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Effect of saponin on the transmucosal passage of β -lactoglobulin across the proximal small intestine of normal and β -lactoglobulin-sensitised rats.

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Abstract

The ability of saponins and glycoalkaloids to permeabilise the mammalian intestinal barrier has been previously demonstrated in vitro, leading to the hypothesis that membranolytic saponins may facilitate transfer to the tissues of otherwise excluded macromolecules. An enhanced uptake of, for instance, potentially allergenic species from the lumen is one of the factors that may affect the induction of food allergy, and its presentation in already sensitised individuals. In the experiments described here, an increase in the transmucosal uptake of the milk allergen β -lactoglobulin (β LG) was assessed in non-sensitised and sensitised Brown Norway rats in the presence of *Gypsophila* saponin. Isolated jejunal loops were exposed in vivo to either β LG followed by saponin, saponin followed by β LG or the two compounds simultaneously. Portal vein blood samples were collected and assayed for β LG and rat mucosal mast cell protease (RCMP II) activity. Mucosal tissue was also examined histologically and assayed for histamine content. Sham-operated animals, exposed to physiological buffer alone, were included as controls and β LG measurements corrected for this component which was negligible. No transfer of β LG occurred in the absence of saponin in non-sensitised rats, whereas a significant enhancement was observed in the presence of saponin. β LG was detected in the portal circulation of sensitised rats exposed to β LG alone; however addition of saponin to the intestinal lumen further enhanced this uptake, possibly by an independent mechanism. Histological examination of the mucosal epithelium exposed to saponin revealed damage, especially at the villus tips. Mucosal histamine and serum RCMP II concentrations were consistent with the differences observed between sensitised and non-sensitised

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animals. It is concluded that exposure to food constituents capable of permeabilising the mucosal epithelium may increase the risk of sensitisation to dietary antigens. © 1997 Elsevier Science Ireland Ltd. All rights reserved

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1. Introduction

One of the functions of intestinal mucosa is to act as a barrier between the external environment and the tissues, whilst at the same time allowing the efficient passage of nutrients into the circulation. The gastrointestinal immune system provides a second, immunological barrier, but it also facilitates the development of systemic tolerance to food antigens. The mechanisms underlying 'oral tolerance' remain poorly understood but they are thought to depend upon the uptake, processing and transport of food-borne antigens from the gut lumen, and the subsequent presentation of 'tolerogens' to suppressor T cells (Lobo-Yeo and Lamb, 1993; Borel et al., 1995). Inevitably, the physical barrier functions of the 'gut' are incomplete, and they may be compromised by mechanical and chemical damage, by the effects of food-borne toxins or by infection. Any significant loss of epithelial integrity could cause proteins to bypass the specialised antigen-presentation pathway, and perhaps lead to inappropriate stimulation of other T cell subsets. This could cause excessive IgG and IgE responses, and ultimately lead to hypersensitivity reactions to food proteins (Brandtzaeg et al., 1989).

A number of different mechanisms can lead to increased intestinal permeability. A relatively high permeability to polyethylene glycol has been reported in patients with atopic eczema (Jackson et al., 1981). Bacterial and viral infections are associated with increased levels of food-borne antigens in the circulation in both humans and animal models, particularly when coupled with malnutrition (Abraham and Ogra, 1994). Some human foods contain constituents that can damage epithelial cells and may thereby disrupt mucosal integrity. In particular, the membranolytic properties of saponins and glycoalkaloids have been demonstrated in vitro using everted jejunal sac

preparations (Johnson et al., 1986; Gee et al., 1989). Glycoalkaloids occur in potatoes at levels which vary according to variety and quality. Saponins occur throughout the plant kingdom (Smockiewiczowa et al., 1978; Price et al., 1987), and may be of increasing concern as the availability of exotic plant foods increases (Ridout et al., 1988; Gee et al., 1993).

In earlier papers we have demonstrated that membranolytic glycosides can increase the permeability of the gut in vitro, and we have suggested that they may increase the transfer of food antigens in vivo (Johnson et al., 1986). The work described here focusses on the effect of a saponin on the passage of the milk protein β -lactoglobulin (β LG) across the intestinal mucosa in an intact animal model. The aim was to determine whether the saponin rendered the epithelium more permeable to the allergen, and hence capable of promoting the induction of an allergenic reaction.

