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Review article

Adjuvants modulating mucosal immune responses or directing systemic responses towards the mucosa

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Abstract – In developing veterinary mucosal vaccines and vaccination strategies, mucosal adjuvants are one of the key players for inducing protective immune responses. Most of the mucosal adjuvants seem to exert their effect via binding to a receptor/or target cells and these properties were used to classify the mucosal adjuvants reviewed in the present paper: (1) ganglioside receptor-binding toxins (cholera toxin, LT enterotoxin, their B subunits and mutants); (2) surface immunoglobulin binding complex CTA1-DD; (3) TLR4 binding lipopolysaccharide; (4) TLR2-binding muramyl dipeptide; (5) Mannose receptor-binding mannan; (6) Dectin-1-binding ß 1,3/1,6 glucans; (7) TLR9-binding CpG-oligodeoxynucleotides; (8) Cytokines and chemokines; (9) Antigen-presenting cell targeting ISCOMATRIX and ISCOM. In addition, attention is given to two adjuvants able to prime the mucosal immune system following a systemic immunization, namely 1α, $25(OH)_2D_3$ and cholera toxin.

mucosal adjuvants / pattern recognition receptors / dendritic cells / domestic animals / systemic immunization

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1. INTRODUCTION

It is generally accepted that initiation of a specific immune response requires activation of a specific defense mechanisms resulting in a pro-inflammatory response. The released cytokines and chemokines assist in activating and directing the adaptive immune response. As a consequence, a vaccine has to induce a pro-inflammatory response to be effective. Most antigens are not immunogenic enough and need help to induce a good immune response. Adjuvants (*adjuvare* (latin) = to help) are substances added to an antigen with the purpose of enhancing its immunogenicity [77]. Whereas a very long list of adjuvants for systemic immunization exists, the number of mucosal adjuvants is far more limited since mucosal adjuvants have to stimulate the mucosa-associated lymphoid tissue underneath the mucosa without disturbing the barrier function of the mucosa.

There is currently no optimal classification system to include all adjuvants [54]. Cox and Coulter [41] described that adjuvants act in one or more of five ways: (1) immunomodulation = modulation of the cytokine response; (2) presentation = preservation of the conformational integrity; (3) induction of CD8+ cytotoxic T-lymphocyte responses; (4) targeting = the ability to deliver the antigen to immune effector cells; and (5) depot generation (Tab. I). Therefore, an interesting classification might be based on their mode of action. However, the complete working mechanism of many adjuvants is not known at the moment, impairing this classification system. It is becoming more and more clear that most non-particulated mucosal adjuvants act by binding to a receptor, whereas the particulated adjuvants act by targeting antigens towards antigen presenting cells (Tab. I). In the present review, these properties were used to classify mucosal adjuvants.

The present review mainly deals with the non-particulated mucosal adjuvants. Several particulated adjuvants will be described in another article [70] of this special issue on "Mucosal immunology in domestic animals". Additionally, we review adjuvants reported to modulate a systemic immune response towards a mucosal one. The latter is particularly interesting for antigens for which a mucosal administration is less feasible due to rapid degradation in the gastrointestinal tract.

2. RECEPTOR-SPECIFIC MUCOSAL ADJUVANTS

Almost all data on mucosal adjuvants for veterinary species are from experimental work and most of these experiments have been performed in pigs, sheep and rabbits. Most of these data are summarized in the present review. The experimental work on horses, cattle, dogs, cats and other veterinary species is far more limited. Data from experiments on the latter species are mainly mentioned in the Tables.

In this chapter extra attention is given to the ganglioside receptor-binding toxins, since they have been most extensively tested for their mucosal adjuvanticity and this in different animal species. However, other adjuvants that have also been shown to enhance and or modulate mucosal immune responses in animals are discussed in the present

Table I. Properties of some adjuvants adapted from Cox and Coulter [41].

Adjuvant	Particle form	Immunomodulation*	Targeting	Receptor	Presentation	CTL induction	Depot
Aluminium salts	+	++++2	+	–	–	–	+
W/O emulsions	+	+1,+2	–	–	–	–	++
O/W emulsions	+	+1,+2	+	–	+++	–	–
ISCOMS	+	+++1,+2	+++	–	++++	++++	–
ISCOMATRIX		+++1,+++2	+++	–	++++	++	–
Saponin	–	+++1,+++2	–	–	–	+	–
LPS/Lipid A	–	++++1	–	TLR4, RP105	–	–	–
Muramyl dipeptide hydrophil	–	++++2	–	TLR2	–	–	–
Muramyl dipeptide lipophil	–	++++1	–	TLR2	–	–	–
CpG ODN	–	++++1	++**	TLR9	–	–	
Carbohydrate polymers	–	++1 or 1 and 2	+++***	Mannose receptor, Dectin-1	–	–	-
Vitamin D3	–	++++2	–	Nuclear vitamin D receptor	–	–	–
CTA1-DD	–	+++2	–	Surface immunoglobulin	–	–	–
Cholera toxin	–	++++2	++++***	GM1, GD1b, ganglioside	+	++	-
LT enterotoxin	–	++++1,++++2	++++***	GM1, GD1b GM2 ganglioside†	+	++	–

*1= Th1-like response, 2=Th2-like response.
** If incorporated in DNA plasmide vaccine.
*** If conjugated.
† In addition asialo-GM1 ganglioside, polyglycosylceramides, and polylactosamine-containing glycoproteins.

review. All these non-particulated mucosal adjuvants are classified based on their receptor-specificity.

2.1. Ganglioside receptor-binding toxins

2.1.1. Cholera toxin and Escherichia coli *thermolabile enterotoxin*

Cholera toxin (CT) and *E. coli* thermolabile enterotoxin (LT) are highly resembling molecules that function as virulence factors in *V. cholerae* and *E. coli* infections, respectively. Both toxins are composed of an A and B subunit of which the B subunit forms a homopentamer structure that binds to epithelial cells. The B subunit of CT and LT binds with high affinity to the glycosphingolipid, GM1-ganglioside (Gal(1-3) GalNAc(1-4)(NeuAc(2-3)Gal(1-4)Glc(1-1) ceramide) [71] and with a lower affinity to GD1b-ganglioside [88]. In addition, LT-B also binds with low affinity to polyglycosylceramides, asialo-GM1, GM2 and polylactosamine-containing glycoproteins [66, 89]. GM1 is present on virtually all cells including enterocytes, dendritic cells (DC), macrophages (MØ), B and T lymphocytes. The A subunit is composed of a globular A1 domain and an A2 domain that interacts with the B subunit. The ADPribosyltransferase activity is facilitated following proteolytic cleavage of the trypsin-sensitive loop between the two domains and reduction of the disulfide bond [71]. Then, the A1 fragment enters the cytosol, enzymatically ribosylates the Gs protein of adenylate cyclase, leading to an increased cAMP production.

