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Immunopotentiation by orally-administered *Quillaja* saponins: effects in mice vaccinated intraperitoneally against rabies

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SUMMARY

Orally fed *Quillaja* saponins amplified the immunopotentiating ability of an intraperitoneally (IP) administered inactivated rabies vaccine in mice. The number of animals surviving rabies infection was markedly higher (90–100%) in groups of animals receiving a combined treatment of oral saponin (SAP) and IP vaccine, compared to groups receiving vaccine alone (25%), or to unimmunized mice (0%). Antibody production was significantly higher in animals fed SAP 2 weeks after primary or secondary sensitization with an IP-injected vaccine. In mice given 2 IP doses of vaccine, 1 week apart, simultaneous feeding of SAP resulted in an enhanced production of rabies-specific (whole Ig) antibodies. On the other hand, animals preconditioned with SAP 3 days prior to administration of the vaccine exhibited greatly increased IgG antibody levels. Moreover, SAP-preconditioned mice vaccinated with a very low dosage produced significantly higher levels of antibodies.

Keywords saponins oral administration immunopotentiation rabies infection intraperitoneal vaccination

INTRODUCTION

Saponins are a class of naturally occurring triterpenoid and steroidal glycosides found in a wide variety of plants and plant foodstuffs (Fenwick & Oakenfull 1983; Price, Johnson & Fenwick, 1987). The use of *Quillaja* saponins as adjuvants is a well established technique for enhancing immune responses to parenterally administered veterinary vaccines against foot-and-mouth disease (Dalsgaard, 1978), rabies (Schneider, Horzinek & Novicky, 1971; Soulebot *et al.*, 1985), and a number of experimental vaccines (Bomford, 1982; McColm, Bomford & Dalton, 1982; Morein *et al.*, 1984). The adjuvant activity of SAP is considered to be due to a slowing of delivery of the antigen into the circulation and its subsequent localization in the spleen (Scott *et al.*, 1984). Unfortunately, many saponins are highly haemolytic (Price *et al.*, 1987), and cause local tissue damage at the injection site (Allison & Byars, 1986). Compared to parenteral administration, however, orally fed saponins are well tolerated at much higher concentrations (Drake *et al.*, 1982; Phillips *et al.*, 1979). In an earlier publication, we presented *in vitro* evidence to indicate that saponins are mitogenic at very low concentrations that are non-toxic to lymphocytes (Chavali, Francis & Campbell, 1987). Elsewhere, we have reported that oral administration of saponins to mice fed inactivated rabies vaccine induces non-specific immune responses, including a significant increase in the in-vitro responses of splenocytes to concanavalin A (Con A) and lipopolysaccharide (LPS) (Chavali & Campbell 1987a), and the induction of secretory soluble factors (Chavali & Campbell 1987b). Enhanced potentiation of both the humoral and the cell-mediated immune responses (CMI) appears to play a major role in offering significant protection against rabies infection in mice fed saponins and vaccine simultaneously (Chavali & Campbell 1987a,b; Maharaj, Froh & Campbell, 1986). These observations prompted us to explore the prophylactic use of saponins in generating improved immune responses to a parenterally administered vaccine and use of saponins in this fashion has been explored in our laboratory in a mouse model.

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MATERIALS AND METHODS

Animals

Swiss female mice (8–10 weeks old) were used in all the experiments. They were allowed continuous access to water and Purina laboratory rodent chow.

Immunostimulants

Saponin (*Quillaja saponaria*) was obtained from Fisher Scientific Co., Fairlawn, NJ, USA. Rabies vaccine (inactivated) was a commercially-available preparation, Endurall-K (Norden Laboratories, Lincoln, NE, USA).

Table 1. Amplification of protective effects of an intraperitoneal vaccine by orally administered saponins

Prophylactic dose	Vaccine given	Therapeutic dose	Survivors/total no. (%)	Time of death (days) (Mean ± s.d.)	Survivors with antibody: No.	Survivors with antibody: Mean titre ± s.d. (FIMT E.U.)
Saline	No	Saline	0/9 (0)	15·5 ± 2·0	—	—
Saline	Yes	Saline	2/8 (25)	13·0 ± 3·0	2	1·56 ± 0·20 (512)
SAP	No	Saline	2/9 (22)	15·0 ± 2·0	2	1·76 ± 0·30 (1024)
SAP	Yes	Saline	13/13 (100)	—	13	1·57 ± 0·27 (512)
Saline	No	SAP	0/11 (0)	14·7 ± 1·5	—	—
Saline	Yes	SAP	13/14 (93)	20	11	1·50 ± 0·25 (512)
SAP	No	SAP	0/13 (0)	15·0 ± 2·0	—	—
SAP	Yes	SAP	15/15 (100)	—	15	1·66 ± 0·14 (1024)

Mice were fed seven daily doses of saline or saponin, before and/or after intracerebral challenge with rabies virus (ERA strain). Animals were given IP inoculations of either vaccine (0·1 ml) or saline (controls) 1 h post-challenge. None of the recorded deaths were due to trauma from IC injection. Antibody titres were measured by ELISA and are expressed in absorbance units. Figures in parentheses following these values (last column) are the corresponding FIMT equivalent units (Barton & Campbell, 1988).