2. Methods

2.1. Saponins

Gypsophila saponin, derived from the roots of the herbaceous plant (*Gypsophila* sp.), was obtained as an impure mixture of saponins from Sigma (UK) and purified by reversed-phase flash chromatography using the procedure described by Johnson et al. (1986).

2.2. Experimental animals

Twenty male Brown Norway rats (ca. 150–180 g), obtained from a commercial supplier (Olac), were housed singly in polypropylene cages with wire bottoms and tops, in an air-conditioned small animal room having an ambient temperature of 21°C, and a 12-h light/dark cycle. The

Table 1

Mucosal histamine and serum RCMP II levels in non-sensitised and sensitised rats exposed to β -lactoglobulin and saponin for specified periods

Type of rat	Agent/time		Serum RCMP II ($\mu\text{g}\cdot\text{ml}^{-1}$) ¹		Mucosal histamine ($\text{ng}\cdot\text{mg}^{-1}$ dry mass)
	1st Exposure	2nd Exposure	1st Exposure	2nd Exposure	
Non-sensitised	β LG/15 min	Saponin/10 min	0.13 ± 0.03	0.30 ± 0.12^a	47.0 ± 13.6^a
Sensitised	β LG/15 min	Saponin/10 min	34.05 ± 11.3	91.1 ± 33.8^b	13.6 ± 1.76^b
Non-sensitised	Saponin/10 min	β LG/15 min	0.64 ± 0.20	0.78 ± 0.21^a	42.9 ± 11.9^a
Non-sensitised	β LG and saponin together/15 min		0.74 ± 0.30^a		45.7 ± 7.98^a

¹Corrected for baseline values obtained after exposure to buffer alone.

^{a,b}RCMP values with different superscripts $P < 0.05$; histamine values with different superscripts $P < 0.05$.

All are means of 5 animals $\pm$ S.E.

animals were allowed free access to water and food (Beekay standard diet, free of ovalbumin and milk proteins) for 6 weeks, by which time their final body weight was between 250 and 300 g.

A further five Brown Norway rats, obtained from the same supplier but housed in small groups under similar conditions, were sensitised to milk protein by intraperitoneal injection of semiskimmed milk (500 μg , containing 1 mg carageenan) on two occasions, 7 days apart. Sensitisation was subsequently confirmed by assaying for the presence of reagenic antibody by passive cutaneous anaphylaxis (PCA) using specific milk antigens (Atkinson et al., 1996).

2.3. Translocation studies

The translocation of β LG across the jejunum was investigated in vivo by luminal exposure and blood sampling via the portal vein according to the protocol outlined in Table 1. Following deep anaesthesia with sodium pentobarbitone, the abdominal cavity of each rat was opened and the proximal small intestine and mesenteric blood supply were exposed. Starting 15 cm from the pyloric sphincter, a 10-cm loop of proximal jejunum was isolated by surgical transection, flushed with saline (0.9%), ligatured at its distal end and fitted with a Y-cannula at the proximal end. Care was taken to retain an intact circulation. At

recorded time intervals, solutions were introduced into the loop via syringes attached to the cannula. Blood samples (ca. 1.2 ml) were taken from the portal vein immediately before each introduction and at the end of the experiment.

The test solutions consisted of Krebs bicarbonate buffer (pH 7.2–7.4) containing D-glucose (28 mM), with or without β LG (75 $\text{mg}\cdot\text{ml}^{-1}$) and *Gypsophila* saponin (20 $\text{mg}\cdot\text{ml}^{-1}$). Loops were initially filled with Krebs-glucose (0.9 ml). A blood sample was removed after 4 min, and after a further 1 min, solutions containing either β LG (0.3 ml) or saponin (0.3 ml), or the two together, were added to the buffer in the loop. Where the test solutions were added separately to the loop, the tissue was exposed to β LG for 15 min and to saponin for 10 min; when the two were added simultaneously the exposure period was 15 min. Final concentrations of β LG and saponin in the loop were 15 mg/ml and 4 mg/ml (approximately 2.7 mM), respectively. At the end of the experiment a final blood sample was taken, and the animal was killed. The loop was removed intact, the contents recovered and the tissue was divided equally for histopathology, and for histamine assay. Five rats were subjected to each combination of treatments. Sera were collected from a further five sham-operated non-sensitised animals, in which the loops were filled with Krebs bicarbonate buffer, to control for any changes due to the manipulative steps.