CT as well as LT are potent immunogens and induce antigen-specific sIgA and serum IgG antibody responses [56, 184]. In addition, both toxins can act as adjuvants for the enhancement of mucosal and serum antibody responses to a mucosal co-administered antigen, resulting in a long-term memory to this antigen [35, 105, 202]. The immune response that is occurring against these toxins is not an advantage. High levels of toxin-specific IgA at the inductive site reduce the adjuvant effect. However, these toxin-specific antibodies do not completely inhibit the mucosal adjuvanticity [192, 193].

Following intestinal administration of LT and CT, both toxins bind to intestinal epithelial cells, which subsequently secrete IL-1, IL-6, IL-10 and IL-1Ra [21, 138]. In addition, CT is reported to increase both the intestinal permeability [131] and the protein uptake [213]. Verma et al. [213] suggested a change in tight junctional permeability following LT administration, which may be due to a change in the cytoskeletal microfilaments. However, the influence of LT and CT on intestinal permeability to macromolecules is controversial since some reports argue against it [98, 152]. What is not controversial is that both toxins can be transported by M cells into the Peyer's patches and are subsequently present within monomorphonuclear cells in the lamina propria [116].

The toxins passing the mucosa will subsequently reach cells of the immune system. The interaction of LT and CT with leukocytes is thought to be of major importance for mediating their adjuvant effects. Effects on DC, MØ, T and B cells will be described. Especially DC seem to be the principal cell type by which LT and CT mediate their adjuvant effect in vivo. Luminal CT attracts DC to the intestinal epithelial layer, where they seem to take up luminal antigens [167]. Furthermore, the toxins induce phenotypic and functional maturation of DC, so upregulating the expression of MHCII, B7.1 and B7.2, downregulating the expression of CD40 and ICAM-1 and increasing the secretion of IL-1β [9, 55, 133, 158]. In addition, a cAMP-dependent upregulation of the chemokine receptors CXCR4 and CCR7 occurs [67], enabling the migration of DC to lymph nodes. Here they can interact with naïve T cells [97, 179] in an enhanced way since their ability to present protein antigen is improved [158]. In vitro, CT-maturated DC are able to prime naive $CD4^+/CD45RA^+$ T

cells and to direct them towards the Th2 phenotype. Inhibition of the expression of the Th1-response promoting cytokine IL-12 has been implicated as the mechanism by which CT mediates this polarization [9, 19, 48]. The ability of CT to activate DC seems to dependent on its specific interaction with GM1 ganglioside [110].

CT and LT exert several effects on MØ, which they also exert on DC, such as enhanced expression of B7.2 [37, 133, 174, 231, 232], enhanced secretion of IL-1 [20, 64] and reduced secretion of IL-12 [19, 174]. In addition, both toxins induce the secretion of IL-10 by MØ, which also promotes a Th2 response [61, 174]. CT also suppresses TNF-α production in response to LPS [24, 29, 38] and as a consequence the NO production is reduced [38].

The effects of CT on T cells also promote a Th2 response. Indeed, the initial event induced by CT in $CD4^+$ T cells involves the upregulation of IL-4 [154, 202, 232]. This results in the secretion of IL-5, IL-6 and IL-10, a typical Th2 response, providing helper signals for the induction of antigen-specific sIgA as well as serum IgG1, IgA and IgE responses in mouse models [91, 230]. In addition CT selectively inhibits proliferation and IFN-γ synthesis of Th1 clones [149, 231] and abrogates IL-12R expression by T cells [19]. In contrast to CT, LT induces both Th1- and Th2-responses with subsequent mucosal sIgA as well as serum IgG1, IgG2a and IgA responses. The LT-induced Th2-response is largely IL-4 independent [232]. This difference in CT and LT adjuvanticity is suggested to rely on differences in either the A [18, 174] or the B subunit [16].

LT and CT have been reported to induce selective apoptosis of $CD8^+$ T cells, naïve cells being more sensitive than activated cells [57, 151, 175].

Binding of CT and LT to B cells leads to the upregulated expression of MHCII, B7.1 and B7.2, CD40, ICAM-1 and IL-2Rα [6, 17, 65, 133]. This activation of B cells enhances their role as MHC II-restricted antigen presenting cells and favors the induction of Th2-dominated responses. In vitro studies indicate that CT facilitates B cell switching to IgA through the action of TGF-β1 and increases the effects of IL-4 and IL-5 on IgG1 and IgA synthesis in lipopolysaccharide (LPS)-triggered spleen B cells [111, 130]. This IgA induction is independent of the A subunit [111].

The induced mucosal immune response is best at the mucosal site directly exposed to the antigen and the adjuvant [159]. This is probably due to an increased expression of homing receptors on endothelial cells [126].

During the last five years, CT was used in an important number of studies in animals as the mucosal model antigen or as the mucosal adjuvant (Tab. II). The most frequently used route is the intranasal one. This route has been used in horses, pigs, rabbits, cattle and sheep and resulted in the first three species in a clear nasal IgA response which was not so in sheep.

Oral immunization with CT as the adjuvant was tested in pigs, chickens and rabbits and mostly induced antigen-specific intestinal IgA responses (Tab. II). The oral use of CT and LT in humans is hampered by their enterotoxicity. As little as 5 μg CT can induce significant diarrhea and 25 μg elicits 20-litre watery diarrhea. However, in all tested animals CT seems to be less toxic. For example in 3- to 4-week-old piglets, a dose of 1 mg CT induced only a pasty to semiliquid diarrhea for 2 h [40], whereas a dose of 50 and 100 μg did not cause clinical signs at all, but still showed a significant adjuvant effect [63, 210]. In pigs, LT loses its enterotoxicity very rapidly with increasing age and 14 times the dose of LT provoking severe watery diarrhea with dehydration in neonatal pigs induces only a pasty to semiliquid diarrhea for 24 h in 3- to 4-week-old pigs [40]. So, there seems to be less need for detoxified variants of both enterotoxins in pigs. Evaluation of the adjuvanticity of different dosages of CT in pigs revealed that 10 μg already significantly enhanced the mucosal IgA and the serum

Table II. CT as mucosal adjuvant in animals.