Experimental procedure

Specific details are given in the Results section. However, experiments adhered to the following basic protocol.

At varying times relative to IP rabies vaccination, mice were force-fed either 0·4 ml saline or saponin (10 mg/0·4 ml saline) via a stomach tube. They were then challenged intracerebrally (IC) with live fixed rabies virus (ERA strain: 10 $MICLD_{50}$ (mouse IC 50% lethal doses)/0·03 ml), and checked daily for deaths over a 28-day observation period. For determination of rabies-specific antibody, animals were bled via a tail vein. Levels of serum rabies-specific antibodies were measured by an enzyme-linked immunosorbent assay (ELISA) as described elsewhere (Barton & Campbell, 1988) but with anti-mouse immunological reagents.

As performed here, the ELISA procedure gave results comparable to those of the fluorescence-inhibition microtest (FIMT), an assay based on measuring virus neutralization (Campbell & Barton, 1988). For purposes of comparison, therefore, antibody titres are given in both ELISA absorbance units and in FIMT equivalent units, calculated as described by Barton & Campbell (1988).

RESULTS

Orally fed saponins amplify the protective effects of an intraperitoneally administered vaccine against rabies infection

All non-immunized animals, except 2 of those preconditioned with saponins, developed rabies and died. Summary data in Table 1 show that the resistance against rabies infection in mice fed SAP alone (22%) was as great as that displayed in the group of animals given a single dose of vaccine (25%). However, the protection afforded by an IP injection of vaccine in mice fed SAP before or after challenge was markedly higher (93–100%) than that in animals given vaccine alone. No naive animals receiving SAP therapeutically survived the challenge.

Orally administered saponins induce early production of enhanced levels of antibodies

Data summarized in Table 1 and previous work (Chavali & Campbell, 1987b; Maharaj *et al.*, 1986), demonstrate that saponins offer significant protection against rabies infection. We examined the effects of orally fed saponins on the dynamics of antibody production in mice given IP injection of vaccine in order to explore possible mechanisms whereby resistance against rabies infection is strengthened. Data presented in

Table 2. Effect of orally fed saponins on antibody production

Antibody class	Vaccine (0·5 ml): Saline	Vaccine (0·5 ml): SAP	Vaccine (0·1 ml): Saline	Vaccine (0·1 ml): SAP
(a) Simultaneous administration				
whole Ig	1·44 ± 0·39 (512)	1·64 ± 0·24* (1024)	1·13 ± 0·56 (128)	1·34 ± 0·34 (256)
IgG	1·63 ± 0·64 (1024)	1·68 ± 0·51 (1024)	0·74 ± 0·54 (64)	0·95 ± 0·58 (128)
IgM	0·46 ± 0·20 (32)	0·48 ± 0·15 (32)	0·27 ± 0·18 (16)	0·27 ± 0·16 (16)
(b) Preconditioning				
whole Ig	1·62 ± 0·23 (512)	1·70 ± 0·21 (1024)	1·49 ± 0·49 (512)	1·61 ± 0·18 (512)
IgG	1·41 ± 0·55 (256)	1·90 ± 0·51* (8192)	1·12 ± 0·54 (128)	1·73 ± 0·61* (2048)
IgM	0·42 ± 0·12 (32)	0·43 ± 0·51 (32)	0·33 ± 0·17 (16)	0·44 ± 0·22 (32)

Animals were force-fed saline or saponin (1 dose), then vaccinated IP: (a) immediately or (b) 3 days later. This procedure was then repeated on the same animals 1 week later. Data represent rabies-specific antibody titres determined by ELISA, after a further 14 days, on serum samples collected from tail veins. For the groups of animals fed saponins, antibody titres determined also on days 28, 42, and 56 after the booster were higher (range: 1·70 ± 0·28 – 2·55 ± 0·60), but did not differ significantly from the controls (range: 1·54 ± 0·48 – 2·10 ± 1·10). Therefore these data have not been included in the table. Each group contained 20–25 mice. Titres are expressed in absorbance units. Figures in parentheses listed with these values are the corresponding FIMT equivalent units (Barton & Campbell, 1988).

* Significantly greater ($P < 0·05$) compared to the control group.

Table 3. Effect of orally administered saponins on antibody production in mice given a single dose of vaccine.

