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Passage of ingested protein into the blood during gastrointestinal hypersensitivity reactions: experiments in the preruminant calf

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SUMMARY

Preruminant calves given a series of feeds of heated soya bean flour (HSF) frequently develop gastrointestinal hypersensitivity to soya bean antigens. The permeability of the intestinal mucosa of calves undergoing hypersensitivity reactions to ingested HSF has been assessed by feeding them milk and quantitating the leakage of β-lactoglobulin into the blood. Maternal β-lactoglobulin did not evoke an antibody response in calves. Therefore its detection in the serum was not influenced by immunological mechanisms that normally remove or exclude antigens from the circulation. In sensitized calves ingestion of HSF caused a dramatic increase in the permeability of the intestine to β-lactoglobulin. The change was transitory and after 24 hr permeability had almost returned to normal. The mucosal barrier was not permanently damaged regardless of the number or the severity of the reactions experienced. Indomethacin was ineffective in counteracting permeability changes. A progressive increase in the sensitivity of the gut to soya bean antigens was accompanied by a rise in the titre of serum antibodies to soya bean proteins. Absorbed β-lactoglobulin was present in the serum in its monomeric form only, and quickly disappeared from the circulation. In an enzyme immunoassay used to measure its concentration absorbed β-lactoglobulin was indistinguishable from the native protein. These results suggest that measurement of intestinal permeability to macromolecules might be useful in the diagnosis of certain forms of food allergy in man.

INTRODUCTION

An essential function of the gastrointestinal mucosa is the exclusion of bacteria, toxins and food antigens from the tissues and the circulation. An effective barrier is formed by the interaction of immunologically specific and non-specific defence mechanisms (Walker, 1976). It is generally believed that in normal circumstances only trace quantities of macromolecules pass unchanged from the gut into the blood. A breach in the mucosal barrier might therefore be expected to have pathogenic consequences (Walker & Isselbacher, 1974).

Inflammatory reactions in the intestinal mucosa can vastly increase the absorption of undegraded protein (Bloch *et al.*, 1979). It is conceivable that this might happen in food allergy. In support of this view, Paganelli *et al.* (1979) observed that egg-sensitive patients developed abnormally high levels of circulating immune complexes and free ovalbumin after oral challenge with egg. However, the quantity of a particular food antigen in the blood is not always a general reflection of mucosal permeability and the ingress of macromolecules from the gut into the tissues. The level of homologous antibodies present has an overriding influence on the concentration of circulating food antigen detectable (Dannaeus *et al.*, 1979).

The present studies were designed to reveal changes in intestinal permeability induced by

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food-allergic reactions in the gastrointestinal tract of the calf. Previous investigations in this laboratory strongly suggested that preruminant calves fed heated soya bean flour (HSF) according to a suitable regimen frequently developed gastrointestinal allergy to soya bean antigens (Kilshaw & Sissons, 1979a). Preliminary studies indicated that the allergenic constituents of HSF were probably the storage globulins glycinin and β-conglycinin (Kilshaw & Sissons, 1979b). The permeability of the gastrointestinal mucosa of calves undergoing hypersensitivity reactions in the gut has been assessed by feeding them with cow's milk and measuring the quantities of β-lactoglobulin appearing in the blood. It was considered likely that most calves would be immunologically tolerant to maternal milk proteins. Therefore the absorption of β-lactoglobulin would be a measure of permeability that could be used repeatedly in the same animal without the intervention of immunological mechanisms that normally exclude or remove food antigens from the blood.

MATERIALS AND METHODS

Animals and feeds. Data were obtained from six Friesian bull calves (A–F) and one Friesian/-Hereford cross (G). The animals were reared from birth on cow's milk and at 2–4 weeks of age each calf was equipped with an abomasal cannula for the administration of experimental feeds. Calves A–C and F were also cannulated in the duodenum and/or ileum to facilitate removal of biopsies and samples of digesta. Beginning 6–8 weeks after birth, single feeds in which HSF (Arkasoy 50; British Arcady, Manchester) was the sole source of protein were administered in order to sensitize the animals to soya bean antigens. Formulation of these feeds has been described elsewhere (Sissons & Smith, 1976). HSF was given six to eight times over a period of 3 weeks and thereafter at irregular intervals of up to 4 weeks. After approximately 1 month calves A, B and C became sensitive to soya bean antigens and experienced diarrhoea within a few hours of ingesting HSF. Calves D, E and G did not react in this manner. Sensitization was not attempted in calf F.

