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Review

Advances in saponin-based adjuvants

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ABSTRACT

Saponins are natural glycosides of steroid or triterpene which exhibited many different biological and pharmacological activities. Notably, saponins can also activate the mammalian immune system, which have led to significant interest in their potential as vaccine adjuvants. The most widely used saponin-based adjuvants are Quil A and its derivatives QS-21, isolated from the bark of Q Molina, which have been evaluated in numerous clinical trials. Their unique capacity to stimulate both the Th1 immune response and the production of cytotoxic T-lymphocytes (CTLs) against exogenous antigens makes them ideal for use in subunit vaccines and vaccines directed against intracellular pathogens as well as for therapeutic cancer vaccines. However, Q saponins have serious drawbacks such as high toxicity, undesirable haemolytic effect and instability in aqueous phase, which limits their use as adjuvant in vaccination. It has driven much research for saponin-based adjuvant from other kinds of natural products. This review will summarize the current advances concerning adjuvant effects of different kinds of saponins. The structure–activity relationship of saponin adjuvants will also be discussed in the light of recent findings. It is hoped that the information collated here will provide the reader with information regarding the adjuvant potential applications of saponins and stimulate further research into these compounds.

Contents

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1. Introduction

Vaccination remains the most cost-effective biomedical approach for the control and prevention of infectious diseases. New generations of vaccines, particularly those based on purified recombinant proteins, synthetic peptides and plasmid DNA, are likely to be less reactogenic and immunogenic than traditional vaccines [1]. Therefore, there is an urgent need for the development of a new and improved vaccine adjuvant [2–4].

Immunological adjuvants were originally described by Ramon [5] as "substances used in combination with a specific antigen that produce more immunity than the antigen alone". Nowadays, the increase of knowledge in the immunology field is leading to a more rational vaccine design aiming to elicit a specific, protective, and long-lasting immunity after vaccination. A rational selection of adjuvants can be driven by the nature of the immune response required (Th1, Th2, antibodies, and CTLs) [6]. In case of toxins, a good humoral immune response is required; however, in case of intracellular bacteria the cell mediated response, mainly cytotoxic T cells and Th1 cells, is the most important. In case of viral infection both humoral and cellular response are fundamental to control the infection. After this, a strategy to elicit the suitable type of immunity should be planned [1].

Adjuvants have significant effects on the nature of the immune responses, and can tilt the immune system in favor to Th1 or Th2 type response [7]. The versatile adjuvant that can induce the appropriate type of immune response to antigens for producing optimal protection against each type of infection would be highly desirable in the vaccine industry [8]. Thus, one of the main challenges for the development of adjuvants is to learn how to selectively induce the appropriate type of immune response against each type of infection. On the other hand, suitable adjuvant should be low toxicity and side effects allowing their license to be used in human or veterinary vaccine formations [9].

While several hundred different adjuvants including mineral salts, microorganism-derived adjuvants, emulsions, cytokines, polysaccharides, nucleic acid-based adjuvants have been tested for the research or usage in novel vaccine design over the last few decades, the vast majority have not been successful in being approved for human use, with limitations including lack of efficacy, unacceptable local or systemic toxicity, difficulty of manufacture, poor stability, and prohibitive cost [9–11]. Meanwhile, exacerbating sub-clinical autoimmune diseases in addition to fever and erosion at the local injected lesion induced by nature of adjuvants has limited their clinical use [12]. For example, freund's complete adjuvant (FCA) causes inflammation, induration or necrosis with disseminated granulomas being reported in the lungs, liver, kidneys, heart, lymph nodes and skeletal muscles of rabbits or rats [13]. For this reason, until recently, only aluminum-based mineral salts (alum) remain the most widely used adjuvant in human vaccines [14]. Alum was the first adjuvants discovered in 1926 [15] and has a good safety record. However, alum is a weak adjuvant for antibody induction to protein subunits and a poor adjuvant for cell-mediated immunity [16]. Moreover, alum can induce immunoglobulin E antibody responses, which is associated with some allergic reactions in human subjects [17]. In addition, alum mainly induces the increases of IgG1, instead of IgG2a and IgG2b, indicating mainly Th2 immunity induced in mouse medol [18]. MF59, consisting of emulsified squalene, was the only adjuvant licensed for human use in addition to alum [19–21]. Similarly, MF59 was also reported to favor Th2 immune response [22].