Species	Route	Dose (µg)	Antigen	Measured antigen-specific response	References
Pig	IN	25	*Ascaris* 16 kDa protein (rAs16) - CT conjugate	Increased serum IgG, nasal IgA, IL-4, IL-10 and protection	[199]
	O	100	CT-B-A2-PRRSV nucleocapsid protein peptide	Increased intestinal IgA, serum IgG and IgA, no vaginal response	[94]
		50	F4 fimbriae-HSA conjugate, HSA-HSA	Increased serum IgA and IgG and salivary IgA	[211]
		50	rFaeG (adhesin of F4 fimbriae)	Increased serum IgA and	[210]
		10, 100	CTB, KLH, OVA or OVA-CTB	Increased IgA in intestine (not for KLH, OVA)	[63]
Sheep	IN	100	PLG + *Toxoplasma gondii* tachyzoite antigen	No clear effect	[187]
	R	25	Keyhole limpet hemocyanin (KLH)	Increased IgG1 and IgA ASC in blood	[161]
		10	*Hemonchus contortus* L3 larvae surface antigen	Very low (total) serum antibody response	[99]
Cattle	IN	100	Limulus hemocyanin (LH)	Increased serum IgA	[166]
Horse	IN	0.2	100 µg CTB, two aerosol boosts with 1 mg CT	Induction of nasal and serum IgA, IgGb and IgGT and serum IgGa	[183]
		1000	Equine influenza virus hemagglutinin (HA) DNA	Increased nasal IgA response	[183]
Rabbit	IN	20	Extract of *Pasteurella multocida (P. mult.)*	Induction of serum IgG and nasal IgA	[189]
		200	*P. mult.* OMP	Induction of serum IgG and nasal IgA	[36]
	O	200	Extract of *P. mult.* in alignate microparticles	Increased serum IgG and induction of nasal IgA	[189]
Cat	R, IN/R	10	FIV-peptides or fixed whole FIV	Induction of serum IgG and IgA	[62]
Chicken	O	50	*Eimeria tenella and* r1PE1 of *E. tenella*	Only with recombinant increased IgA and IgG in intestine, serum.	[72]
		4, 20, 100	Inactivated infectious bursal disease virus	No effect	[93]
	IC	50	r Ea1A of *Eimeria acervulina*, CT-rEa1A conjugate	Increased serum antibody response slightly better with the conjugate	[215]
	O, R	10	Mycotoxin aflatoxin B1 (AFB)	No enhanced serum IgG, no fecal IgA response	[226]

R: rectal; O: oral; IN: intranasal; IC: intra-caecal; OMP: outer membrane proteints; FIV: feline immunodeficiency virus.

IgA and IgG responses against coadministered CT-B. However, when using keyhole limpet hemocyanin or ovalbumin as antigen, even 50 µg CT could not induce an antibody response, whereas when added to OVA-CT-B conjugate, a clear response was observed [63]. This indicates that not only the adjuvant and its dose but also the antigen are important in mucosal immunization. In experiments we performed, 50 µg CT did enhance the IgG and IgA response following oral immunization of pigs with a human serum albumin (HSA)-HSA conjugate (HSA-HSA conjugate) or a HSA-F4 conjugate (purified F4 fimbriae of enterotoxigenic *E. coli* (ETEC)) [211]. However the most pronounced effect was observed for the latter conjugate. A good adjuvant effect was also seen following oral immunization with the recombinant adhesin of F4 [210]. These observations seem to indicate that CT is especially suitable for antigens that bind to (target) the mucosa.

2.1.2. CT-B and LT-B

An important strategy for utilizing the immune-stimulatory properties of LT and CT has been the use of the non-toxic B subunits. However, the results obtained with LT-B and CT-B alone as mucosal adjuvants are highly inconsistent. Studies have shown that neither LT-B nor CT-B enhance immune responses to mucosal co-administered protein antigens when given orally [165, 231], whereas some other reports have suggested that LT-B and CT-B could display mucosal adjuvant activity when intranasally administered (large doses) in combination with proteins (large doses) [46, 47, 52, 165]. Antibody responses can be observed when LT-B or CT-B are directly conjugated to the antigen itself [165] and are given either orally or intranasally (Tab. III). However, it is interesting to note that the holotoxin stimulates stronger responses on a dose-by-dose basis following intranasal delivery compared to the B subunit [52, 108].

On the contrary, there are studies reporting the use of recombinant LT-B and CT-B subunits to induce tolerance following oral delivery, but only in the complete absence of holotoxin [227]. Hereto, the B-subunits need to be directly coupled to the antigen so that they can function as a carrier. Following GM1-receptor-mediated uptake, the antigen reaches immature antigen presenting cells, resulting in the induction of TGF-ß-secreting regulatory T cells. In contrast to CT, rCT-B and the catalytically inactive holotoxin do not cause significant maturation of human DC [179]. As for CT, CT-B suppresses the production of TNF-α in response to LPS, but in contrast to CT it may also suppress production of other pro-inflammatory cytokines [29].

2.1.3. CT and LT mutants

Another attempt to dissociate the enterotoxicity of LT and CT from their adjuvanticity has been the construction of mutations in the enzymatically active A subunit. This subunit consists of two chains, A1 and A2, joined by a proteolytically sensitive peptide (Arg192). Well characterized mutations in the A subunit are lysine for serine at position 63 (LT(S63K)) and arginine for alanine at position 72 (LT(A72R)). Both mutations differ in their residual enzymatic activity and this activity is positively correlated with their adjuvanticity with LT(A72R) having the highest activity [10, 132]. Both mutants are active in domestic animals when applied intranasally (Tab. IV). Dickinson and Clements [49] constructed LT(R129G) with a mutation of arginine to lysine in the proteolytic cleavage site. This mutant has a reduced enterotoxicity, shows in vitro absence of ADP-ribosyltransferase activity, but still has its mucosal adjuvanticity when given intranasally, orally or rectally [31, 155, 237]. Yuan et al. [237] used this adjuvant intranasally in gnotobiotic pigs at a dose of 5 µg and although the adjuvant enhanced the IgA ASC response, it did not induce a protective intestinal immunity. The dose of 5 µg was chosen since 10 µg produced diarrhea in 85% of neonatal piglets. We used this adjuvant for oral immunization of conventional weaned piglets

Table III. CT-B and LT-B as mucosal adjuvants in animals.

