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Review:

Adjuvant effects of saponins on animal immune responses*

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Abstract: Vaccines require optimal adjuvants including immunopotentiator and delivery systems to offer long term protection from infectious diseases in animals and man. Initially it was believed that adjuvants are responsible for promoting strong and sustainable antibody responses. Now it has been shown that adjuvants influence the isotype and avidity of antibody and also affect the properties of cell-mediated immunity. Mostly oil emulsions, lipopolysaccharides, polymers, saponins, liposomes, cytokines, ISCOMs (immunostimulating complexes), Freund's complete adjuvant, Freund's incomplete adjuvant, alums, bacterial toxins etc., are common adjuvants under investigation. Saponin based adjuvants have the ability to stimulate the cell mediated immune system as well as to enhance antibody production and have the advantage that only a low dose is needed for adjuvant activity. In the present study the importance of adjuvants, their role and the effect of saponin in immune system is reviewed.

Key words: Adjuvants, Saponins, Immunity, Immunostimulators, Steroids, Triterpenoid
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INTRODUCTION

Infectious diseases have always been a scourge to human beings and their companion animals. Many infectious diseases are ubiquitous and often fatal. Vaccination is the most efficacious and valuable tool in the prevention of infectious diseases, provided that they are administered prophylactically in anticipation of pathogen exposure. Vaccines mainly capitalize the immune system's ability to respond rapidly to micro-organisms after a second encounter and have been described as 'weapons of mass protection' (Cohen and Marshall, 2001; Curtiss, 2002). The goal of vaccination is to stimulate a strong, protective and long-lasting immune response to the administered antigen. For the achievement of these objectives, potent adjuvant and novel vaccine strategies are required to make the vaccine sufficiently immunogenic to initiate a potent immune response (Fearon, 1997; Bomford, 1998). Saponin based adjuvants have the unique ability to enhance immunity.

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IMPORTANCE OF VACCINE ADJUVANT

Adjuvants

The word adjuvant is derived from the Latin word adjuvare, which means help or aid or to enhance (Vogel, 1998). Singh and O'Hagan (2003) reviewed that immunological adjuvants were originally described by Ramon in 1924 as "substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone". This broad definition encompasses a very wide range of materials (Vogel and Powell, 1995). The first adjuvants were developed in the 1920's and a number of substances including chemicals, microbial components and mammalian proteins have been used to boost the immunity (Cox and Coulter, 1992; 1997).

Importance of adjuvants

These are a group of structurally heterogeneous compounds that enhance or modulate the immunogenicity of the poorly immunogenic vaccine proteins or peptides (Gupta *et al*., 1993; Vogel, 1995). Most vaccines traditionally consist of live attenuated pathogens, whole inactivated/killed organisms or

inactivated toxins containing many immunopotentiators essential for activating integrated protective immune responses. An adjuvant can be used for increasing the immunogenicity of poor antigen, improving the efficacy of vaccine in new born and reducing the amount of antigen or the number of immunizations (McElrath, 1995). Compared to injection of antigen alone, injection of antigen plus an adjuvant generally permits use of a much smaller quantity of the antigen and greatly enhances the antibody titer (Kaeberle, 1986).

Roles of adjuvant in modulation of immune responses

Adjuvants can be used to improve the immune response to vaccine antigens for several different purposes, including: (1) increasing the immunogenicity of weak antigens; (2) enhancing the speed and duration of the immune response; (3) modulating antibody avidity, specificity, isotype or subclass distribution; (4) stimulating cell mediated immunity; (5) promoting the induction of mucosal immunity; (6) enhancing immune responses in immunologically immature or senescent individuals; (7) decreasing the dose of antigen in the vaccine to reduce costs or (8) helping to overcome antigen competition in combination vaccines (Singh and O′Hagan, 2003). Adjuvants were initially thought of as agents capable of promoting and sustaining antibody response. However, new evidence has shown that adjuvants influence the titer, duration, isotype, and avidity of antibody, and affect the properties of cell-mediated immunity (Hunter *et al*., 1995).