Saponins are natural glycosides of steroid or triterpene which exhibited many different biological and pharmacological actions such as immunomodulatory, antitumor, antiinflammatory, molluscicidal, antiviral, antifungal, hypoglycemic, hypocholesterolemic [23,24]. Saponins have a diverse range of properties, which include sweetness, bitterness [25–27], foaming, emulsifying [28], and haemolytic properties [23,29]. Saponins have wide applications in beverages and confectionery, as well as in cosmetics [30,31] and pharmaceutical products [23]. They are believed to form the main constituents of many plant drugs and folk medicines, and are considered responsible for numerous pharmacological properties [32]. Notably, saponins can activate the mammalian immune system, which has led to significant interest in their potential as vaccine adjuvants [33]. The lead candidate saponin adjuvants are Quil A and its derivatives QS-21 [34], which have been included as adjuvant in vaccine formulations against HIV in guinea pig and human [35–37], cancer in human [7,38], malaria in Aotus monkeys and mice [39], respiratory syncytial virus [40], cytomegalovirus [41], *T* [42], and visceral leishmaniasis in mice [43–45]. The unique capacity of Quil A and QS-21 to stimulate both the Th1 immune response and the production of CTLs against exogenous antigens makes them ideal for use in subunit vaccines and vaccines directed against intracellular pathogens as well as for therapeutic cancer vaccines [46,47]. However, *Q* saponins have serious drawbacks such as high toxicity, undesirable haemolytic effect and unstability in aqueous phase, which limits their use as adjuvant in human vaccination [48–52]. Meanwhile, the overexploitation of the *Q.* bark has caused important ecological damage and a considerable shortage of the available supplies [53]. Therefore, many saponins from other kinds of natural product have been screened and shown to possess the adjuvant activities during the last decade. There have been several reviews in recent years of published reports about saponin-based adjuvants [54,55]. Most of them, however, deal with the chemical structure and adjuvant activities of *Q* saponins. The purpose of the present review was to summarize adjuvant effects of different kinds of saponins and their structure–function relationship and try to understand the molecular mechanism of their activity, as far as the available literature permits. It is hoped that the information collated here will provide the reader with information regarding the adjuvant potential applications of saponins and stimulate further research into these compounds.

2. Saponins with the adjuvant properties

2.1. Q A fi

The literature dealing with the adjuvant properties of saponins almost exclusively focuses on the extracts of *Q* Molina [54,55]. *Q.* extract as adjuvants was first described in the 1930s, and later used to improve a foot-and-mouth disease vaccine. In 1978, Dalsgaard first obtained an enriched mixture of saponins (Quil A) from this extract, and found Quil A stimulated both humoral and cellular immunity as well as to induce differential antibody isotypes [56]. Quil A had been used commercially in a veterinary foot-and-mouth disease vaccine as well as in some experimental vaccines [57–59]. However, its toxicity precludes expanded use in human vaccines.

Since Quil A was a heterogenous mixture of saponins when analyzed using RP-HPLC (Fig. 1) [60], it was possible that the various components may produce different levels of adjuvanticity and toxicity that could be exploited to produce useful adjuvants for human vaccines. The purification and structure–function relationships of adjuvant-active saponins have been the subject of interest. The first detailed immunological study of saponin fractions isolated by RP-HPLC using bovine serum albumin as the antigen showed that 10 of the fractions including the major peaks QS-7, QS-17, QS-18, and QS-21 had adjuvant activity [61]. While the adjuvant and physical properties of these saponins are similar, their toxicity varies considerably. QS-18 is lethal in mice at doses as low as 25 μg, while QS-21 shows only some lethality at 500 μg [60]. These results and others