Adjuvant	Species	Route	Dose (µg)	Antigen	Measured antigen-specific response	References
CT-B	Pig	O	1000	CT (50 µg) and KLH	No KLH specific antibody response	[63]
				PRRSV N peptide and CT (100µg)	No N-peptide antibody response in serum, intestine, vagina	[94]
				CT-B-A2-N-myc or CT-B-myc9 and CT (100µg)	Induction of anti-N-peptide serum IgG, IgA or anti-myc serum IgG	[94]
	Cattle	SC/IN	10/15	Bovine herpesvirus-1 glycoprotein D	No positive effect of CT-B adjuvant	[241]
	Rabbit	IN		Influenza HA	High Serum IgG, nasal IgG	[73]
	Fox	IN	100	OVA	No OVA-specific antibody response	[170]
	Chicken	O	200	CT-B-BSA glutaraldehyde-conjugated	Induction of BSA specific serum IgG	[135]
				CT-B and BSA	Increased BSA-specific serum IgG, IgM, IgA	[136]
		IN	20	Inactivated Newcastle disease virus	Induced IgA and neutralizing antibody titers in nasal washes and enhanced protection.	[190]
CT-B-A2	Horse	IN	300-5000	CT-B-A2-SeMF3(peptide) of *Str. equi*	Induction of serum IgA, Igb, IgGa and IgG(T) and nasal IgA, IgGb	[177]
LT-B	Chicken	O, R	100	AFB-BSA-rLT-B conjugate or AFB-BSA mixed with rLTB	No enhanced serum IgG, no faecal IgA response	[226]
	Rabbit	O	± 1000	LT-B-*E. coli* STa carbodiimide-conjugated	Induction of ST- and LT-B- specific serum IgG and mucosal IgA antibody responses	[114]

O: Oral; SC: subcutaneous; IN: intranasal; R: rectal; KLH: keyhole limpet hemocyanin; PRRSV: porcine reproductive and respiratory syndrome virus; OVA: ovalbumin; BSA: bovine serum albumin; Str.: *Streptococcus*; AFB: Mycotoxin aflatoxin B1; STa: thermostable enterotoxin a.

with F18 fimbriae in a dose of 25 μg and could only see a weak immune response and no clinical signs (unpublished results, Tab. IV). Another mutant that lacked the nick site in the A subunit by deleting a tripeptide (Arg192-Thr193-Ile194), was insensitive to activation by trypsin. This mutant has no ADP-ribosyltransferase activity, but has a strong adjuvant activity (LT triple-aa deletion mutant) [235] and enhanced nasal and serum antibody responses in pigs and cattle following intranasal immunization (Tab. IV).

2.2. Surface immunoglobulin-binding CTA1-DD

Another approach to detoxify CT is linking the enzymatically active A subunit domain of the toxin to a molecule with another cell-binding specificity than the natural B subunit. Agren et al. [2] did this by genetically fusing the gene of the ADP-ribosyltransferase active A1 subunit of CT to a gene encoding a synthetic analogue of the *Staphylococcus aureus* protein A. The latter is called D and the fusion protein, CTA1-DD, containing a dimer of D. CTA1-DD targets the ADPribosyltransferase towards B cells. The fusion protein binds to naïve and memory B cells of all isotypes, with adjuvant effects similar to CT [3]. This protein is non-toxic to mice even when given at 1000-fold the toxic dose of CT. Both CT and CTA1-DD have been observed to enhance antibody and cell-mediated immune responses, the fusion protein exerting these effects primarily via B cells [4, 180]. CTA1-DD mostly functions when applied nasally, but not when given orally. However, the authors used CTA1-DD as an adjuvant in 5-week-old pigs intranasally immunized with F18 fimbriae and only observed weak priming of the antibody response (unpublished results, Tab. IV).

2.3. Pattern Recognition Receptor binding adjuvants

Several molecular structures of microorganisms are recognized by so called pattern recognition receptors (PRR). These PRR are among others present on MØ and DC. Some PRR are mainly involved in opsonization and thus enhance phagocytosis of microorganisms such as the mannose receptor, scavenger receptors and Dectin-1. Others, such as Toll-like receptors (TLR) seem to function exclusively as signaling receptors resulting in the following: (1) activation of innate immune responses, (2) expression of co-stimulatory molecules and (3) via activation of DC, an enhanced T cell stimulatory potential as well as (4) production of immunomodulatory cytokines, essential for T cell priming and effector T cell differentiation [157, 185].

It is therefore not surprising that several of the molecules of microbial origin shown in the past to have adjuvant activity, have more recently been identified to act via these PRR.

2.3.1. TLR4- and radioprotective 105 (RP105)-binding

In 1956, Johnson et al. [103] demonstrated the adjuvant activity of lipopolysaccharide (LPS) from Gram-negative bacteria. LPS exerts its activity mainly via TLR4 on antigen-presenting cells [139, 178]. In fact it is a multi step mechanism. LPS first binds in the blood or extracellular fluid to the soluble LPS binding protein (LBP) with its lipid A moiety [197]. LBP is a lipid transferase that catalyzes the LPS' transfer from the outer membrane of the bacteria to CD14, another LPS binding molecule. CD14 is expressed on the myelomonocytic cells as a glycosylphosphatidylinositol-anchored (GPI-anchored) membrane molecule (mCD14) or is present as a soluble molecule in the circulation (sCD14) [228]. mCD14 enables LPS to get close to TLR4-MD-2, suggesting that LPS needs to be transferred from CD14 to TLR4-MD-2 [44, 150]. MD-2 is an extracellular molecule associated with the extracellular leucine-rich-domain of TLR4. Association of TLR4 with MD-2, is required for the expression of TLR4. As most other

Table IV. LT and CT mutants as mucosal adjuvants in animals.

Adjuvant	Species	Route	Dose (μg)	Antigen	Measured antigen-specific response	References
LT triple-aa deletion mutant	Pig	IN	40, 200, 1000	*Erysipelothrix rhusiopathiae* SPA 46.5 kDa or *Bordetella bronchiseptica* sialic acid-binding HA	Increased nasal IgA and serum IgG; 200 μg the highest IgA responses and best protection	[235]
	Cattle	IN	1000	Intimin (*E. coli* O157:H7) C terminal 64 kDa	Increased nasal and salivary IgA, serum and colostral IgG1	[235]
LT(R192G)	Pig	IN	5	Rotavirus-like particles (VLP) containing VP2 and VP6	Rotavirus-specific IgA (memory) B cells in intestinal tissues, but no protection	[237]
		O/IN or IN/O or IN	5	Attenuated rotavirus oral/VLP intranasal with LT mutant	One oral followed by two IN immunizations induces strongest B-cell responses in GALT, high protection	[238]
		O	25	F18 fimbriae of *Escherichia coli*	Weak IgM response, no protection	*
	Cat	IN	10	P24Gag of feline immunodeficiency virus	Increased IgG in serum and IgA in saliva and vaginal washes	[122]
LTR72	Rabbit	IN	50	Influenza/A HA in esterified hyaluronic acid (HYAFF) microspheres or soluble HA	Increased serum IgG	[181]
LTK63	Micro-pig	IN	100	Influenza/A HA in esterified hyaluronic acid (HYAFF) microspheres or soluble HA	Increased nasal IgA, serum IgG for the HYAFF formulation	[181]
	Rabbit	IN	25	Influenza/A HA in esterified hyaluronic acid (HYAFF) microspheres	Increased serum IgG	[181]
CTA1-DD	pig	IN	100	F18 fimbriae	Slightly increased serum F18-specific Ig	*

O: oral; IN: intranasal; HA: hemagglutinin; SPA: surface protective antigen; * unpublished results.