Classification of adjuvants

Different scientists used different criteria for classifying the adjuvants. Cox and Coulter (1992) classified adjuvants into particulate and non-particulate groups. According to Vogel (1998) adjuvants can be classified according to their sources, mechanism of action or physiochemical properties. Edelman [1997; reviewed by Allison and Byars (1991)] classified adjuvants into three groups: (1) immunostimulatory adjuvants, (2) carrier adjuvants and (3) vehicle adjuvants. Adjuvants are also classified: (1) according to their route of administration like mucosal or parental; (2) divided into alum salts and other mineral adjuvants, tensoactive agents, bacterial derivatives, vehicles and slow release materials or cytokines (Byars and Allison, 1990); (3) divided into the groups: gel-based adjuvants, tensoactive agents, bacterial products, oil emulsions, particulate adjuvants, fusion proteins or lipopeptides (Jennings *et al*., 1998).

Most common adjuvants

Mostly aluminum or oil adjuvants are used in vaccine, but these chemical adjuvants have many disadvantages, such as side effects, strong local stimulation and carcinogenesis, together with complicated preparations or failure to increase immunogenicity of weak antigen and so on (Bowersock and Martin, 1999; Gupta *et al*., 1995; Gong and Wu, 1996). Comparative studies in humans and animals showed that aluminum is a weak adjuvant for antibody induction to recombinant protein vaccines and induces a Th2, rather than a Th1 response (Gupta, 1998). Some other group of adjuvants including oil emulsions, lipopolysaccharides, polymers, saponins, liposomes, cytokines, ISCOMs, Freund's complete adjuvant, Freund's incomplete adjuvant, alums, bacterial toxins etc., have been evaluated and clinical trials are under investigation, although the mechanism of action of adjuvants often remain poorly understood (Vogel, 1995; Edelman, 1997).

EFFECTS OF SAPONINS ON ANIMAL IMMUNE SYSTEM

Saponins

Saponins are steroid or triterpenoid glycosides found in wild or cultivated plants, lower marine animals and some bacteria (Riguera, 1997; Yoshiki *et al*., 1998). Saponins contain a steroidal or triterpenoid aglycone to which one or more sugar chains are attached (Oda *et al*., 2003). These are found widely in the plant kingdom with the triterpenoid saponins predominant mostly in cultivated crops, while steroid saponins are common in plants used as herbs or for their health-promoting properties according to Fenwick *et al*.(1991). Triterpenoid saponins have been detected in many legumes such as soybeans, beans, peas, lucerne, etc., and also in alliums, tea, spinach, sugar beet, quinoa, liquorices, sunflower, horse chestnut and ginseng. Steroid saponins are found in oats, capsicum peppers, aubergine, tomato seed, alliums, asparagus, yam, fenugreek, yucca and ginseng. Saponins are tensoactive glycosides containing a hydrophobic nucleus of triterpenoid structure with car-

bohydrate chains linked to the nucleus (Kensil, 1996).

One example of an extensively studied group of triterpenoid saponins is produced from the bark of *Quillaja saponaria*, a tree native to the Andes region (Francis *et al*., 2002). Experiments demonstrating the physiological, immunological and pharmacological properties of saponins have aroused considerable clinical interest in these substances. In animal system, various studies have shown the effect of saponins on cell membrane, animal growth and feed intake, protein digestion, cholesterol metabolism, animal reproduction, the immune system, nervous system functioning or cytostatic effects on malignant cells or molluscicidal effect or virucidal activity or effect on protozoa (Francis *et al*., 2002).