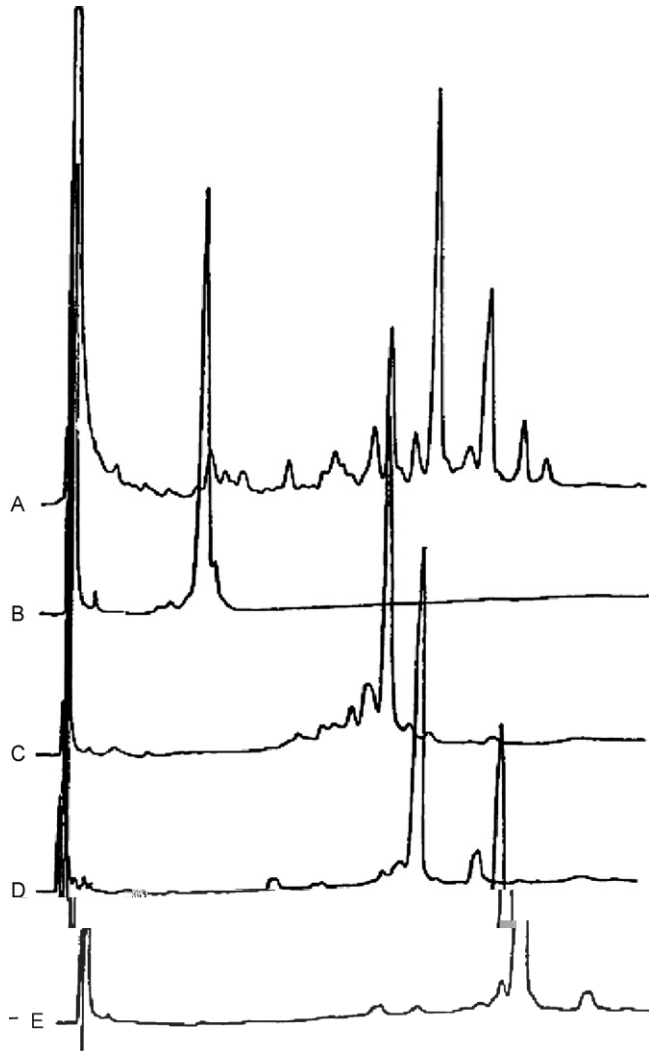

Fig. 1. HPLC chromatograms of an aqueous extract of *Q.* bark treated by ultrafiltration (A), saponin QS-7 (B), saponin QS-17 (C), saponin QS-18 (D) and saponin QS-21 (E). Gradient was 30–40% 0.1% TFA/acetonitrile/30 min, 40%/15 min at a flow rate of 1 ml/min. A total of 100 μg of purified saponin or 200 μg bark extract (dry weight) was used per injection [60].

led to the development of QS-21 as an effective adjuvant with a recombinant subunit vaccine against feline leukemia virus (FeLV) which is commercially available [60,62]. The addition of QS-21 to a denatured recombinant FeLV-A envelope glycoprotein expressed in *E* resulted in the protection of cats against a challenge with infectious FeLV. In effect, several authors have shown that *Q* saponins (including QS-21) stimulated the production of CTLs and induces Th1 cytokines (IL-2 and IFN-γ

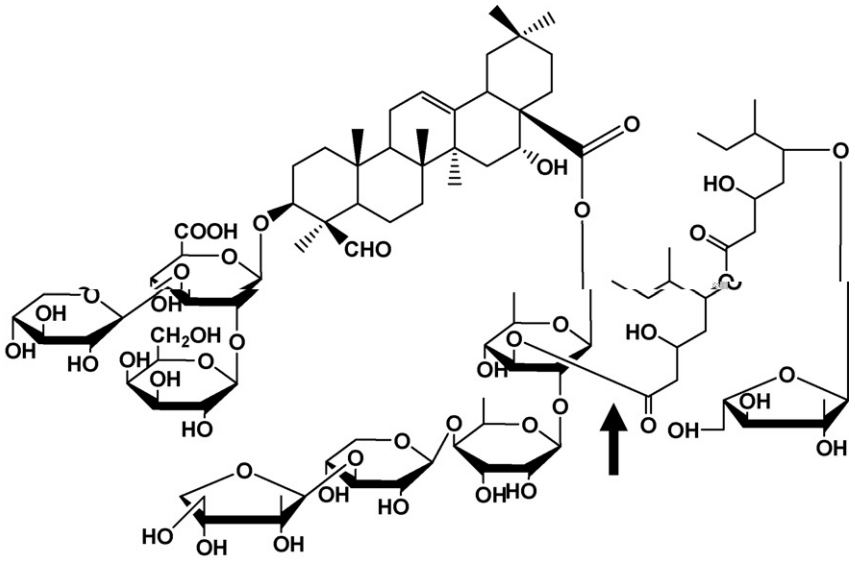

Fig. 2. Chemical structure of QS-21 from Q . The arrows point the local of the acyl side chain responsible for Th1 type responses, CTL stimulation and toxicity.