Blood was sampled for β-lactoglobulin determinations after feeding calves with milk (40 g milk/kg body weight) or with milk mixed with HSF (66 g HSF/kg milk).

Quantitation of β-lactoglobulin in the serum. The concentration of β-lactoglobulin in bovine serum was determined by an enzyme immunoassay. Reagents were prepared as described by Voller, Bidwell & Bartlett (1976). The wells of microtitre plates (Dynatech, M29) were coated by incubation overnight at 4°C with 0·2-ml aliquots of the IgG fraction of a rabbit antiserum specific for β-lactoglobulin (1 μg/ml). After washing, duplicate test samples at suitable dilutions in 1 : 4 normal bovine serum (devoid of β-lactoglobulin) were added and incubated in the plate for 6 hr at room temperature. After further washing, the conjugate anti-β-lactoglobulin–alkaline phosphatase (1 : 1,600) was added and the plate incubated for 1 hr at 37°C and overnight at 4°C. A final wash was followed by addition of substrate. Reactions were terminated after 40 min at room temperature by addition of 3 M NaOH, optical densities (400 nm) measured and results determined by reference to a standard curve (0·5–10 ng/ml β-lactoglobulin). β-lactoglobulin that had been absorbed from the gut showed close parallelism with the standard curve for the native protein.

Serum from calves less than 2–3 months old contained a high molecular weight component (mol. wt > 150,000) in their serum which cross-reacted antigenically with β-lactoglobulin. On serial dilution this material did not give results in the enzyme immunoassay in parallel with the standard curve for β-lactoglobulin, reflecting inferior binding avidity with the antiserum. The component was not found in foetal calf serum and it gradually declined in concentration during the first 2–3 months after birth. Results reported here were obtained at a stage when this material was undetectable or present at very low concentrations.

Antibody determinations. Antibodies specific for soya bean proteins and for β-lactoglobulin were measured by an enzyme immunoassay similar to that described by Weits *et al.* (1978) using porcine antisera specific for bovine IgG, IgA and IgM. Results are expressed as titres, defined as the highest doubling dilution of serum to give an OD 400 nm of at least 0·1 above background. Sera were also tested by passive cutaneous anaphylaxis as previously described (Kilshaw & Sissons, 1979a).

Column chromatography. The molecular size of β-lactoglobulin absorbed from the gut was

determined by gel filtration on a Sephadex G-100 column (2·5 × 70 cm) calibrated with bovine serum albumin, ovalbumin, β-lactoglobulin (in dimeric form) and α-lactalbumin. Fractions (5 ml) were assessed spectrophotometrically (OD 280 nm) to determine total protein and then diluted 1 : 10 and analysed for β-lactoglobulin by enzyme immunoassay (OD 400 nm).

RESULTS

Effect of HSF on the permeability of the gut to β-lactoglobulin

Calf A was challenged with two feeds of HSF within a period of 16 hr. Before the challenge and 6 hr after the second feed the animal received milk and the resulting concentrations of β-lactoglobulin in the blood were determined. The experiment was performed before and after the calf had been sensitized to soya bean antigens. Results obtained before sensitization are shown in Fig. 1a. On 3 consecutive days before challenge with HSF the levels of β-lactoglobulin in the blood after feeding with milk were low and showed little variation; values after challenge with HSF were similar. In contrast, after sensitization permeability to β-lactoglobulin was considerably increased by ingestion of HSF (Fig. 1b). Assessment of permeability 24 hr after HSF showed a return towards normal values.

A similar experiment was performed in calf B after sensitization. Again, ingestion of HSF caused a substantial increase in intestinal permeability to β-lactoglobulin with a partial return to normal 24 hr later (Fig. 2a). Calf C was sensitized and then challenged with a single feed of HSF followed by milk 4 hr later; absorption of β-lactoglobulin was dramatically increased (Fig. 2b). Intestinal permeability always returned to normal after reactions to HSF regardless of their severity. Calf A repeatedly experienced such reactions over a period of 10 weeks yet before each challenge absorption of β-lactoglobulin was normal.