Effects on immune system

Saponin based adjuvants have the ability to modulate the cell mediated immune system as well as to enhance antibody production and have the advantage that only a low dose is needed for adjuvant activity (Oda *et al*., 2000). Saponins induce a strong adjuvant effect to T-dependent as well as T-independent antigens. Saponins also induce strong cytotoxic CD8+ lymphocyte responses and potentiate the response to mucosal antigens (Kensil, 1996). However, saponins are surface active agents and cause haemolysis of red blood cells in vitro, although haemolysis does not appear to be correlated with adjuvant activity (Kensil, 1996). The study results of Oda *et al*.(2000) suggested that the adjuvant activity of saponins did not relate with haemolytic activity. It was considered that not only the functional groups themselves, but the overall conformation of such functional groups, affected adjuvant activity of saponins. Saponins have been widely used as adjuvants for many years and have been included in several veterinary vaccines. The adjuvant action of saponins was, however, not so pronounced in some of the non-mammalian species tested (Cossarini-Dunier, 1985; Grayson *et al*., 1987).

Saponin not only has stimulatory effects on the components of specific immunity, but also presents some non-specific immune reactions such as inflammation (de Oliveira *et al*., 2001; Haridas *et al*., 2001) and monocyte proliferation (Delmas *et al*., 2000; Yui *et al*., 2001).

Mechanism of action

The mechanisms of immune-stimulating action of saponins have not been clearly understood, but many explanations have been put forward. Saponins reportedly induce production of cytokines such as interleukins and interferons that might mediate their immunostimulant effects (Jie *et al*., 1984; Kensil, 1996). It is likely that they interact with antigen-presenting cells to induce many of these responses (Barr *et al*., 1998). The incorporation of saponins into cell or endosomal membranes might expose the incorporated antigen to cytosolic proteases. According to Bangham *et al*.(1962), saponins have been shown to intercalate into cell membranes, through interaction with cholesterol, forming 'holes' or pores. It is currently unknown if the adjuvant effect of saponins is related to pore formation, which may allow antigens to gain access to the endogenous pathway of antigens presentation, promoting cytotoxic T-lymphocyte (CTL) response (Sjölander *et al*., 2001).

It was believed that the adjuvant activity of saponins could be related to branched sugar chains or aldehyde groups (Bomford *et al*., 1992) or to an acyl residue bearing the aglycone (Kensil, 1996). Latter, soyasaponins and lablabosides were found to show strong adjuvant activity despite lacking acyl residues and possessing only un-branched sugar chains (Oda *et al*., 2000). Also, most of the escins that have acyl residues and branched sugar chains did not show adjuvant activity. Adjuvant activity and toxicity, but not the cholesterol-binding capacity, of QH-B, a *Quillaja* fraction, decreased on peroxidate oxidation due to alterations in the structure of the sugars, galactose and xylose. Modification of the apiose moiety may influence adjuvant activity but not toxicity in vivo (Ronnberg *et al*., 1997). Saponins with an acyl residue or oxide-ring moiety tend to show haemolytic activity (Oda *et al*., 2000). Oda *et al*.(2000) concluded that not only the functional groups themselves, but the overall conformation of such functional groups, affected adjuvant activity of saponins. Soyasaponins, lablabosides and purified *Quillaja* saponin-21, which possess adjuvant action, have only two to four O atoms, equally distributed around the aglycone, and may retain the typical amphipathic features. On the other hand, escins without adjuvant activity have seven O atoms, with five localized around one side of the aglycone, thus reducing its hydrophobic and adjuvant nature. In a study of structural and biological activity of protopanaxatriol-type saponins (PTS) from the roots of *Panax notoginseng*, Sun *et al*.(2006a)

concluded that the number, length and position of sugar side chains, and the type of glucosyl group in the structure of PTS could not only affect their haemolytic activities and adjuvant potentials, but have significant effects on the nature of the immune responses.