GPI-0100 (semi-synthetic saponin from Quil A) were superior to QS-21 alone for induction of IgM and IgG antibodies against MUC1 and/or GD3 and their corresponding IFN-γ release and DTH against KLH.

2.2. G

Ginseng saponins (ginsenosides) are believed to be the active substances in the root of *P* . Ginseng extract significantly increased the blood polymorphonuclear leukocyte phagocytosis and intracellular killing [83], and lymphocyte proliferation [84], and IFN-γ and TNF production [85]. Ginseng extract could also enhance specific antibody response against diphtheric toxoids in mice [86] and increase IgG and IgM antibody responses in mice immunized with sheep red blood cells (SRBC) [87]. Rivera et al. [88] reported that ginseng extract potentiated the antibody response to porcine parvovirus (PPV) in guinea pigs. Adjuvant effect of ginsenoside on bacterial antigens can be obtained by evaluating the enhancing effect on vaccinating pigs against *E* infections [89]. Ginsenosides may induce Th1 and Th2 immune isotype, varying according to the antigen and the species [90]. Ginsenoside Rb_1 induced balanced Th1 or Th2 type of immunity of PPV vaccines [91], while ginsenoside Rg_1 enhanced Th2 lineage development from the naive $CD4^+$ T cell both by increasing Th2 specific cytokine secretion and by repressing Th1 specific cytokine production [92].

2.3. P

The roots of *P.* (Burk.) F. H. Chen has been used in traditional Chinese medicine for treatment of cardiovascular diseases, inflammation, different body pains, trauma, and internal and external bleeding due to injury [93]. Over 50 different saponins isolated from *P.* belong to the dammarane-type saponins, which are the main bioactive principles in this drug and account for 12% of the total root [94]. These saponins include ginsenosides, notoginsenosides and gypenosides and are composed of a protopanaxadiol and of protopanaxatriol glycosides [95,96]. Although some of its chemical constituents were similar to those present in two other well-known species in the same plant genus—*P.* and *P.* , notoginsenosides are the inherent constituents in *P.* . *P.* saponins were shown to display a slight haemolytic effect and enhance significantly a specific antibody and cellular immune response against OVA in mice [97]. From this extract, Sun et al. isolated eleven immunological adjuvant-active saponins, notoginsenosides K, R_1, R_2, R_4 and U, as well as ginsenosides Rb_1, Rd, Re, Rg_1, Rh_1, Rh_4 [98–101]. Yoshikawa et al. also examined the adjuvant effect of eleven notoginsenosides (A, C, D, G–N), two ginsenosides (Rb_1, Rg_1), and five quinquenosides (I–V) from *P.* g and *P.* , and found that notoginsenosides D, G, H and K could increase the sera IgG level in OVA-immunized mice, and notoginsenosides A, C, I, L, and N and quiquenosides III–V tended to show this activity [102]. In order to further elucidate the mechanism responsible for adjuvant activity of *P.* saponin, ginsenoside Rd was evaluated for inducing Th1 or Th2 immune responses in mice against OVA, and was proved to increase a antigen-specific antibody and cellular response and elicit a Th1 and Th2 immune response by regulating production and gene expression of Th1 cytokines and Th2 cytokines [103].

2.4. P *fl*

The saponins from the root of *P.* *fl* increased a specific antibody and cellular response against OVA in mice, and could be a promising balanced Th1 and Th2 directing immunological adjuvants [104]. The further purification of this extract afforded four adjuvant-active saponins, platycodin D, D2, D3, and platycoside E. These four saponins all significantly enhanced the Con A-, LPS-, and OVA-induced splenocyte proliferation, serum OVA-specific IgG, IgG1, IgG2a, and IgG2b antibody titers in the immunized mice. Platycodin D and D2 were found to promote the mRNA expression of cytokines IL-2, IFN-γ, IL-4, and IL-10 and transcription factors T-bet and GATA-3 in Con A-stimulated mice splenocytes, suggesting that these saponins could simultaneously elicited a Th1 and Th2 immune response by regulating gene expression of Th1/Th2 cytokines and transcription factors [105,106]. Platycodin D and D2 .atyTDa0(s) 3yromisi4(e)-10(d)-160.3(r)-44e rr3ant-acti3veroing57 -2(hep-37

