichia coli*, ††Group A Streptococcus) [91–97].

vaccination outcomes [48,49].

2.1. Antigen formulations for sublingual delivery: improving immunogenicity

The recognition of an antigen by the immune system depends on its ability to be identified as a danger by immune sentinels. The intrinsic antigen immunogenicity is then a parameter to take into account while formulating a vaccine, for example by adding an appropriate adjuvant to promote the desired immune response.

With time, as advances were made in pathogens characterization, comprehension and also genetic engineering, modified innocuous viruses and bacteria were used as vectors for vaccines antigens. Chosen parts of a target microorganism's genetic material could thus be incorporated in these non-pathogenic vectors then expressing identifiable markers [23,50–52]. Viral vectors are still broadly used for this application and present promising capacities at inducing a strong immune response while being safer than attenuated and inactivated viruses. Although used for a certain time in non-mucosal vaccination, formulations based on adenoviral or baculovirus vectors are still experimental for buccal vaccines [50,52] and require adjuvant addition [52] such as alpha-Galactosylceramide (α-GalCer) alone or combined to CpG-containing synthetic oligodeoxynucleotides (CpG-ODN) to induce a strong immune response [53]. More recently, virosomes were evaluated for sublingual vaccination against influenza [21]. Likewise, this vaccine needed adjuvant *c*-di-AMP addition to induce the required protection against pathogens, and particularly a mucosal immune response. Similarly, vaccines containing immunogenic modular virus-like particles (VLPs) [54,55] were also developed to mimic virus assembly. The effectiveness of sublingually administered Group A streptococcus vaccines based on freeze-dried microbially produced VLPs was further enhanced by co-administration of CT adjuvant, thus increasing salivary SIgA levels against the J8 peptide [55].

While pathogen-mimicking antigens could raise concerns due to their excessive immunogenicity, which can be problematic for immunocompromised people, research is also focusing on vaccine formulations containing recombinant proteins. Although having a superior safety profile, these antigens present a lower intrinsic immunogenicity and often need to be co-administered with adjuvants to induce efficient immune responses. Other strategies have been developed to enhance antigen immunogenicity by linking them directly to an immunostimulatory or immune-targeting component [19,20,56–58]. One example is CTB [19,20] which improves the biodisponibility and uptake of chemically-linked protein antigens by APCs through GM1 receptor linking. Other works also described nanofiber-forming polypeptides, hydrophilic polymers (polyethyleneglycol: PEG) or repeated amino-acid short sequences (Proline, Alanine, Serine: PAS) to enhance antigen immunogenicity due to improved penetration properties [59]. These authors stress the importance of PEGylation or PASylation of antigenic peptides to generate strong immune responses by enhancing antigen penetration through the mucus layer. The mucus is indeed forming a dense glycoproteins network [40,60,61], hindering the passage of proteins through the mucosa; and its presence on the sublingual epithelium surface is then acting as a physical barrier preventing most contacts between antigens and APCs. Such polymeric delivery systems thus increase the encounter of the antigens by APCs [62], therefore enhancing vaccination efficacy. Another way of circumventing such physical barrier could then reside in the use of innovative delivery systems enhancing antigen retention and penetration.

2.2. Transmucosal delivery systems: releasing antigens directly to immune cells

Liquid vaccine formulations can be delivered using needle-free injectors such as MucoJet system [63] or existing injectors repositioned (or adapted) for sublingual vaccination such as Syrijet, originally used to deliver anesthetics in dentistry, thus already approved for human use [31]. With both devices, the vaccine is propelled through the mucosa allowing its efficient delivery directly across the buccal epithelium, thus improving contacts with APCs (Fig. 1). Jones et al. compared the efficacy of topical sublingual vaccination to the needle-free sublingual injection of a modified vaccinia Ankara (MVA) vector and a recombinant trimeric gp120 protein adjuvanted by dmLT against heterologous simian HIV (SHIV) in rhesus macaques [31]. While topical application led to undetectable mucosal antibodies, the needle-free injection induced a protective immunity against pathogenic SHIV by generating a strong systemic IgG response as well as mucosal (vaginal, rectal, salivary) SIgA and IgG responses leading to broad protection against multiple clades of HIV-1. Furthermore, mucosal antibodies protective effect was demonstrated by a higher infection rate after rectal challenge of animals that presented the higher levels of contraction in rectal IgG response at pre-challenge time-point. These results underline the importance to focus not only on magnitude but also on long-lasting effect of the induced response.