TLR, TLR4 is a type I transmembrane protein with a conserved cytoplasmic domain called the Toll/interleukin (IL)-1 receptor (TIR) domain [156]. This domain is essential for signaling. Stimulation of TLR results in dimerization and conformational changes, which initiate the signaling via TIR and leads to activation of NF-κB. The subsequent signaling pathway was reviewed recently [191].

LPS is also capable of activating B cells. This does not occur via TLR4-MD-2, but via a structurally related cell surface complex, radioprotective 105 (RP105), which associates with MD-1 [142, 143]. This complex does not signal via TIR. Another transmembrane protein, CD19, was shown to be important for delivering the RP105 signal [233].

Stimulation with LPS results in production of pro-inflammatory cytokines such as IL-1 and TNF-α but also in the release of colony stimulating factors (CSF) and increase in MHC class II expression. Ligation of RP105-MD-1 on B cells induces resistance against apoptosis, upregulation of co-stimulatory molecules and proliferation [27]. LPS itself has only experimentally been used as an adjuvant due to its high toxicity. Several studies have been conducted with chemically modified forms of lipid A. One of the best studied is monophosphoryl lipid A (MPL). MPL is derived from the LPS of *Salmonella minnesota*. Like LPS, MPL is thought to act via TLR4 on antigen-presenting cells and has been shown to induce the release of pro-inflammatory cytokines, but also of IL-2 and IFN-γ. It has been used in vaccines against melanomas and breast cancers, against several infections such as hepatitis B virus infection and against allergies. For the latter it has been approved in Europe [30, 225]. MPL is most effective when used in liposomes.

2.3.2. TLR2-binding

Muramyl dipeptide (MDP) (N-acetylmuramyl-L-alanyl-D-isoglutamine) is derived from the cell wall of mycobacteria and is one of the active components in the Freund complete adjuvant. MDP acts on TLR2 and was used for intravaginal, oral, intragastric or intraduodenal immunizations of mice, giving an enhanced mucosal immune response [101, 195].

There exists several analogues of MDP. One analogue, adamantylamide dipeptide, constructed by linking amantadine to MDP, has been shown to induce in rabbits a serum IgG and IgA response as well as fecal IgA when given orally in a dose of 100 mg together with 5 mg ovalbumin. The response was observed 10 days after a booster immunization [12]. Recently, a new analogue was constructed, with changes in both the sugar and the peptide parts of the molecule. This analogue shows an enhanced adjuvant activity and suppressed adverse side effects following subcutaneous administration. The introduction of lipophilic residues has improved its incorporation into liposomes. However, evaluation of this MDP analogue in mucosal immunizations has not yet been performed [201].

2.3.3. Mannose receptor-binding

Mannan, a polymannose, can be derived from the cell wall of yeasts. Oxidatively coupled to recombinant protein antigens and given intranasally, but not intraperitoneally, to mice it markedly enhanced the production of IgA, IgG1 and IgG2a in serum and IgA locally in the lung and at remote mucosal sites, including tears, vaginal and salivary secretions. The response was better with CT as the adjuvant. It is thought that the enhanced immune response results from binding to the mannose receptor on phagocytes [186].

2.3.4. Dectin-1-binding

ß 1,3/1,6 glucans are also present in the cell wall of yeast, but in addition, they can be derived form the cell wall of fungi, some bacteria, algae, seaweed and corns [203].

Many studies in mammalians demonstrate that soluble and particulate ß 1,3/1,6 glucans are immunological response modifiers and can be used in the therapy of neoplasia, infectious diseases and immunosuppression. Their main direct target cells appear to be DC, monocytes/MØ, neutrophils and natural killer cells. Glucan treatment of monocyte/ MØ induces the production of TNF-α, IL-1, platelet-activating factor and arachidonic acid metabolites, such as PGE_2 and LTB_4 [1, 50, 58]. The direct interaction of ß-glucans with their target cells is mediated via PRR. Until now, five different PRR have been identified which bind ß-glucans or are involved in the interaction with the glucan: Dectin-1 [22], the lectin-binding domain of the ß chain (CD11b) of the complement receptor 3 [173], the lactosylceramide receptor (CDw17) [219], some scavenger receptors [171] and TLR2/6 [68]. Dectin-1 is thought to synergize with TLR2 to induce TNF-α and IL-12 production and very recently it was demonstrated that Dectin-1 activation of DC even results in production of IL-10 and IL-2 [169].

Several studies have demonstrated the adjuvant effect of glucans when co-administered with either bacterial, fungal, protozoa or viral antigens [13, 144, 168]. In all these studies, ß-glucans were administered via the parental route. Experiments in our laboratory with ß-glucans in pigs have shown that their addition to the feed for a period of two weeks immediately after weaning drastically reduced the excretion of ETEC upon challenge. Supplementation of glucans to piglet's feed can also have a modulating adjuvant effect on simultaneously administered antigens. Different glucans can have distinct immunomodulating effects. One glucan shifted the systemic antibody response against intramuscularly administered bovine thyroglobulin towards IgA while the other suppressed the antigen-specific proliferation of peripheral blood leukocytes [78]. Furthermore, the glucan administration resulted in an enhanced priming of an F4-specific intestinal immune response in piglets when given together with an oral immunization with F4 fimbriae[1]. In dogs, the effect of oral supplementation of glucans on a systemic immunization seems to be different from this in pigs in that an increased antigen-specific IgM response was observed whereas the IgA response should have had a tendency to be lower[2].

2.3.5. TLR9-binding

Cytidine-phosphate-Guanosines (CpG) are unmethylated dinucleotides present at a frequency of approximately 1 on 16 nucleotides in bacterial DNA, whereas they are underrepresented (1/50 to 1/60) and methylated in the vertebrate (mammalian) genomes [25, 160]. Because of these differences, a nonself pattern recognition mechanism has evolved in the vertebrate immune system using PRR enabling them to encounter invading pathogens via their unmethylated CpG-dinucleotides [119]. Hemmi et al. [86] demonstrated that TLR-9 is required for the immune activation by CpG-dinucleotides. This receptor is highly expressed on plasmacytoid precursor DC, B cells, and cells of the monocytes/MØ lineage [53, 104, 239]. Whereas a lot of studies demonstrated an intracellular presence, more recent studies also show cell surface expression. Upon IFNγ treatment, both TLR9 mRNA expression and responsiveness to CpG DNA are upregulated in PBMC, whereas LPS upregulates cell surface expression [53].