from the root of *P.* increased specific antibody levels in mice immunized with ovalbumin and hens immunized with rotavirus. In mice, there was a preferential increase of the IgG2a subclass, high IL-2 and IFN-γ production. These two fractions were less toxic than Quil A at the same dose [111]. Katselis et al. [112] evaluated the immunological activity of eight pure saponins from the root of *P.* in mouse models with OVA. Among eight saponins, PS1, onjisaponin A and onjisaponin B significantly increased the IgG2a subclass antibody and IL-2 production. Among the hot water extracts from 267 different types of Chinese and Japanese medicinal plants screened for the adjuvant activity, the root of *P.* contained the most potent adjuvants when combined with nasal influenza or diphtheria–pertussis–tetanus (DPT) vaccine, and its active substances were identified as onjisaponins A, E–G. These four onjisaponins provided safe and potent adjuvants for intranasal inoculation of influenza HA and DPT vaccines [113]. We have recently isolated six adjuvant-active saponins, onjisaponins A, B, polygalasaponin XXVII, XXXII, and tenuifolisaponin A, B from the root of *P.* [114]. Tenuifolisaponin A and B significantly enhanced ORF2-specific IgG, IgG1 and IgG2b antibody titers in the mice immunized with porcine circovirus type 2 ORF2-based DNA vaccine by up-regulating expressions of cytokine IL-2, 4, 10 and INF-γ mRNA [115].

2.6. O

The other adjuvant-active saponins isolated in recent years were shown in Table 1.

3. Structure–activity relationship of saponins with the adjuvant properties

Saponins are present in a wide range of plant species and in some marine organisms [147]. Saponins are complex molecules consisting of non-sugar aglycone coupled to sugar chain units [148]. Saponins are often subdivided into two main classes, the triterpenoid and the steroid saponins [149], which are both derived from the 30 carbon atoms containing precursor oxidosqualene [150]. The difference between the two classes lies in the fact that the steroid saponins have three methyl groups removed (i.e. they are molecules with 27 C-atoms), whereas in the triterpenoid saponins all 30 C-atoms are retained. Saponins have one or more linear or branched sugar chains containing glucose, galactose, glucuronic acid, xylose, rhamnose or methylpentose, attached to the aglycone via a glycosidic ether or ester link. In some saponins, the presence of acylated sugars has also been detected. According to the number of sugar chains attached to the aglycone, the saponins can be monodesmosidic saponins (with a single sugar chain), or bidesmosidic saponins (with two sugar chains). In the monodesmosidic saponins, the sugar chain is typically attached by a glycosidic ether linkage at the C-3 of the aglycone. In addition to the C-3 linked sugar chain, bidesmosidic saponins have a second sugar chain bound at C-28 (triterpene saponins) or at C-26 (steroid saponins) by an ester linkage. Because of the typical lability of esters, bidesmosidic saponins are readily converted into their monodesmosidic forms by mild hydrolysis. The bidesmosidic saponins may have potent biological and pharmacological activities in animals [151].

3.1. S

The adjuvanticity of saponin depends on its structure comprised of hydrophilic sugar side chains and hydrophobic aglycone backbone [29]. The adjuvant activity of saponins was also thought to be related to branched sugar chains or aldehyde groups [144] or to an acyl residue bearing the aglycone [63].

3.1.1. E

The adjuvant activity of saponins was thought to be related to aldehyde groups in the aglycones [47,144]. QS-21 derivatives that were modified at the carboxyl group on an anionic sugar, glucuronic

Table 1
Specification of the other adjuvant-active saponins isolated in recent years.