Quillaja saponaria

The bark extract of the tree *Quillaja saponaria* was found to have adjuvant activity. The extract contains a number of components that have been purified and found to exhibit adjuvant activity, individually (Jacobsen *et al*., 1996). *Quillaja* and other saponins, either as crude mixtures or as purified compounds, have been reported to increase immune-cell proliferation in vitro (Chavali *et al*., 1987; Plohmann *et al*., 1997; Lacaille-Dubois *et al*., 1999). Purified *Quillaja* saponins boosted antibody production without producing any reaginic antibodies (So *et al*., 1997). Chavali and Campbell (1987a; 1987b) found that mice fed *Quillaja* saponins exhibited increased cell proliferation in spleen and mesenteric lymph nodes and increased and prolonged natural killer (NK) cell activity, suggesting that saponins act directly on T-helper cells of the mucosal immune system to induce secretion of soluble mediators. Other studies also suggest that *Quillaja* saponin has mitogenic activity and induces T cell and B cell proliferation (Chavali *et al*., 1987; Mowat *et al*., 1991).

Quil A is a saponin fraction derived from an aqueous extract from the bark of *Quillaja saponaria*. Fractions purified from this extract by reverse phase chromatography, mainly QS-21, have been studied as alternatives to alum when strong cellular responses are required for a particular vaccine (Allison and Byars, 1991; Kensil *et al*., 1991; Takahashi *et al*., 1990). Adjuvants that are surface-active agents rely on surface free energy of cells to bind to hydrophobic surfaces and bring about their immunostimulatory properties (Allison, 1979). Quil A and QS-21 (saponin-type adjuvants), have hydrophobic domains from which their surface-active properties arise (Hunter *et al*., 1995). Quil A has been used successfully for veterinary applications (Dalsgaard, 1987). It is a natural product composed of more than 23 different saponins and is generally considered too toxic for human use. In addition to severe local reactions and granulomas, toxicity includes severe haemolysis reflecting the affinity of saponins for cholesterol present in erythrocyte membranes, resulting in membrane solubilization and haemolysis (Kensil *et al*., 1991; Warren and Chedid, 1988; Dalsgaard, 1987).

Kensil *et al*.(1991) further tested separated fractions, finding decreased toxicity associated with QS-7 and QS-21 fractions. Increased antigen specific IgG responses were observed in mice intradermally vaccinated with antigen and 20 μg of QS-7, QS-17, QS-18, or QS-21, compared to antigen alone. QS-21 is less toxic than Quil A itself (Kensil *et al*., 1995).

QS-21 has been shown to be a potent adjuvant for CTL induction, and induces Th1 cytokines (IL-2 and IFN-γ) and antibodies of the IgG2a isotype (Kensil, 1996). While according to Evans *et al*.(2001), in a trial with HIV-1 env antigen, QS-21 was able to allow a significant dose reduction for the antigen and also enhanced proliferative T-cell responses but not CTL. QS-21 has also been claimed to perform as an adjuvant for DNA vaccines, following both systemic and mucosal administration (Sasaki *et al*., 1998).

However, ascribed to high toxicity, haemolytic effect and instability, *Quillaja* saponins have been restricted for use in human vaccination (Marciani *et al*., 2000; 2001; Liu *et al*., 2002).

Ginseng

Ginseng is another example of a saponin containing plant which is the best known traditional Chinese medicine. The major constituents of ginseng are the saponins (Wang *et al*., 1979). Sapogenins have been identified from the ginseng plant and have been extracted from its root (Jin, 1996). Ginseng extract has been reported to have modulatory effects on phagocytic cells, lymphocytes and antibody production in humans and animals. Increase in the human immune responses was reported by Scaglione *et al*.(1990), after oral administration of ginseng extract. Wu *et al*.(1991) reported that ginseng extract enhanced the proliferative response of human blood lymphocytes to phytohemagglutinin (PHA) at lower concentrations, but inhibited the response at higher concentration. Ginseng extract has potential as a stimulator of the immune system of dairy cows and treatment can activate the innate immunity of cows and may contribute to the cow's recovery from mastitis (Hu *et al*., 2001). Ginseng extract influenced the zymosan-induced chemiluminescence (CL) in the blood in a dose dependent manner and significant increase of CL in milk was also seen for the cell treated with 10^2 μg/ml ginseng solution in comparison