Weeks after booster	Antibody class	Treatment			
		Saline (Od)	SAP (−3d)	SAP (Od)	SAP (+3d)
(a) Group 1 (vaccine 0·5 ml)					
2	whole Ig	1·11±0·38 (128)	1·32±0·28* (256)	1·10±0·51 (128)	1·27±0·27 (256)
	IgG	1·08±0·35 (128)	1·30±0·29* (256)	1·16±0·48 (256)	1·32±0·28* (256)
4	whole Ig	1·43±0·20 (256)	1·53±0·22 (512)	1·38±0·48 (256)	1·53±0·22 (512)
	IgG	1·24±0·25 (256)	1·33±0·30 (256)	1·29±0·43 (256)	1·40±0·22 (256)
6	whole Ig	1·41±0·32 (256)	1·47±0·36 (512)	1·31±0·53 (256)	1·48±0·26 (512)
	IgG	1·31±0·36 (256)	1·49±0·48 (512)	1·35±0·55 (256)	1·46±0·35 (512)
(b) Group 2 (vaccine 0·02 ml)					
2	whole Ig	0·48±0·41 (32)	0·85±0·36* (64)	1·04±0·41* (128)	0·82±0·22* (64)
	IgG	0·38±0·32 (16)	0·63±0·28* (32)	0·68±0·26* (64)	0·72±0·27* (64)
4	whole Ig	0·49±0·26 (32)	0·86±0·28* (64)	1·03±0·39* (128)	0·84±0·30* (64)
	IgG	0·46±0·32 (32)	0·81±0·28* (64)	0·88±0·28* (64)	0·75±0·33* (64)
6	whole Ig	0·46±0·32 (32)	0·91±0·28* (128)	1·04±0·46* (128)	1·11±0·31* (128)
	IgG	0·47±0·37 (32)	0·81±0·28* (64)	0·83±0·39* (64)	0·83±0·33* (64)

Mice were fed saline or saponin 3 days before (−3d), 3 days after (+3d) or along with (Od) a single IP injection of vaccine. Three dose levels of vaccine were tested (0·5 ml, 0·1 ml and 0·02 ml), and each group contained at least 20 mice. There was no significant difference between the antibody titres in animals vaccinated with 0·5 ml or 0·1 ml; for simplicity, therefore, only results on animals vaccinated with 0·5 ml and 0·02 ml doses are shown. Titres of rabies-specific antibodies were determined by ELISA on serum samples from tail veins. Values are in absorbance units, with the corresponding FIMT equivalent units (Barton & Campbell, 1988) in parentheses. Levels of IgM antibodies determined on the 2 and 4 week samples did not differ significantly from the respective controls and therefore these data are not shown in the table.

* Significantly different from saline controls ($P < 0·05$).

Tables 2 and 3 suggest that, as early as 2 weeks after immunization, the increase in the synthesis of rabies-specific antibodies was significantly higher in animals fed saponins compared to the controls. Antibody (whole Ig) production was significantly elevated, following simultaneous feeding of SAP for animals given a higher dose (0·5 ml) of vaccine compared to levels detected in mice receiving either a lower dose (0·1 ml) or saline (Table 2a). On the other hand, preconditioning of animals with saponins (3 days prior to immunization) resulted in a marked enhancement of IgG antibodies, irrespective of the amount of vaccine given (Table 2b).

Results presented in Table 3 illustrate the antibody generation affected by saponin fed before, concomitantly, or subsequent to, IP vaccination. In animals given a higher dose of vaccine, oral administration of saponins 3 days before or 3 days after immunization resulted in a significant increase in the formation of IgG class antibodies compared to those given SAP simultaneously or to the controls. In contrast, for mice immunized at low dosage levels of vaccine antibody production was markedly higher for animals fed saponins, before, during, or after immunization, compared to the controls.

DISCUSSION

The route of administration of a vaccine or an antigen can greatly influence both the magnitude and the specificity of antibody production (Allison & Byars, 1986). Such immune responses undergo appreciable modulation in the presence of an adjuvant/immunopotentiator (e.g., saponin). An unpurified *Quillaja* saponin, when administered subcutaneously, enhances

T-dependent (TD) immune responses to sheep red blood cells (SRBC) and dinitrophenyl-keyhole limpet haemocyanin (DNP-KLH) (Bomford, 1982). On the other hand, intraperitoneal administration of a purified saponin fraction (Quil A) (Dalsgaard, 1978), markedly promotes T-independent (TI) immune responses to trinitrophenyl (TNP)-LPS, TNP-Brucella and TNP-Ficoll (Flebbe & Braley-Mullen, 1986a). The differences observed in these two studies can be attributed to the relative purity of the saponin preparations used (Chavali *et al.*, 1987) and to the expected influence of route of administration on antibody production (Allison & Byars, 1986). In sharp contrast to the above findings, orally fed crude saponins potentiate responses of lymphocytes to Con A (TD) and LPS (TI) (Chavali & Campbell, 1987a). Indirect evidence has been cited to suggest that intraperitoneally injected Quil A mediates immunopotentiation by expansion (division) of immune competent cells (Flebbe & Braley-Mullen, 1986b). In support of this suggestion, we have documented direct evidence that orally fed SAP induces clonal expansion as shown by a significant increase in cell proliferation *in vivo* (Chavali & Campbell, 1987a).