To investigate the kinetics of these permeability changes calf A was fed with a mixture of milk and HSF and blood β-lactoglobulin was measured over 8 hr (Fig. 3). HSF increased the permeability of the gut 2 hr after feeding, levels of β-lactoglobulin were maximal at 4–6 hr and declined thereafter. Fig. 4 shows similar results from an experiment in calf C; 24 hr after challenge

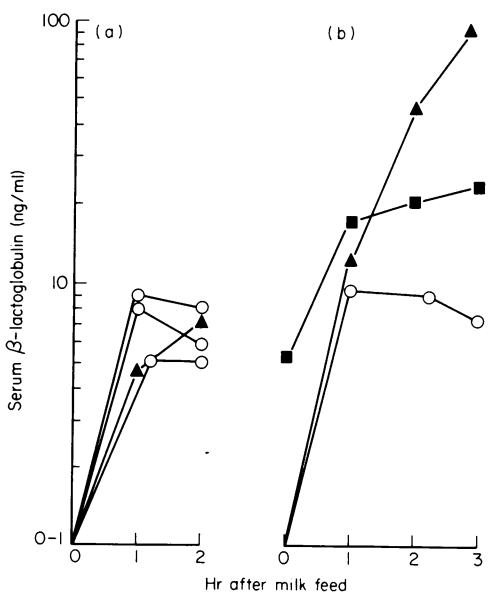

Fig. 1. Serum β-lactoglobulin levels in calf A following milk feed; (o——o) before challenge with HSF; (▲——▲) 6 hr or (■——■) 24 hr after HSF. (a) Before, (b) after sensitization to soya bean antigens.

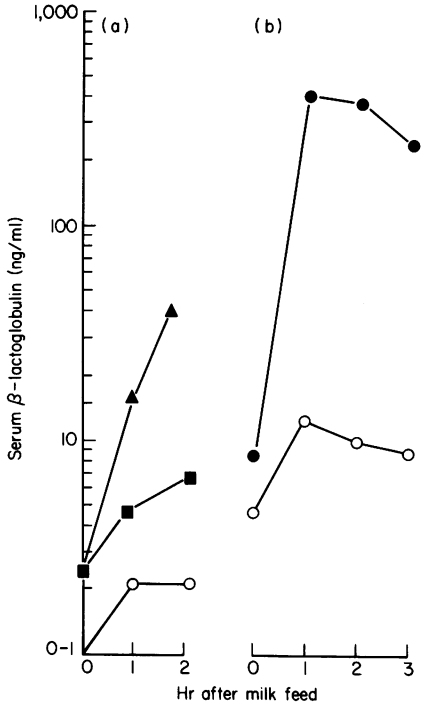

Fig. 2. Serum β-lactoglobulin levels in sensitized calves following milk feed; (o——o) before challenge with HSF; (●——●) 4 hr, (▲——▲) 6 hr or (■——■) 24 hr after HSF. (a) Calf B, (b) Calf C.

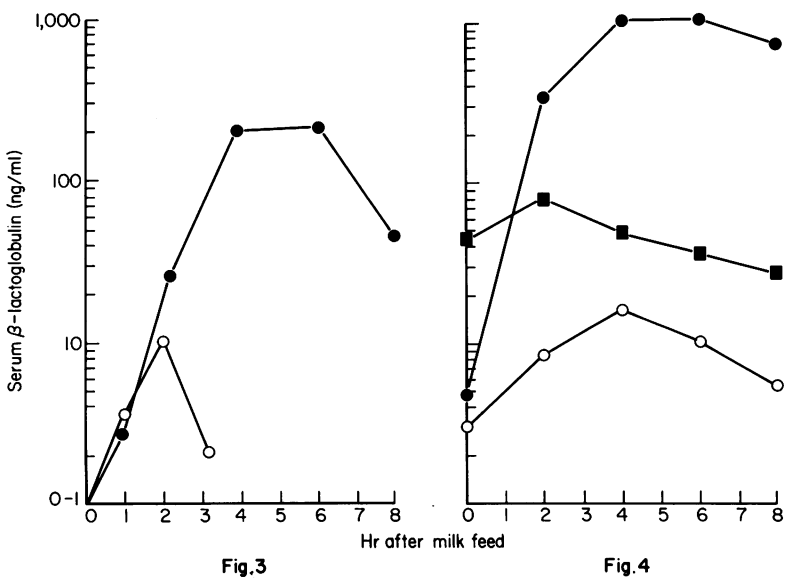

Fig. 3. Serum β-lactoglobulin levels in sensitized calf A after feeding with milk alone (o——o) or with milk mixed with HSF (●——●).