with control. A significantly higher proportion of phagocytic cells in blood were also recorded in ginseng treated group (Hu *et al*., 1995). Oral administration of ginseng extract has been found to enhance the antibody response and blood lymphocyte proliferation in human (Scaglione *et al*., 1990; Wu *et al*., 1991). A double blind clinical trial conducted by Scaglione *et al*.(1990) revealed that blood polymorphonuclear leukocytes (PMNL) phagocytosis and intracellular killing were significantly increased by the administration of 100 mg of an aqueous ginseng extract or standardized ginseng extract (G115) twice daily for 8 weeks. Ginsenoside Rg1 has been found to increase the lymphocyte proliferative responses to mitogenic stimulation and IL-2 production in aged rats but not in young rats (Liu and Zhang, 1995; 1996) and human (Liu *et al*., 1995).

Ginseng extract has also been reported to enhance, specific antibody response against diphtheric toxoids in mice (Yang *et al*., 1983); increase IgG and IgM antibody responses in mice immunized with sheep red blood cells and oral administration of ginseng extract at the same time (Jie *et al*., 1984). Sun *et al*.(2006a) reported the ovalbumin (OVA) specific IgG, IgG1, IgG2a and IgG2b antibody levels in serum were significantly enhanced by seven PTS from the root of *Panax notoginseng* compared with OVA control group ($P<0.01$ or $P<0.001$). The Rb1 fraction of ginseng elicits a balanced Th1 and Th2 immune response. In a study conducted by Rivera *et al*.(2005), porcine parvovirus (PPV) vaccines containing Rb1 was evaluated for inducing Th1 or Th2 type of immunity in mice. Study revealed the production of large amounts of cytokines including IFN-gamma, IL-2, IL-4, IL-10 and TNF-alpha and stimulated titers of antigen specific IgG1, IgG(2a) and IgG(2b). Smolina *et al*.(2001) reported that administration of the root or culture of *Panax ginseng* plant in mice increased PMNL and macrophage phagocytosis, and induced production of IFN-γ and TNF. Wang *et al*.(2001) found that an aqueous extract containing mainly oligosaccharides and polysaccharides from *Panax quinquefolium* stimulated the proliferation of normal mouse spleen cells, of which the major responding subpopulation was identified as B-lymphocytes. Sun *et al*.(2006b) reported that *Panax notoginseng* saponin could reduce leukocyte adhesion in venules under the inhibitory effect on the expression of adhesion molecules (CD11b and CD18) on neutrophils.

Immunostimulating complex (ISCOM)

These are comprised of antigen, cholesterol, phospholipid and saponin. ISCOM-based vaccines have been shown to promote both antibody and cellular immune responses in a variety of experimental animal models (Sanders *et al*., 2005). *Quillaja* saponins can combine with lipids and antigens to form ISCOM, which compose antigen and adjuvant in the same particle and have unique adjuvant properties (Morein *et al*., 1984; Lovgren *et al*., 1987).

ISCOMs have been reported to induce antibody responses or protective immunity in guinea-pig, turkey, cat, rabbit, dog, seal, sheep, pig, cow, horse or monkeys (Mowat *et al*., 1999) and induced specific CTL responses (Coulter *et al*., 1998). Cholera toxin A1 (CTA1-DD) and an unrelated antigen can be incorporated together into the ISCOM, resulting in greatly augmented immunogenicity of the antigen. The combined vector was a highly effective enhancer of a broad range of immune responses, including specific serum Abs and balanced Th1 and Th2 CD4(+) T cell priming as well as a strong mucosal IgA response (Helgeby *et al*., 2006). To study responses generated by nasal vaccination with an ISCOM-based vaccine for equine influenza (EQUIP F) in horses revealed that significantly enhanced levels of virus-specific IgA were detected in the nasal washes from vaccinated ponies (Crouch *et al*., 2005).