The results of the present study demonstrate that orally administered *Quillaja saponaria* saponins potentiate immune responses in mice given an intraperitoneal injection of vaccine. Saponins, administered orally in repeated doses prior to a challenge with live virus, were able to protect 20–25% of the test animals against rabies infection (Table 1, Chavali & Campbell, 1987b). The current investigations suggest that orally-fed saponins (prophylactically or therapeutically) amplify the protective effects of an intraperitoneally administered rabies vaccine (Table 1). These findings confirm similar observations by Singh *et al.* (1983) who noted that orally fed *Panax ginseng* extract, a rich source of a complex mixture of saponins, amplifies the protective effects of an interferon-inducer against infection by Semliki Forest virus.

Host defence mechanisms against an infection include the development of both cell-mediated and humoral immunity. The virus-specific and non-specific immune responses play a major role in curtailing the spread of rabies (Wiktor, Doherty & Koprowski, 1977a,b) and other infections (Doherty, 1985). In mice fed inactivated rabies antigen, non-specific cell-mediated immune responses are further augmented in the presence of saponins (Chavali & Campbell, 1987a,b). The marked ability of orally fed saponin together with inactivated rabies antigen to enhance humoral immune responses has been correlated with a significant increase in protection against live rabies challenge (Chavali & Campbell, 1987a; Maharaj *et al.*, 1986). The dynamics of immunoglobulin synthesis depend, however, on the physicochemical nature of the antigen and the amount, route, and timing of its administration (Allison & Byars, 1986). Data included in Tables 2 and 3 indicate that, 2 weeks after a primary or secondary sensitization, the levels of rabies-specific antibodies are significantly higher in animals fed saponin compared to the controls. Differences in the time of exposure of animals to vaccine and saponins (stimuli) may have influenced the specificity and/or kinetics of antibody production, which are reflected in their capacity to bind to the ligand (G-protein) in the ELISA employed in this investigation. Therefore it is possible that significant amounts of whole Ig antibodies (with no corresponding increase in the IgG class) were detected in animals fed saponins simultaneous with an IP vaccination (Table 2a). Following oral administration of SAP to animals, 3 days before (Table 2b), or 3 days after (Table 3) an IP injection of the antigen, markedly higher levels of IgG antibodies (with no concomitant increase in the whole Ig) were detected. Antibodies of the IgG class, but not IgM, offer significant protection against rabies infection (Turner, 1978). IgM antibodies are restricted in their ability to diffuse into the tissues, and remain largely in the circulation. Thus it has been argued that IgM antibody would be of limited value in curtailing rabies infection where neural rather than viremic spread is important in pathogenesis (Murphy, 1977). In the present study, we were unable to detect any effect of orally fed *Quillaja* saponins on the synthesis of IgM antibodies (using an ELISA technique). However, in an earlier publication (Jie, Cammisuli & Baggiolini, 1984), orally fed *Panax ginseng* saponins were found to have a significant effect on primary and secondary IgM responses to SRBC.

Administration of an appropriate adjuvant enhances the potency of a vaccine, while decreasing the amount of antigen and the number of injections required to obtain protective immunity. Presently, there is a considerable interest in adjuvants and other agents with immunostimulatory properties to elicit greater immune responses to weaker antigens such as aqueous and subunit vaccines (Allison & Byars, 1986). The data presented in Table 3b show clearly that orally fed saponins potentiate immune responses in mice given a single IP injection of a very low dose of antigen.

A conclusion to be drawn from this study is that orally fed saponins greatly reinforce the protective effects of intraperitoneally administered rabies vaccine. Improved resistance against some viral infections may be achieved in light of the ability of saponins to effect an early, and significantly increased, production of antigen-specific antibodies. The implications of greatly elevated levels of IgG antibodies in animals preconditioned with saponins merits further investigation in view of the potential value in protection against infection. Investigation of the immunopotentiating activities of orally fed saponins in animals given subcutaneous or intramuscular injections of the vaccine (challenge) also warrants further study. Such studies may be of particular significance in the post-exposure therapeutic strategies for treating rabies and other viral infections in both man and other animals.

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