The biological activity of these CpG-dinucleotides can be mimicked by chemically synthesized CpG-oligodeoxynucleotides (CpG-ODN). CpG-ODN are chemically synthesized single stranded DNA sequences that are able to stimulate MØ, NK cells, DC and B cells. They were originally synthesized in a specific motif in which the CpG-dinucleotide is flanked preferentially by two purines, adenine (A) or guanine (G) at the 5'-end, and two pyrimidines, cytosine (C) or thymine (T) at the 3'-end,

[1] Cox et al., unpublished results.
[2] Stuyven et al., unpublished results.

making for example AGCpGTT. The experimental use and success of some DNA-vaccination trials is partly due to the presence of CpG-motifs in the DNA expression vectors [8, 200, 212].

The immunostimulating effects of CpG-ODN also depend on the sequence of the nucleotides flanking the CpG-dinucleotide as well as the target species. Optimal CpG-ODN motifs have been reported for several animal species (Tab. V). Comparing these motifs, it was obvious that recognition of a GTCGTT motif is highly conserved. A GACGTT motif however, was optimal for inbred strains of mice and rabbits [164].

Upon contact with CpG-ODN, DC and MØ produce IL-12. This in turn activates NK cells, which subsequently produce IFN-γ. IFN-γ suppresses Th2 cells but induces MØ to produce more IL-12. All this results in a Th1 microenvironment. Besides IL-12 and IFN-γ, there is also increased secretion of IL-18, which also modulates towards Th1 [112, 113, 127, 234]. In this Th1-environment, CpG activated NK cells have an increased lytic activity [223]. The effects on APC function seem to differ for DC and MØ. In vitro CpG-ODN cause a decrease in the synthesis of MHC II molecules by peritoneal MØ resulting in down regulation of antigen presentation [32]. However treatment of DC with CpG-ODN induces maturation, increased expression of both MHC II and co-stimulatory molecules and a transient increase in antigen processing followed by a decline [100]. Stimulation of B cells with CpG-ODN results in proliferation [117], IL-6, IL-10 and IgM secretion [234], enhanced expression of activation markers including CD69, CD86, IL-2R (CD25) and IFN-γR [134], and of MHCII in pigs [208]. Proliferation has been observed in mice [117], humans [83], cattle [23] and pigs [208].

Not all CpG-ODN have immunostimulatory properties and some may even have neutralizing effects when co-administered with stimulating CpG-ODN. This is the case with CpG-dinucleotide sequences in the genome of adenoviruses in which the CpG-dinucleotide is preceded by a C and/or followed by a G [118].

Horner et al. [90] demonstrated in mice that CpG given intranasally together with ß-galactosidase was as good as CT for induction of antigen-specific mucosal IgA responses (Th2-responses), whereas a systemic Th1-response was obtained. Several other studies using other antigens confirmed the adjuvant effect following intranasal administration leading to a Th1-biased immune response, but did not always demonstrate the strong IgA response seen by Horner et al. [137]. More recently, the adjuvant effect was also demonstrated for oral immunization [69] and intravaginal immunization [7]. One of the problems with the oral immunization is that the CpG-ODN are rapidly degraded in the gastrointestinal tract. Therefore Wang et al. [220] synthesized oligonucleotides consisting of a novel 3'-3'-linked structure and synthetic stimulatory motifs and called these second-generation immunomodulatory oligonucleotides or IMO. These IMO were more stable in the murine gastrointestinal tract resulting in a stronger immune response, making them a potentially interesting intestinal adjuvant. Table V gives an overview of CpG-ODN with immunostimulatory properties for domestic animals.

2.4. Cytokine or chemokine receptor binding adjuvants

Several cytokines and chemokines have been tested in mice as mucosal adjuvants such as IL-1, IL-5, IL-6, IL-12, IL-15, RANTES, lymphotactin, MCP-1 (monocyte chemoattractant protein 1), MIP-1α, (macrophage inflammatory protein 1α), MIP-1ß, MIP-2, HNP-1 (human neutrophil peptide 1), HNP-2, and HNP-3 [188]. They can be administered as a soluble protein or as a gene encoded by a DNA vaccine. Limitation of the protein form is that high concentrations are needed due to their short half-life time. This problem can be overcome by administering it as a DNA vaccine. DNA vaccination will result in the production of

Table V. Immunostimulatory CpG-ODN motifs for several animal species.

Species	Name	ODN sequence 5'-3'	Reference
Sheep	2007	tcgtcgttttgtcgttttgtcgtt	[164]
	2135	tcgtcgtttgtcgttttgtcgtt	[164]
	2216	ggGGGACGATCGTCgggggG	[140]
	Immunomer 6	5'-Tctgargttct-l-tcttgragtct-5'	[107]
	Immunomer 7	5'-tctgtrgttct-l-tcttgrtgtct-5'	[107]
Goat	2135	tcgtcgtttgtcgttttgtcgtt	[164]
	Immunomer 6	5'-Tctgargttct-l-tcttgragtct-5'	[107]
	Immunomer 7	5'-tctgtrgttct-l-tcttgrtgtct-5'	[107]
	Oligo 4	ctatctgtcgttctctgt	[107]
Horse	2135	tcgtcgtttgtcgttttgtcgtt	[164]
Pig	2007	tcgtcgttgtcgttttgtcgtt	[164, 236]
	D19	ggTGCATCGATGCAGggggg	[106]
	D25	ggtgcatcgatgcagggggg	[106]
	D32	ggTGCGTCGACGCAGggggg	[106]
	A2	gctagacgttagcgt	[112, 204]
	H	Ttttcaattcgaagatgaat	[51]
	I	attcatcttcgaattgaaaa	[51]
	2216	ggGGGACGATCGTCgggggG	[51]
	PCV-2/2	acttcggcagcggcagcacc	[84]
	PCV-2/3	accctgtaacgtttgtcaga	[8]
	PCV-2/4	ctgtgtgatcgatcgatatccatt	[84]
	PCV-2/5	gttttcgaacgcagcgccga	[84]
	Immunomer 6	5'-Tctgargttct-l-tcttgragtct-5'	[107]
	Immunomer 7	5'-tctgtrgttct-l-tcttgrtgtct-5'	[107]
Dog	No. 2	GGtgcatcgatgcagGGGGG	[121]
	1968	tcgtcgctgttgtcgtttctt	[224]
	2005	tcgtcgttgtcgttgtcgtt	[224]
	2006	tcgtcgttttgtcgttttgtcgtt	[224]
	2007	tcgtcgttgtcgttttgtcgtt	[224]
	2012	tgtcgtttgtcgtttgtcgtt	[224]
	2014	tgtcgttgtcgttgtcgtt	[224]
Cat	n.i.	gttcttcggggcgttctttttaagaacgcccc	[125]
	n.i.	gaagaacgttttccaatgatttttcattggaaaac	[125]
	1968	tcgtcgctgttgtcgtttctt	[224]
	2005	tcgtcgttgtcgttgtcgtt	[224]
	2006	tcgtcgttttgtcgttttgtcgtt	[224]
	2007	tcgtcgttgtcgttttgtcgtt	[224]
	2012	tgtcgtttgtcgtttgtcgtt	[224]
	2014	tgtcgttgtcgttgtcgtt	[224]
Cattle	2006	tcgtcgttttgtcgttttgtcgtt	[162]
	2007	tcgtcgttgtcgttttgtcgtt	[162]
	2059	tcgtcgttttgtcgtttgtcgtt	[240]
	2135	tcgtcgtttgtcgttttgtcgtt	[162]
	2216	ggGGGACGATCGTCgggggG	[140]
Chicken	2006	tcgtcgttttgtcgttttgtcgtt	[229]
	2007	tcgtcgttgtcgttttgtcgtt	[79]
	#1	tcgatcgacgttgagggggg	[85]
	#17	gtcgttgtcgttgtcgtt	[85]
	n.i.	gctagacgttagcgt	[218]
	n.i.	tccatgacgttcctgacgtt	[221]
Rabbit	2000	tccatgacgttcctgcagttcctgacgtt	[164]
	2007	tcgtcgttgtcgttttgtcgtt	[95]
	ISS	tgactgtgaacgttcgagatga	[196]