Astragalus saponin

Astragalus (*huang qi*), a Chinese traditional herb, is thought to strengthen and boost the immune system by improving the ability of the macrophages. It contains numerous triterpene saponins (astragalosides I~X, isoastragalosides I~IV and soyasaponin I). Most of the modern research on astragalus has focused on its immune-enhancing effects. Laboratory studies have found astragalus to increase macrophages, T-cell transformation, NK cell activity, interferon production, and phagocytosis. The study also documented increased levels of IgA and IgG antibodies in nasal secretions after two months of treatment (Chang and But, 1986).

Astragalus saponin is believed to induce the cellular and humoral immune responses with slight hemolytic activity. Yang *et al*.(2005) reported very low haemolytic effect (0.66%) with 500 μg/ml concentration of *Astragalus membranaceus* saponins (AMS) induced in mice and significantly enhanced OVA-specific IgG, IgG1 and IgG2b antibody titers in

mice serum. Peripheral lymphocyte proliferation and serum antibody titer increased in chicken vaccinated with Newcastle disease have also been found (Kong *et al*., 2004). A ten-fold potentiation in the in vitro antitumor activity of rIL-2 generated lymphokine-activated killer (LAK) cells was recorded by Wang *et al*.(1992). Astragalus was shown to potentiate the LAK cell inducing activity of rIL-2 against an Hs294T melanoma cell line. Fifty U/ml of rIL-2 with Astragalus extract F3 was more effective than 500 U/ml rIL-2 alone (Chu *et al*., 1994).

Yesilada *et al*.(2005) studied the effect of 13 cycloartane- and 1 oleanan-type triterpene saponins isolated from Turkish species included *Astragalus brachypterus*, *Astragalus cephalotes*, *Astragalus microcephalus*, and *Astragalus trojanus*, as well as methanol extracts from the roots of three *Astragalus cephalotes*, *Astragalus oleifolius* and *Astragalus trojanus* on in vitro cytokine release. All triterpene saponins tested showed a prominent IL-2 inducing activity between 35.9% and 139.6%. Among the extracts the highest score was obtained for *Astragalus oleifolius* (141.2%).

Astragalus herbal mixture stimulated macrophages to produce interleukin-6 and tumor necrosis factor (Yoshida *et al*., 1997).

Others

The root of a herbaceous plant *Achyranthes bidentata* (Amaranthaceae family) ("Niu Xi" in Chinese, Radix Achyranthes Bidentatae) is a well-known traditional Chinese medicine containing saponins (Wang and Zhu, 1996; Li *et al*., 2005) and has been reported as immunostimulator (Li *et al*., 2003; Chen *et al*., 2005). *Achyranthes bidentata* saponins (ABS) modulated immune responses and the haemolytic activities have been observed in mice. ABS significantly enhanced the OVA-specific IgG, IgG1, IgG2b antibody titers with slight haemolysis in mice (Sun, 2006).

Avicins, a family of triterpenoid saponins from *Acacia victoriae* (Bentham) represent a new class of plant stress metabolites capable of activating stress adaptation and suppressing proinflammatory components of the innate immune system in human cells by redox regulation (Haridas *et al*., 2004).

Saponins from soybean have been separated into six (Khalil and El-Adawy, 1994) or eight (Oda *et al*., 2003) fractions of soyasapogenol and soyasaponins groups. Oda *et al*.(2000; 2003) found that the soyasaponins exhibited high adjuvant activity while the soyasapogenol group exhibited low activity. Soyasaponin also exhibited low hemolytic activity.

Codonopsis (*Codonopsis pilosula*, *dang shen*) is a less expensive, milder substitute for Asian ginseng and contains saponins. Its uses are similar to those of Asian ginseng, with laboratory experiments having shown that codonopsis may enhance phagocytosis of the reticuloendothelial system, thus improving immune system function (Steven and Yue, 1992).

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