2.4. Assay for β LG

After clotting, all blood samples were centrifuged, and the sera were removed to fresh tubes and stored at -20°C . Samples were assayed for β LG content by a two site ELISA immunoassay technique, with polyclonal anti- β LG antibodies as capture antibodies, and the F(ab')_2 fragments from the same IgG antibodies (acetylcholinesterase labelled; AChE), as tracer (Wal et al., 1995b). Anti- β LG antibodies were prepared from rabbit immune serum by separation of the total immunoglobulin fraction on a Protein A (Prosep A, Bioprocessing) column. AChE-labelling of purified immunoglobulins was performed according to Grassi et al. (1989). Microplates (maxisorp, Nunc) were coated with a $10\text{ }\mu\text{g}\cdot\text{ml}^{-1}$ solution of purified immunoglobulins in 50 mM phosphate buffer, pH 7.4 (200 μl per well, overnight incubation at room temperature). Plates were subsequently washed and saturated with 0.1 M phosphate buffer, pH 7.4, containing 0.15 M NaCl, 0.1% human serum albumin and 0.01% sodium azide (EIA buffer) and incubated overnight at 4°C . Under these conditions plates can be stored for several weeks.

Immediately prior to use the plates were thoroughly washed. Serum samples were diluted (1:5 to 1:10⁴) and 100 μl applied to wells in triplicate, followed by AChE antibodies (100 μl). After overnight incubation at 4°C , plates were washed, enzyme substrate was added (Elleman's reagent, 200 μl), and the absorbance was read at 414 nm after 1 h. Serum from sham-operated animals was used as a control, and a range of β LG standards (0–50 $\text{ng}\cdot\text{ml}^{-1}$, in control rat serum) was prepared to obtain a standard curve for quantification. Under the above conditions the limit of detection, defined as the mean of the non-specific binding + 2 S.D., was 0.1 $\text{ng}\cdot\text{ml}^{-1}$. Background absorbance was measured with negative controls. The range of the dose response curve was 0.5–50 $\text{ng}\cdot\text{ml}^{-1}$ with an accuracy of ca. 10%.

2.5. Rat mucosal mast cell protease II (RCMP II) ELISA assay

The level of RCMP II levels in serum samples was assessed using reagents obtained from More-

dum Animal Health Ltd. (Edinburgh, UK). Mouse monoclonal anti-RCMP II (1 ng/ml in 50 mM carbonate buffer pH 9.6) was bound to an ELISA plate (M129 B, Dynatech Laboratories Ltd., Billingshurst, Sussex, UK) for 24 h at 4°C . Excess antibody and other non-reacting chemicals were removed by washing the plate five times with 0.05% Tween 20 in phosphate buffered saline (PBS) after this and each subsequent incubation. Residual binding sites were blocked with 4% sheep serum albumin in carbonate buffer for 2 h at 37°C . RCMP II standard was diluted to 64 ng/ml, and the sera to be examined were diluted 40-fold in PBS. They were then titrated using log 2 dilutions, and incubated overnight at 4°C . Plates were incubated with peroxidase labelled sheep F(ab')_2 anti-RCMP II in 0.05% Tween 20 in PBS for 1 h at room temperature. Substrate (*o*-phenylene diamine, 1.08 mg/ml in citrate phosphate buffer with 20 volumes H_2O_2 , 1 $\mu\text{l}/\text{ml}$) was added to develop the colour. The reaction was stopped using 2.5 M H_2SO_4 and the optical density read on a Luminar ELISA reader at 490 nm. The level of RCMP II in serum was calculated against the standard curve.