l = glycerol linker in immunomers; r = 1-(2'-deoxy-β-D-ribofuranosyl)-2-oxo-7-deaza-8-methyl-purine; n.i. = not indicated; small letters = phosphorothionate binding; CAPITALS = phosphodiester binding.

the cytokine/chemokine over a period of weeks to months. However, mucosal application of DNA vaccines needs further optimization in large animals in order to be a feasible method. Due to these limitations, the number of cytokines/chemokines tested as mucosal adjuvants in veterinary species is still very limited. One of the rare examples is the use of IL-1 in rabbits. IL-1 enhances APC activity and IgG and IgA production by B cells. In rabbits, IL-1 was shown to increase the immune response against *Streptococcus sobrinus* when given together at the palatine tonsils [115].

3. ANTIGEN-PRESENTING CELL TARGETING MUCOSAL ADJUVANTS

Besides the non-particulated mucosal adjuvants that mainly exert their effect via binding to a receptor, there are several particulated adjuvants, which have as an important property that they transport the antigen through the mucosal barrier towards AGP cells. Most of these adjuvants are reviewed in the article on mucosal delivery of vaccines [70]. Here, we only highlight the saponin-based mucosal adjuvants used in domestic animals.

The rough extract derived from the bark of a Chilean tree *Quillaja saponaria Molina* containing a mixture of triterpenoid glycosides is called saponins. This extract has adjuvant effects, but its toxicity is too high. Quil A is a part of this rough extract with less toxicity but still containing a mixture of saponins with strong adjuvant activity [42]. Quil A has been used in rectal immunizations of cats with FIV antigens [62] and aerosol immunization of pigs with killed *Actinobacillus pleuropneumoniae* [128]. Whereas the aerosol immunization in pigs induced a marked IgA response in serum, broncheoalveolar and nasal fluids, the rectal immunization in cats induced a weak antibody response but a strong lymphocyte proliferation. Fractionation of Quil A resulted in 28 fractions of which QS21 and ISCOPREP 703 have been extensively analysed [110]. QS21, one of the best-tested fractions, has been shown to induce IL-2 and IFN-γ, both Th1 cytokines [15, 176].

Several saponin fractions have been used to prepare ISCOM [147]. ISCOM are 40 nm large particles made up of saponins (Quil A), lipids and antigen held together by hydrophobic interactions between these three components. The compulsory elements to form the ISCOM structure are cholesterol and saponin. Cholesterol is the ligand that binds to saponin forming 12 nm rings. These rings are fixed together by lipids (phosphatidylcholine) to form the spherical nanoparticles. Hydrophobic or amphiphatic antigens can be easily incorporated into this complex. The amphiphatic nature of the antigen is necessary for the interaction with the Quil A/cholesterol matrix. Using phosphatidylcholine enables incorporating a greater variety of antigens. So, ISCOM are a versatile and flexible delivery system in which all molecules/components are exchangeable apart from cholesterol and one saponin. The first ISCOM used Quil A as saponins [147]. Later on, more purified fractions of Quil A, such as ISCOPREP™, were used to form these complexes [172]. ISCOM are preferentially targeting DC [182]. However, all antigen-presenting cells take up antigen from ISCOM more efficiently [216]. Following contact with the ISCOM, the expression of MHC class II molecules is upregulated [14] and pro-inflammatory cytokines and IL-12 are released enhancing Th1-like responses [217]. However, Th2 cytokines can also be induced [87]. Besides CD4+ T cells, also CD8+ cytotoxic T cells become activated [194]. Nevertheless, the immunological properties of ISCOM can easily be varied since a large variety of saponins with different properties exists [172] and since other hydrophobic or amphiphatic molecules with immunomodulatory properties can be integrated into the ISCOM.

Whereas most studies use the systemic route for administering ISCOM, it has been demonstrated that mucosal applications can

be effective. Studies with CT as the antigen have demonstrated the potential to use ISCOM for oral and nasal administration. This was confirmed for the nasal route using influenza [129], RSV [92] and envelope proteins of herpes simplex [146] and for the oral route using rabies virus ISCOM [34] or ovalbumin [145]. Today, mucosal administration in domestic animals is still limited. Intranasal immunization has been performed in pigs against rotavirus [80], in sheep against influenza hemagglutinin [39] and in dogs against *Echinococcus granulosus* surface antigens or ovalbumin [26]. In all three studies, an enhanced mucosal IgA response was observed. However, protection was either not obtained [80] or not evaluated [26]. Oral administration has been analyzed in pigs [96, 153] and sheep [209] with rotavirus as the antigen and it resulted in good IgA responses with partial protection.

ISCOPREP™ is also present in the ISCOMATRIX® adjuvant [148]. This is an adjuvant that is identical to the ISCOM except that it does not contain antigen. This adjuvant can be mixed with antigens and when applied at a mucosa should have some of the advantages of ISCOM such as the preferential targeting of antigen presenting cells. The ISCOMATRIX has been analyzed in oral [209] and intranasal immunization in sheep [39] and in oral immunization in pigs [96] and it induced good mucosal IgA responses. However, the response obtained in sheep differed from that of an ISCOM vaccination in that the ISCOMATRIX induced a Th2-like response, whereas the ISCOM vaccine induced a mixed Th1-/Th2-like response.