Species	Saponin type[a]	Features	Ref.
A saponins	3-MD, 3,28-BD	Slight haemolytic; promote OVA-specific splenocyte proliferation, and IgG, IgG1 and IgG2b antibody titers.	[116]
A saponins	3-MD, 3,28-BD		[117]
A	3-MD, 3,6 (25)-BD, 3,6,25-TD	Slight hemolytic; enhance OVA-specific splenocyte proliferation, and IgG, IgG1 and IgG2b antibody titers; promote the peripheral lymphocyte proliferation and serum antibody titer in chicken vaccinated with Newcastle disease vaccine; activate macrophages.	[118–120]
C saponins	3-MD, 3,28-BD	Mucosal adjuvant; potentiate specific IgG and IgA antibody responses to cholera toxin and OVA; increase mucosal permeability.	[121,122]
Escins	3-MD	Low toxicity; induce lower antibody responses to OVA than QS-21.	[29]
G saponins	3-MD	Slight haemolytic; enhance OVA-specific splenocyte proliferation, IgG, IgG1 and IgG2b antibody titers, and IL-12 production from lymphocytes and macrophages.	[123,124]
Gypenosides	3-MD, 3,21-BD	Slight haemolytic; increase OVA-specific splenocyte proliferation, IgG, IgG1 and IgG2b antibody levels, release of IL-2 from splenocyte and IL-1 from macrophages.	[125–127]
Jujubosides	3-MD	Less or no haemolytic; increase OVA-specific antibody response.	[128]
Kinmoonosides	3,28-BD	Activate T and B cells; enhance OVA-specific IgG, IgG1 IgG2a and IgG2b antibody levels.	[129,130]
Lablabosides	3,28-BD	Induce the production of large IgG1 and little IgG2a antibody response to ADV antigen.	[131,132]
Periandradulcins	3-MD	Slight haemolytic; increase IgG, IgG1, IgG2a and IgG2b response to FML antigen.	[67]
P (CP05)	3,28-BD	Induced an equally potent DTH to FML and IgG2b response, and a slight lower IgG, IgG2a and IgG3 titers compared with QS-21.	[133,134]
Q saponins	3-MD	Low toxicity; enhance bovine herpesvirus type 1 specific IgG, IgG1 and IgG2a antibody levels.	[135]
Saikosaponins	3-MD	Slight haemolytic; enhance OVA-specific splenocyte proliferation, and serum IgG, IgG1 and IgG2b antibody levels; increase level of IL-1 and cellular lysosomal enzyme, induce cytostatic activity and expression of Fc receptor and Ia antigen of macrophages.	[136–142]
Soyasaponins	3-MD, 3,22-BD	Little haemolytic; induce a stronger antibody response to OVA than QS-21, predominantly the IgG1 isotype but little IgG2a.	[143–145]
Taurosides	3-MD	Induce strong humoral immune responses to HIV-1 envelope glycoproteins rgp160 and rgp120.	[146]
Trigoneosides	3,26-BD	No haemolytic; increase OVA-specific antibody response.	[29].

[a] Described on the basis of the number and position of sugar chain(s). MD: monodesmoside; BM: bisdesmoside; TD: tridesmoside.

acid, by reacting the glucuronic acid carboxyl group of QS-21 with free amino groups, retained adjuvant activity for antibody stimulation, in contrast, QS-21 derivatives modified at an aldehyde on the triterpene did not show adjuvant activity for antibody stimulation or for induction of cytotoxic T-lymphocytes [47]. These results stress the pivotal role that the aldehyde group plays in the adjuvant properties of *Q* saponins. One possible mechanism involving the aldehyde might be the formation of a Schiff base with a free amino group on the surface of an immune cell target [152]. Palatnik de Sousa et al. [153] suggested that the proportion of conformational isomers of the triterpen-aldehyde is crucial for the integrity of the Th1 adjuvant response and it seems that axial aldehyde are more important in humoral immune response while equatorial aldehyde are more relevant to the cellular protective immune response. Although the similarities in the potency of the humoral response induced by the QS-21 and CP05–FML formulations are related to their similarities in composition and structure, the presence of the aldehyde group in QS-21 but not in CP05 could then explain the stronger induction of the typical Th1 IgG2a subtype in QS-21 [44]. The study on the saponins from the root of *P.* showed that a carboxyl function at position 23 instead of an aldehyde group can be just as effective for inducing adjuvant activity [113].