2.6. Histamine assay

The mucosa was scraped from the proximal half of each loop and extracted by homogenisation in perchloric acid (0.4 mM; 1 ml). The extract was centrifuged and the supernatant assayed for histamine activity using a continuous flow fluorometric analysis (Lebel, 1983). The weight of the dried pellet was recorded to normalise the activity against mucosal tissue weight.

2.7. Mucosal morphology

To monitor changes in mucosal morphology and histology, the distal half of each loop was fixed in formalin-phosphate buffer (4% formaldehyde in 1 M phosphate buffer pH 6.8), wax-embedded and examined after haematoxylin and eosin staining. The sections were examined by an experienced pathologist, with special emphasis on, (1) infiltration of eosinophilic granulocytes, (2) villus atrophy, and (3) degenerative changes of epithelial cells

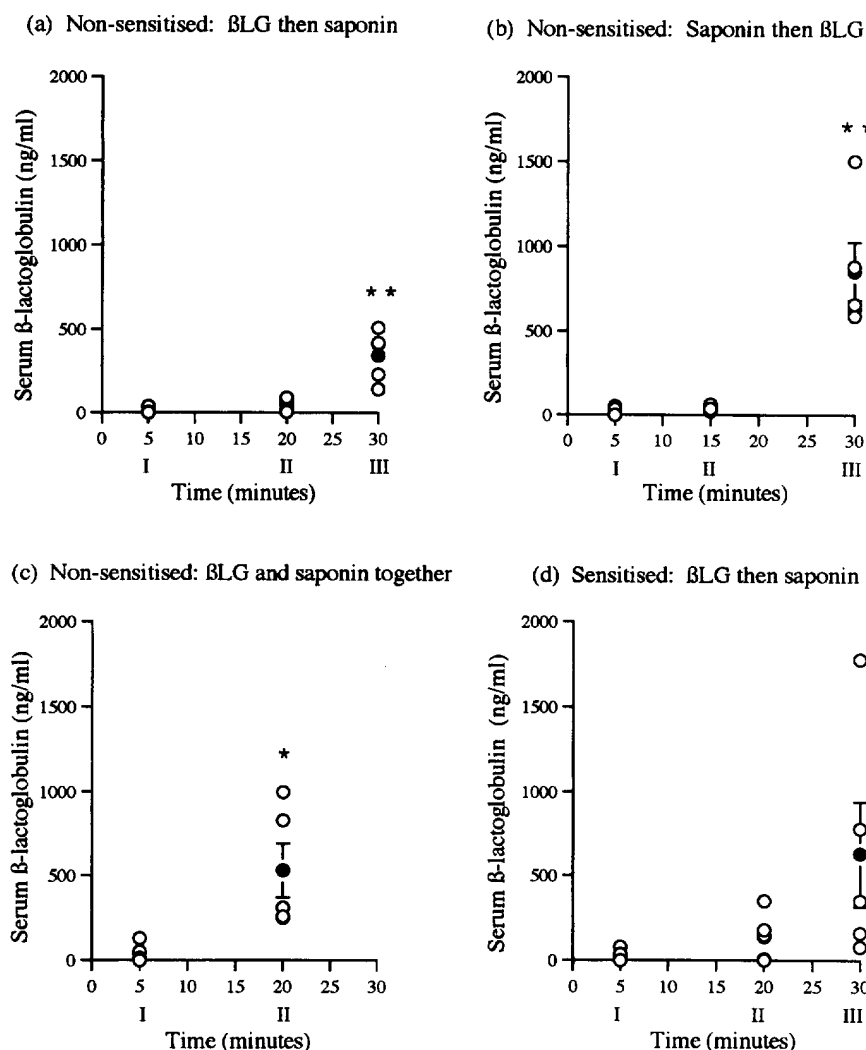


Fig. 1. Serum β -lactoglobulin levels in samples of portal blood from, (a) non-sensitised rats exposed intraluminally to β LG followed by saponin, (b) non-sensitised rats exposed to saponin then β LG, (c) non-sensitised rats exposed to β LG and saponin together, and (d) sensitised rats exposed to β LG then saponin. Scattergrams represent the effect of exposure of the jejunal mucosal epithelium to buffer (I) followed by the test compounds as specified (II, III). Means (●) of 5 animals at each time point are $\pm$ S.E. * $P < 0.05$; ** $P < 0.02$ compared to other time points within individual plots.

at the tips of the villi, and increased exfoliation of epithelial cells.