Fig. 4. Serum β-lactoglobulin levels in sensitized calf C after feeding with milk mixed with HSF (●——●) or with milk alone 24 hr before (o——o) and 24 hr after (■——■) this feed.

β-lactoglobulin was still present in the blood but its concentration was only slightly increased by further ingestion of milk suggesting that intestinal permeability had returned to normal.

HSF had very little effect on intestinal permeability in unsensitized calves. Table 1 shows β-lactoglobulin levels after feeding milk with HSF to animals never before exposed to soya protein compared with the values obtained for milk alone.

During the course of this study three calves (D, E and G) failed to become sensitized to soya bean antigens. In contrast to the reactions of calves A, B and C they experienced neither diarrhoea nor an increase in intestinal permeability when challenged.

Effect of indomethacin on intestinal permeability

Inhibitors of prostaglandin synthesis, including indomethacin, have been reported to alleviate symptoms of food intolerance in man (Buisseret *et al.*, 1978) and to protect against anaphylactic cardiovascular shock in calves (Burka & Eyre, 1974). For this reason the effect of the drug on intestinal hypersensitivity in the calf was investigated.

Indomethacin (Merck, Sharp & Dohme) in doses of 2–5 mg/kg dispersed in 500 ml 0·9% NaCl was infused into the abomasum of calf A 1 hr before feeding with a mixture of milk and HSF. Doses of neither 2 mg/kg nor 5 mg/kg effectively counteracted the increase in permeability (Table 2). Indomethacin itself caused a very slight rise in permeability in the absence of HSF. The doses of indomethacin tested were relatively high compared with those normally recommended for man (Wade, 1977) and they caused drowsiness.

Table 1. Effect of HSF on intestinal permeability in unsensitized calves

		Blood β-lactoglobulin (ng/ml) at:	
Calf	Feed	2 hr	4 hr
F	Milk	12·0	12·8
	Milk + HSF	14·8	14·4
G	Milk	26·0	23·5
	Milk + HSF	30·0	28·0

Table 2. Effect of indomethacin on changes in intestinal permeability induced by HSF (calf A)

		Blood β-lactoglobulin (ng/ml) at:	
Feed	Dose of indomethacin	2 hr	4 hr
Milk + HSF	None	n.d.	620
Milk + HSF	2 mg/kg	165	530
Milk + HSF	5 mg/kg	63	505
Milk	2·5 mg/kg	15	8
Milk	None	6	5

n.d. = Not determined.

Changes in the sensitivity of the intestine in relation to antibody levels

Successive feeds of HSF caused progressively larger increases in intestinal permeability and rising titres of serum antibodies (IgG, IgA and IgM) to soya bean proteins (Table 3).

Antibodies to β-lactoglobulin were not detected or were present at trace levels only.

Molecular size of β-lactoglobulin in the blood and its rate of clearance

A 3-ml sample of serum taken from calf A 4 hr after feeding with milk and HSF, having a β-lactoglobulin concentration of 620 ng/ml, was analysed on a Sephadex G-100 column. β-lactoglobulin was detected by enzyme immunoassay in only one peak with a molecular weight of 18,100 daltons (Fig. 5). This value closely approximates to the molecular weight of the monomeric form of the protein (Andrews, 1964).

Absorbed β-lactoglobulin was quickly cleared from the blood. Its decline in concentration over a period of 16 hr indicated a half-life of approximately 4 hr. The low molecular weight of the monomer suggests that it would rapidly equilibrate with extravascular fluids and be excreted in the urine.