4. MODULATION OF A SYSTEMIC IMMUNE RESPONSE TOWARDS A MUCOSAL ONE

Parenteral immunization with an antigen generally induces a systemic and not a mucosal immune response. However, it has been demonstrated that a mucosal immune response can be induced by a systemic immunization using an appropriate immunomodulator as adjuvant. Two adjuvants for which priming of the mucosal immune system has been observed when applied systemically are the steroid hormone $1\alpha,25(OH)_2D_3$ (Vitamin D3) and CT.

4.1. Vitamin D3 (Vit D3)

It was first demonstrated in mice that an intramuscular, subcutaneous [45, 59] or intradermal [60] immunization with a microbial antigen and aluminium hydroxide as the adjuvant induces a mucosal immune response when $1\alpha,25(OH)_2D_3$ (Vit D3, calcitriol), the active metabolite of vitamin D, is added as an additional immunomodulator. Mice show enhanced antigen-specific IgG and IgA in serum and all tested mucosal secretions (tears, oral, vaginal and colorectal secretions) [45] and increased numbers of IgA and IgG secreting cells (ASC) in systemic (the local draining lymph node and spleen) and mucosa-associated lymphoid tissues (Peyer's patches, mesenteric lymph nodes). Vit D3 modulated the production of cytokines in the local draining lymph node. Indeed, when cells from the local draining lymph node of a mouse treated with Vit D3 were stimulated in vitro with anti-CD3ε, an enhanced production of IL-4, IL-5 and IL-10 (Th2-cytokines) and a reduced production of IL-2 and IFN-γ (Th1-cytokines) was seen [45]. So, in the local draining lymph node, a switch towards a Th2-cytokine profile occurred, as is necessary for an IgA response. Furthermore, migration of antigen-pulsed dendritic cells from the local draining lymph nodes towards the Peyer's patches was seen, where the activation and differentiation of antigen-specific B cells for a mucosal immune response is initiated [59, 60].

Vit D3 exerts its effects on the immune system by binding to a nuclear receptor (nVDR), which is present in activated Th, cytotoxic T cells (CTL) as well as in activated B cells [11, 141, 163]. Via this receptor, Vit D3 modulates the production of

cytokines [123] by reducing the transcription and secretion of among others IFN-γ [33], IL-2 [5], IL-8 [82], IL-12 [43] and GM-CSF [198]. IL-12 is the most important cytokine for promoting differentiation of Th0 cells towards Th1 cells. Furthermore, Vit D3 favours the induction of $CD4^{+}CD25^{+}$ regulatory T cells and enhances the production of Th2 cytokines such as IL-4 and IL-10, which in turn inhibits Th1 responses [45]. Vit D3 is also known to stimulate TGF-ß [223] that is involved in mucosal immunity, isotype-switching towards IgA as well as IgG2b [124]. So the steroid hormone can be classified as a Th2-modulating adjuvant.

In pigs, intramuscular injection of human serum albumin (HSA) in incomplete Freund adjuvant (IFA) with 2 µg Vit D3 transiently enhanced the antigen-specific serum IgA and IgM responses, the IgA titres in saliva, feces and nasal secretions and the antigen-specific IgA and IgG ASC in the local draining lymph nodes [204]. Furthermore, slightly higher numbers of HSA-specific IgA ASC appeared in the Peyer's patches (PP) of the Vit D3 injected pigs, indicating priming of the gut-associated lymphoid tissue [207]. Thus, when suckling piglets were intramuscularly immunized with fimbriae (0.1 mg) of F4+ ETEC strains in IFA supplemented with Vit D3, these piglets showed a secondary F4-specific serum IgA response and a reduced $F4^{+}$-ETEC fecal excretion upon challenge 4 days post-weaning [206].

In turkeys, IM immunization with the recombinant major outer membrane protein (rMOMP) of *Chlamydophila psittaci* in IFA with 2 µg Vit D3 also resulted in an enhanced serum IgA response, but not in an increased protection against a challenge infection [214]. In humans, no enhanced IgA response could be observed after IM injection of 1µg Vit D3 co-administered with an influenza vaccine [120].

4.2. Cholera toxin

Transcutaneous immunization by topical administration of CT to the skin has been demonstrated to induce a systemic antibody response against both itself and co-administered proteins in mice [74], humans [76], sheep, cats and dogs [81]. In mice [75] and humans [76] it was shown that this immunization route also induces a mucosal immune response. In mice, even comparable IgA titers were obtained as following an oral immunization [102]. In sheep transcutaneous immunization could induce a systemic immune response but had little or no effect on the mucosa-associated lymphoid tissue [28]. Indeed, the serum IgG1, IgG2 and IgA responses against a co-administered antigen as well as the IgG1 and IgG2 responses in lung wash fluid were lower and the IgA response was absent in comparison with the response following intramuscular injection. Antibodies against the adjuvant were also only detected in serum (IgG1, IgG2, IgA) and lung wash fluid (IgG1), but not in other mucosal secretions. So, extended research is needed to determine the usefulness of the transcutaneous route for priming of the mucosal immune system in domestic animals.

5. GENERAL CONCLUSIONS

The advantage of mucosal vaccines for prevention of numerous infectious diseases is indisputable. Nevertheless, the number of available mucosal vaccines is still very limited. This is due to important difficulties that are encountered by using the mucosal route: (1) the antigen should pass the mucosal barrier in sufficient amount, (2) mucosal tolerance mechanisms should be overcome, (3) protective immune mechanisms should be activated and (4) this should ideally occur with minimal/or no influence on mucosal functionality. Mucosal adjuvants are crucial in reaching these goals. Recent insights that probably all non-particulated mucosal adjuvants activate/modulate mucosal immunity via receptor-mediated mechanisms in which activation of innate immunity is an important step will help in developing new vaccination strategies. In most studies only one adjuvant is used. An ideal mucosal

adjuvant should carry the antigen through the epithelial layer without disturbing this layer, conserve the native conformation of the antigen, attract antigen presenting cells allowing the antigen to be taken up in optimal circumstances, enhance its presentation and activate immune mechanisms resulting in a protective IgA response. This ideal adjuvant does not exist, but it will be one of the challenges to come close to this ideal picture. Hereto, combining adjuvants acting via different mechanisms will be inevitable. So, increased efforts are needed to elucidate the mechanisms of action of adjuvants and to find new mucosal adjuvants differing in action mechanism and effects.

Another challenge for veterinary medicine is the different animal species. Results in mice are often not extrapolatable to other species and this also accounts for data obtained in one domestic animal species. But not only species differences but also the antigen can influence the effect of the adjuvant. Therefore, adjuvanticity (and adverse effects) remains to be analyzed in the target species using the intended antigen(s) and the intended route of administration. As can be learned from this review, most data on veterinary mucosal adjuvants have been obtained from experiments in swine and sheep, but even in these species information is too limited. There is an urgent need for more studies in the different domestic animal species.

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