2.8. Statistics

The significance of differences between means was assessed by analysis of variance coupled with Student's *t*-test, using Minitab software (Minitab Inc, State College, PA, USA).

3. Results

The concentrations of β LG in sera from individual non-sensitised animals, after exposure to β LG in the presence or absence of saponin are given in Fig. 1a–c. There was no evidence of β LG transfer to the circulation in the absence of saponin (Fig. 1a II), and the addition of saponin alone to the loop (Fig. 1b II) did not elevate

serum β LG levels above the baseline of 21 ± 9 . However, when the saponin was administered either simultaneously with β LG (Fig. 1c), or after a 15 min exposure to β LG (Fig. 1a), its presence in the loop caused plasma β LG levels to rise significantly compared to the control, to 530 ± 160 and 342 ± 68 ng·ml⁻¹, respectively ($P < 0.01$), indicating an increase in the permeability of the intestinal epithelium to proteins. In the group of animals exposed first to saponin and then, 10 min later, to β LG (Fig. 1b), serum β LG increased to similar levels (852 ± 170 ng·ml⁻¹, $P < 0.01$) as those observed when both compounds were added simultaneously to the loop.

Sensitisation to β LG was confirmed by PCA and, when the area of dye extravasation was measured, animals in this group had ADE values in the range 79–227 mm². Baseline β LG concentrations were very similar (24 ± 15 ng/ml) to those found in non-sensitised animals. In most cases exposure of the jejunal mucosal epithelium of these rats to β LG alone resulted in a measurable transfer of protein to the circulation (Fig. 1d). There was a tendency for the subsequent addition of saponin to the loop to raise circulating β LG levels even further, although the response was somewhat variable and therefore not statistically significant.

Increases in serum RMCP II levels after addition of β LG or saponin to the lumen in non-sensitised rats were minimal and not statistically different from the background concentration (< 1 μ g/ml). In sensitised animals however, concentrations rose from a baseline of around 1 μ g·ml⁻¹ to levels ranging from 17–203 μ g·ml⁻¹ (Table 1). Histamine levels in the mucosal tissue of non-sensitised rats remained high and, when expressed in terms of ng·mg⁻¹ dry mass of tissue, were in good agreement with those previously found in conventional rats by Wal et al. (1985). They were also consistent with the low serum RMCP II values obtained for these animals. Conversely, in the sensitised animals, the lower histamine concentrations in the tissue following antigenic challenge probably resulted from mast cell degranulation, the release of histamine, and its subsequent degradation in the intestinal lumen by diamine oxidase. These findings can therefore be

seen as consistent with raised RMCP II levels and greater permeability of the mucosa to β LG in these animals in the absence of saponin.

Whereas the control tissue from sham-operated animals did not show any degenerative changes (Fig. 2a), histological examination of tissue samples from intestinal segments exposed to saponin revealed signs of epithelial damage, infiltration of eosinophilic granulocytes and slight to moderate villus atrophy. Damaged epithelial cells were most evident at villus tips in nonsensitised animals, where the mucosa had been exposed to saponin before addition of β LG (Fig. 2b and c). However, significant degenerative changes were also seen in non-sensitised animals exposed to saponin at a later stage.

4. Discussion

The mucosal surfaces of the gastrointestinal tract provide an important interface between the external environment and the internal milieu. Gut-associated lymphoid tissue provides a second immunological barrier that plays a major role in the defence of the host against infection by this route (Abraham and Ogra, 1994). In our previous studies, using isolated preparations of proximal intestinal tissue, we established that exposure of the intact small intestinal epithelium to saponins and glycoalkaloids led to a loss of integrity sufficient to enable macromolecules to cross the epithelium (Johnson et al., 1986; Gee et al., 1989; Gee et al., 1995). These findings suggested that food constituents increasing mucosal permeability may favour the uptake of larger amounts of, for instance, allergenic protein, and thereby facilitate sensitisation or exacerbate challenge reactions in already sensitised individuals, leading to inappropriate immune responsiveness to food. In a separate publication (Atkinson et al., 1996) we have demonstrated that exposure of Brown Norway rats to *Gypsophila* saponin via the oral route can significantly increase the number of animals producing antigen-specific reagenic antibody in response to parenterally administered ovalbumin with a carageenan adjuvant. Using a small intestinal preparation with intact circulation, the hy-