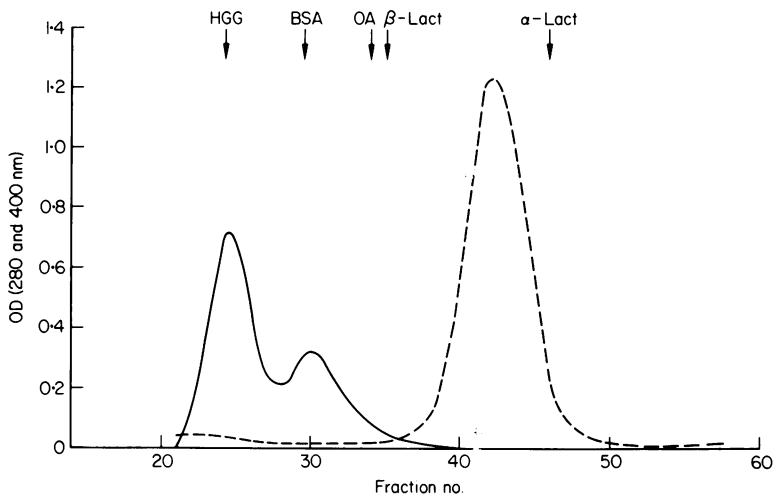

Fig. 5. Sephadex G-100 fractionation of a serum sample taken from calf A 4 hr after ingestion of milk mixed with HSF, having a β-lactoglobulin concentration of 620 ng/ml. (——) OD 280 nm (total protein), (– – – –) OD 400 nm (enzyme immunoassay for β-lactoglobulin).

Table 3. Changes in the sensitivity of the intestine to HSF over 8 weeks in relation to antibody titres (calf A)

Permeability: blood β-lactoglobulin (ng/ml at 4 hr)*	Antibodies to soya protein				Weeks after first HSF feed	Total no. HSF feeds
	IgG	IgA	IgM	PCA†		
46	1:2,560	1:80	1:80	−ve	9	12
210	1:2,560	1:80	1:80	−ve	11	13
620	1:10,240	1:160	1:160	−ve	13	15
1,290	1:40,960	1:1,280	1:160	−ve	17	20

* Calf was challenged with a mixture of milk and HSF.

† PCA test in calves (72 hr latent period).

DISCUSSION

Three calves became sensitized when fed HSF and experienced substantial increases in intestinal permeability when challenged while three failed to respond. The reason for these differences in susceptibility is unknown. In agreement with previous studies (Kilshaw & Sissons, 1979a) the reactions strongly suggest gastrointestinal hypersensitivity. At first, calves did not respond adversely to HSF, then, after a series of feeds reactions developed and became progressively worse; sensitivity was retained for several weeks without re-exposure to soya bean antigens.

An increase in the sensitivity of the gut to HSF was accompanied by increasing titres of IgG, IgA and IgM antibodies in the serum. It is not clear whether IgG antibodies were important in causing the reactions that have been described or were merely a consequence of increased penetration of soya bean antigens through the mucosa. In contrast to previous studies (Kilshaw & Sissons, 1979a) antibodies were not detected by PCA. This inconsistency may be due to differences in the sensitivity of recipient calves in the PCA test; such variability has been observed by ourselves and by others (Beadle & Pay, 1975).

The immunological mechanism responsible for permeability changes in our experiments remains uncertain. Intestinal hypersensitivity reactions in the calf have been ascribed to the action of complement-fixing IgG1 antibodies (Barratt, Strachan & Porter, 1978) and more rarely to IgE (Barratt & Porter, 1979). An increase in gut permeability was detected 2 hr after infusing HSF into the abomasum of a sensitized calf and was maintained for at least 6 hr. The speed of onset of permeability changes may have been influenced by the rate of abomasal emptying, and the kinetics do little to elucidate the mechanism. They are, however, at variance with IgE-mediated hypersensitivity in the rat intestine in which permeability has been shown to increase and return to normal within 45 min of ingestion of antigen (Byars & Ferraresi, 1976).

The failure of indomethacin to influence mucosal permeability argues against a role for prostaglandins in the reactions that have been described. In inflammation prostaglandins are thought to cause vasodilatation rather than increased vascular permeability (Williams & Peck, 1977) and this could explain the ineffectiveness of indomethacin in our experiments.

The choice of β-lactoglobulin for permeability studies was convenient because it did not evoke an antibody response in calves and therefore its permeation through the mucosa was not subject to immunological mechanisms for antigen exclusion and clearance. In addition, it was more suitable for revealing permeability changes than lower molecular weight markers (Kingham & Loehry, 1976).

Measurements of intestinal permeability have provided a sensitive diagnostic test for food allergy in our calves. It is conceivable that a similar approach could be of value in man. Some food-allergic patients experience an increase in intestinal permeability even when their main clinical symptoms are eczema and asthma (Paganelli *et al.*, 1979; Brostoff & Carini, 1979). For permeability studies in human infants a human milk protein such as α-lactalbumin might be a suitable marker.

It is possible that the absorption of abnormally large quantities of food antigens and bacterial products during hypersensitivity reactions in the gut might perpetuate the hypersensitive condition and possibly initiate secondary pathogenic reactions either locally or in peripheral tissues. The preruminant calf appears to be a suitable experimental subject for further investigation of these events.

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