An alternative to needle-free injectors to deliver sublingual vaccines is the use of microneedle devices. Contrary to subcutaneous vaccines for which it has been extensively studied, this strategy is novel for sublingual administration [64]. Some microneedles arrays have nonetheless demonstrated their ability to deliver attenuated influenza virions [65], liposomes [66] as well as VLPs and DNA [67] through buccal mucosa. These arrays indeed offer a wide versatility in terms of properties which are adaptable to different vaccine formulations types (as extensively reviewed by Creighton et al. [64]) and permit to improve sublingual vaccines efficacy. When using such microneedles devices, antigens and adjuvants are directly released through epithelium, thus ensuring a controlled dose delivery, and a greatly reduced saliva wash-out. Microneedles arrays therefore represent an interesting platform to deliver sublingual vaccines even if further studies are needed to evaluate their impact on immediate local inflammation and activation of the innate immune system after buccal delivery [64]. However, a randomized one-session clinical trial (NCT03629041) was recently performed by applying a 700 μm long stainless-steel solid microneedle patch on various regions of the oral cavity (lip, buccal/cheek, tongue, hard palate and gingiva) of 30 volunteers. This study concluded on the safety and significantly reduced pain as compared to a hypodermic needle [68], a favourable result for vaccine administration by microneedle arrays.

2.3. Mucoadhesive formulations: lengthening contact time between antigens and mucosa

The development of semi-solid or solid vaccines represents another option to maintain vaccine formulations at the sublingual site, and therefore avoid antigen dilution and improve protective immune responses [69]. These scaffolds are often films [70–72], tablets [73] or gels [47,74] with adapted fabrication processes preserving the fragile properties of the molecules they carry. In particular, protein antigens can easily be denatured or degraded by harsh conditions needed during the production of the majority of tablets, pills and films (*i.e.* high pressure, high temperature, freeze drying or harsh chemical conditions) [69]. Consequently, vaccine components bioactivity and integrity need to be thoroughly evaluated after fabrication process to ensure that the solid form does not alter antigen conformation as wells as the nature and intensity of the induced immune response, as illustrated by Bajrovic et al. [70] and Amacker et al. [73]. These studies point out that the integrity of each component of a formulated vaccine has to be carefully assessed to get a vaccine with the desired properties. Films or tablets offer the advantage to preserve various enzymes, viruses, and bacteria bioactivity within their matrix while also leading to a better overall thermostability (no loss of bioactivity after several month of storage at room temperature, at 37 °C or 40 °C and after repeated freezing and thawing cycles). Moreover, solid or semi-solid formulations present specific properties, especially stability and mucoadhesion provided by

the integration of specific additives or excipients such as adhering polymers, stabilizing detergents, or permeation enhancers such as chitosan or cyclodextrins [62,75]. This versatility generates almost infinite possibilities in terms of formulation with a wide variety of properties adaptable to vaccine components as well as physico-chemical considerations (stability, degradation rate, mucoadhesion…). Each of these parameters can be tuned to optimize the vaccine-induced immune response as reviewed for parenteral vaccines by Moyer et al. [49]. This review stresses the importance of vaccine formulations on vaccination outcomes and the need to include them in the early development of vaccines while maintaining a high degree of safety.