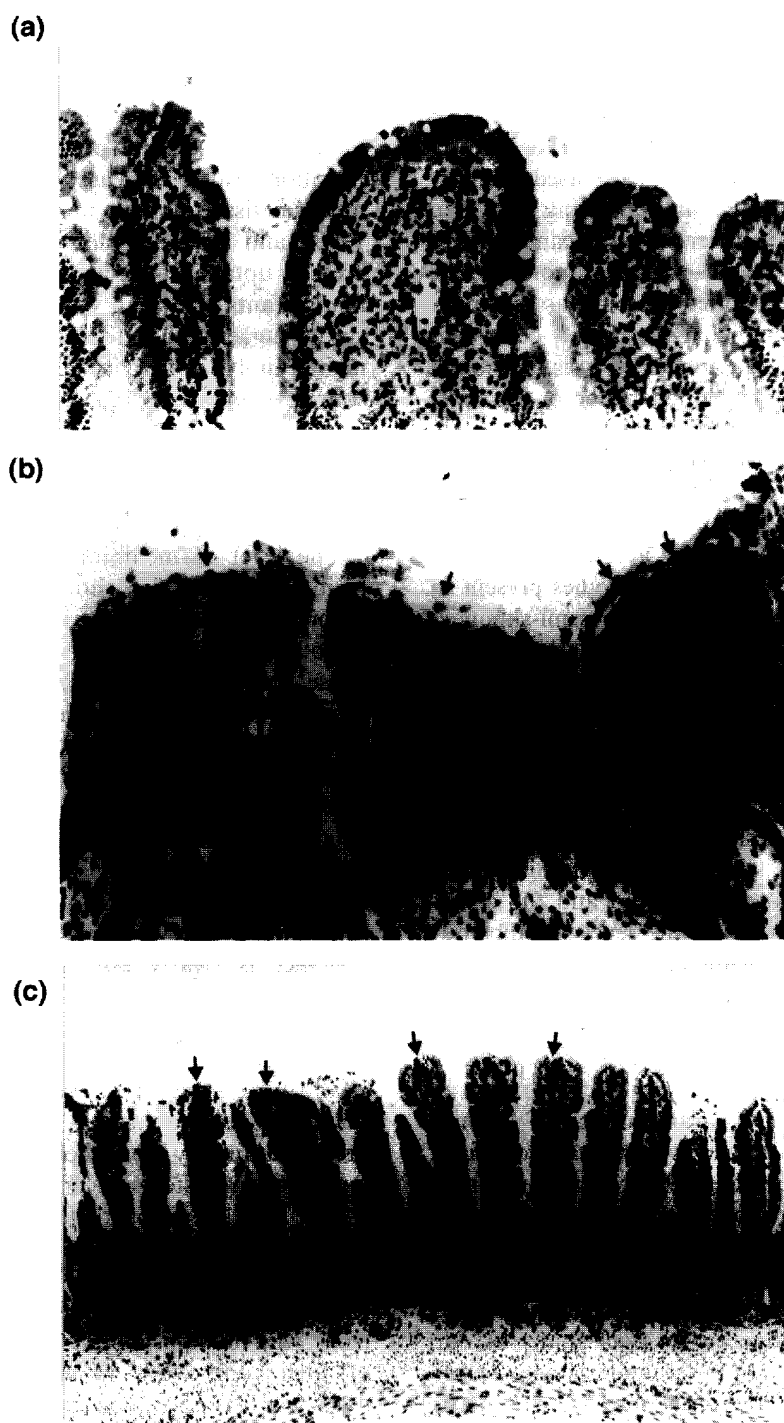


Fig. 2. Light microscopy of sections of isolated loops of rat proximal jejunum after exposure in vivo to Krebs' bicarbonate buffer containing 28 mM glucose (a) without, or (b) and (c) with *Gypsophila* saponin (approx 1 mM) and β LG. Arrows indicate villous atrophy with enterocyte detachment at the tips. (a) and (b) $\times 200$; (c) $\times 100$.

pothesis that the presence of intraluminal saponin can increase the permeability of the mucosa in vivo, and facilitate the transfer of β LG into the portal circulation has now been confirmed.