It was reported that the adjuvant activity of saponins also relates to the acyl residue bearing the aglycone [63]. In contrast to the majority of saponins from other species, *Q* saponins are acylated. The three most predominant saponins (QS-17, QS-18 and QS-21) are acylated at the 4-hydroxyl position of fucose with two linked 3,5-dihydroxy-6-methyloctanoic acids containing a glycosylation site at the 5-OH position of one of the acyl chains. It was proved that the remarkable property of *Q* saponins to stimulate CTL production against exogenous proteins appears to depend on their lipophilic acyl side chain [51]. Deacylated QS-18 and QS-

Another treatment of Riedel de Haen saponin (R) and Quil A with H_2SO_4 gave rise to their sapogenin fractions, which showed much slighter in vivo toxicity and reduced hemolytic potential without affecting their aldehyde and Th1 cellular immune response [153]. Thus, the presence of a monoterpene hydrophobic moiety could favor interactions between the saponin and membrane cholesterol promoting the haemolysis. On the other hand, the size of the attached glicidic chains also modulates the hemolytic activity of saponins. The saponin from *P.* , for instance, has a single sugar chain which is composed of two residues of glucuronic acid attached to carbon C-3 via oxygen. The hemolytic effect of this triterpenoid saponin was further reduced by removal of the glycosidic moiety [67]. The saponin and sapogenin fractions isolated from *B. fl* and Riedel De Haen or Quil A [153] also showed that the removal of the glicidic moiety abolished the undesirable hemolytic activity but still maintaining the adjuvant potential. Sun et al. reported that the number, the length and the location of sugar side chains, and the type of sugar in sugar moiety all could affect the haemolytic activity of protopanaxadiol-type and protopanaxatriol-type saponins [156,157]. The hemolytic potentials of platycodigenin-type saponins could decrease with the increased number of monosaccharide of the glycidic moieties at the C-3 of the aglycone [105]. Therefore, it is considered that not only the functional groups and the glycidic moieties themselves, but their overall conformation affects haemolytic activity of saponins.

4. Conclusion and perspective

This review has summarized the current development of saponin-based adjuvant for potential use in human or veterinary vaccines. More and more researches have focused on improved saponin-based adjuvant which may increase the effectiveness of current vaccines. There is, however, no universal ideal adjuvant for each vaccine and they should be adapted according to specific criteria to have the best balance between safety and efficacy. Until recently, Quil A remains most widely used in research or production for novel vaccines, and most studies on the mechanisms or structure–activity relationship of saponins are focused on Quil A or its purified compounds. However, several drawbacks of Quil A and its purified saponins have limited their clinical use in vaccine designs. The researches have paid more and more attentions to other kinds of saponins extracted from natural products or traditional Chinese medicines.

Several saponins have been showed to possess excellent adjuvant effect with relatively lower haemolysis, making them ideal adjuvant candidate for future use. Consequently, these kinds of adjuvants also need elaborated research to show their detailed mechanism of protection against different diseases and their specific enhancement of humoral or cellular immune response should be further confirmed in various novel vaccines for human or veterinary use. ISCOM™ and ISCOMATRIX™ combine the advantages of a particulate carrier system with the presence of an in-built adjuvant (Quil A) and consequently have been found to be more immunogenic than other colloidal systems such as liposomes and protein micelles [167]. Critically, formulation of ISCOM™ and ISCOMATRIX™ vaccines retained the adjuvant activity of the saponin, while removing its haemolytic activity, producing no toxicity. They also required substantially less antigen and adjuvant to induce immunity in the host than vaccination with simple mixtures of free antigen and saponins [33]. Many studies have demonstrated the ability of ISCOM™ and ISCOMATRIX™ vaccines to induce strong antigen-specific antibody and cell-mediated immune responses to a wide range of antigens in a number of animal models [168–170]. As such, the adjuvant properties of these other saponins deserve further investigations when incorporated into ISCOM™ and ISCOMATRIX™.

Although some saponins have a strong adjuvant activity when administered parenterally, in general, they have a low or no activity when delivered orally. This low oral activity may be due to (i) the relatively low doses of saponin delivered to the gastrointestinal tract, and (ii) the saponin's breakdown to non-absorbable byproducts by gastric and intestinal secretions and the intestinal flora [55]. Nevertheless, some ingested saponins (i.e. licorice) show significant pharmacological activity that indicates some gastrointestinal absorption occurs. Thus, how or what the routes of injection influence the adjuvant effect of saponin remains to be resolved. Other remaining questions include the levels of IgE generated with these saponin-based vaccines and the crucial question of longevity of the immune response that is generated.

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