3. Future perspectives in the development of sublingual vaccines

3.1. Make the most of imaging technologies

Whole-mouse *in vivo* imaging technologies allow labelled compounds visualization through complete animal body with a non-invasive monitoring over time. Hence, sublingually administered fluorescent proteins can be monitored to visualize antigen trafficking and lymphatic drainage. With this technique, fluorochrome-conjugated protein residency time can be followed at immunization site [59] or near-infrared dye labelled antigen trafficking can be monitored through whole mouse body [55,57,76]. After administration of a fluorescent compound, fluorescent signal decreases over time at sublingual site and appears in the liver and gastrointestinal tract but not in other organs [55]. Using such fluorescence imaging, it was possible to analyse organs *ex vivo* 4 h after administration, thus leading to the observation of a strong fluorescent signal in the draining submandibular LNs. Authors justified the monitoring *ex vivo*, due to the interior location of the organs or the proximity with the immunization site, preventing detection of a possible lymph node signal.

The study of cellular processes taking place in LNs during the initiation of adaptive response is of great interest to optimize vaccine formulations. As an example, lymph node observation by tissue clearing recently highlighted the presence of antigen-specific germinal centres (GCs) [77] and an extended GC activity by the design of a particulate vaccine [78] administered by injection. As GC formation takes place within B cell follicles in LNs, which are specific sites where B cells maturate, adapting this technique for the observation of sublingual mucosa draining LNs (*i.e.* submandibular or cervical LNs) would greatly facilitate the design of optimized formulations. Light sheet microscopy or flatbed fluorescence scanner are some examples of imaging advances improving the visualization or quantification of a fluorescent signal from a labelled antigen in whole cleared organs. The combination of cutting-edge imaging techniques as well as improvements of deep penetration signals in animals, sensitivity, and resolution could then greatly contribute to the *ex vivo* and *in vivo* study of sublingual antigen trafficking.

In addition to whole body imaging tools, laser scanning confocal microscopy has higher sensitivity and definition to observe antigens in tissues. Indeed, it is possible to colocalize FITC-labelled bacteria (intact or fragmented) with ovalbumin-stimulated CD11c-positive DC cells. This double staining permitted to reveal bacteria phagocytosis by DCs both in the oral mucosa and draining cervical LNs [79]. A similar observation was also performed with fluorescent PLGA-PEG nanoparticles which were internalized by specialized APCs (MHC-II-positive cells) in both mouse and pig sublingual mucosa. The same nanoparticles were then detected in porcine regional draining LNs; an interesting observation for the induction of humoral response, which is associated with GC formation [71]. After mice sublingual immunization with ovalbumin and CT, GC-localized cells ($GL7^+$, $B220^+$ and $CD38^-$ cells) were detected in both spleen and cervical LNs as determined by flow cytometry and by confocal microscopy [80]. Furthermore, since DCs regulate both immunity and tolerance, it is also possible to observe the phenotypic heterogeneity of these cells by immunohistochemistry. Indeed, FITC painting of the sublingual mucosa revealed morphologically different DC subsets in the deeper lamina propria. Round-type MHC-II-positive cells, with less dendrites, expressing high levels of the CD206 marker and low levels of CD11c integrin, exhibited a lower lymph node migratory capacity and tolerogenic functions [81]. These findings highlight the potential of imaging technologies to determine morphological changes of cells, heterogeneity of phenotypes or particular location of specific subsets in tissues. It also displays how, when conjugated to single-cell techniques such as flow cytometry, it can contribute to a superior understanding of the involved mechanisms.

3.2. Sustained delivery systems: a possible approach for sublingual delivery?

Imaging technologies and in particular whole-body imaging are particularly helpful in the elaboration of formulations or delivery systems by allowing the *in vivo* follow up of the vehicle or vaccine compounds after administration. Such technologies are then particularly interesting for the development of systems lengthening vaccine retention time. As recently shown with injectable formulations administered *via* an osmotic pump, a sustained vaccine delivery is a promising approach to enhance vaccination efficacy [82–84].