In the non-sensitised rats the presence of *Gypsophila* saponins evidently increased the permeability of the epithelium, allowing β LG to cross the intestinal barrier in significantly larger amounts than in the absence of saponins. The translocation of the luminal protein was accompanied by signs of epithelial disruption consistent with tissue damage caused by cell lysis. Although the small intestinal epithelium is one of the most rapidly proliferating tissues of the body, and acute damage caused by food-borne saponins might therefore be expected to be rapidly repaired, our results suggest that a state of high permeability is maintained for some minutes at least.

The specificity of reagenic antibodies present in BN rats sensitised parenterally to semi-skimmed milk resembles the distribution of IgE responses in the sera of allergic patients, demonstrating that the BN rat is a suitable model for studying allergy (Atkinson and Miller, 1994; Wal et al., 1995a). β LG was taken up by sensitised rats, even in the absence of saponin, probably due to increased permeability as a result of mast cell activation following antigen absorption. The increased serum mast cell protease activity, and the corresponding decline in histamine levels in the mucosal epithelium following antigenic challenge, are indicative of allergen uptake. The mechanism by which saponin further increases β LG absorption in these animals is thought to be independent of an immune mechanism. There is some controversy as to whether an increased translocation of luminal antigens is to be expected in previously sensitised animals. Fargeas et al. (1995) recently reported that β LG failed to appear in the sera of orally sensitised guinea pigs, probably because of the formation of a β LG-mucosal IgA immune complex. Our findings may reflect a species difference, or they might be due to the fact that, (a) our animals were sensitised by parenteral immunization, rather than by the oral route, which may in turn result in an absence of antigen specific IgA, or (b) the antigen challenge was via the jejunum rather than the colon.

It has previously been recognised that the integrity of the intestinal mucosal barrier can be compromised by a variety of factors including malnutrition and infection. For example in the neonatal mouse both rotavirus infection and malnutrition lead to increased levels of ovalbumin in mucosal tissue and in the serum (Uhnnoo et al., 1990), and a similar mechanism may lead to increased uptake of dietary macromolecules in human infants (Heyman et al., 1984). The present study suggests that food-borne substances with membranolytic activity are another potential source of increased mucosal permeability. Exposure to membranolytic food constituents may therefore be undesirable even at levels which might be judged safe by the standards of conventional toxicology.

The level of membranolytic saponins in new fruit and vegetables should ideally be assessed before they enter the food chain. Common leguminous vegetables appear to pose little risk because their principal saponin component is soya saponin, which we have previously shown to have virtually no membranolytic effect against the gut (Gee et al., 1989). However, unconventional foods are constantly appearing in the diets of European countries. Quinoa, which is a saponin-rich staple crop from South America, is one case in point. Although the saponin content of this commodity can be reduced to negligible levels by processing, vigilance is clearly essential, particularly if the food is intended for use in infant diets (Gee et al., 1993). Moreover, our previous studies on the membranolytic effect of individual saponins from alfalfa have shown that the ability of these compounds to permeabilize the small bowel cannot be predicted directly from their activity in other biological assay systems (Oleszek et al., 1994).

The level of exposure to membranolytic cytotoxins which can lead to an increased risk of sensitisation and adverse reactions to dietary allergens in humans remains to be established in trials with human subjects. The principle source of such compounds in conventional European diets would appear to be potatoes, and potato products containing high levels of glycoalkaloids (Davies and Blincow, 1984; Gee et al., 1995). A search for an epidemiological link between expo-

sure to glycoalkaloids from such products and an increased incidence of true food-allergy would provide a valuable means of assessing the risk in a human population. Further information could possibly be provided from very carefully controlled clinical studies in which sensitised individuals undergo oral challenge in the absence or presence of membranolytic agents such as saponins.

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