By prolonged exposure of antigens to APCs, a stronger and more durable antibody response can be generated by a mechanism improving the immunodominance of the intact antigen [83]. In addition, sustained delivery systems were shown to promote dose-sparing effects by enhancing the immune response with a single low dose of vaccine [75]. This type of delivery system thus permits to avoid multiple dose administrations and reduces undesired side effects due to repetitive doses. Interestingly, slow delivery of vaccines was shown to promote broader immune responses with a greater ability to induce neutralizing antibodies, which is a sought-after correlate of protection for many infectious diseases [83] . Indeed, at the same dose, a prolonged antigen exposure to APCs permits an enhanced immune response by altering responding specific B cells repertoire and leading to a more diverse cell population. In fact, with a sustained antigen delivery, follicular dendritic cells (FDCs) which present antigens and antibodies to B cells in GCs, have access to new and intact antigens for a longer period of time as compared to a bolus administration. With this latter, more antigen breakdown products will indeed be presented to B cells leading to a lower probability of inducing neutralizing antibodies. Moreover, GC response kinetic is also a critical parameter since B cells enter into selection and have a few days to be recruited. With sustained delivery, this window of time is lengthened, offering more time for B cells to expand their repertoire thus leading to a more diverse pool of cells therefore increasing probability of obtaining a significant number of neutralizing antibodies. The study of GCs induction and response kinetics after sustained antigen delivery in the sublingual compartment could thus highlight yet undeciphered immune mechanisms. As a prolonged retention of a biomaterial underneath the tongue seems difficult to imagine in humans, solution could reside in a hybrid system where a particulate antigen could be delivered slowly by a ‘depot effect’ in the epithelium, as highlighted in reviews about parenteral vaccination [48,85].

A combination of imaging technologies and novel biomaterials leading to sustained buccal delivery of antigen could be key parameters to explore in the near future; and may certainly impact the prospects of sublingual vaccination.

3.3. Hybrid delivery systems with multiple properties: the future of sublingual vaccination?

As described, saliva wash-out, immune tolerance as well as physical properties of the sublingual mucosa (mucus and epithelium crossing) represent the main hurdles encountered when evaluating liquid

sublingual vaccines, thus leading to a lack of reproducible results. With mucoadhesive elements such as cellulose derivatives [67,71], solid delivery systems actually offer solutions to combine limited dilution together with the delivery of particulate antigens. Such platforms then present multiple interesting properties involving mucoadhesion [71], mucus permeation and lengthened contact time [59], leading to enhanced immune responses based on efficient nanoparticular antigen delivery to draining LNs, higher quantities of specific antibodies in blood and secretions and/or a specific T-cell response induction [59,67,71]. In addition, solid vaccine platforms such as microneedles arrays [66,67], tablets [73] or films [71] can integrate a wide variety of antigen types from VLP [67,73] to liposomes [66] or even nanoparticles [71]. Those antigen nanoformulations have demonstrated their ability to stimulate the immune system by their microorganisms-mimicking shape [86–88] while being much safer than live or attenuated pathogens. By their versatility, those antigens can also integrate immunostimulating agents which can be encapsulated inside nanoparticular devices or exposed at their surface[85,88–90]. Moreover, they can present a synergistic effect when presented in a solid delivery system by lengthening the antigen exposure to the immune system, thus reducing immune tolerance and overcoming reduced immune responses found in sublingual vaccination. Similarly, to liquid vaccines, solid platforms can integrate various components with immunostimulating or stabilizing properties [70]. Hybrid delivery systems can also increase vaccine thermostability at room temperature [66,70,73] and are therefore very promising for applications in developing countries where the cold chain is often difficult to maintain. With a thermostable and easy-to administer vaccine approach, immunization coverage might be extended, holding great promises to enhance global access to vaccines.

4. Concluding remarks

As advances are made in the development of new adjuvants, immunomodulators, vectors and antigen formulations, new interrogations also arise. Immunization frequency, immune response kinetic and longevity, antigen uptake, mucosa transport as well as cell maturation are still partially opened mechanistic questions which need to be answered to fully appreciate the potential of sublingual vaccination for human beings. Yet, sublingual delivery system development is paving the way for a better understanding of fundamental mucosal immunological mechanisms and probably opens perspectives for translation to the clinic.

Declaration of Competing Interest

The authors hereby declare that there was no conflict of interest in the present study.

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