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Intestinal hypersensitivity reactions in the rat

I. UPTAKE OF INTACT PROTEIN, PERMEABILITY TO SUGARS AND THEIR CORRELATION WITH MUCOSAL MAST-CELL ACTIVATION

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SUMMARY

We have confirmed previous observations that intestinal anaphylaxis induced in rats previously sensitized to ovalbumin (OVA) is associated with an increased uptake of an unrelated 'bystander' protein, bovine serum albumin (BSA) fed 1 hr previously. In this study, this enhanced protein uptake was associated with an increased lactulose/rhamnose excretion ratio after administration of these sugars, although there was no correlation between the two measurements. One hour after antigen challenge the serum levels of rat mast-cell protease II (RMCPII), a specific marker for mucosal mast-cell secretion, were significantly higher than both the pre-challenge levels and those of sham-challenged controls ($P < 0{\cdot}002$). There was a significant positive correlation between the serum levels of RMCPII and the lactulose/rhamnose excretion ratios ($P < 0{\cdot}05$), but no such correlation existed between RMCPII and BSA levels in the challenged rats. In other studies the urinary lactulose/rhamnose ratios of rats with cetrimide-induced gut damage were found to be significantly increased, although BSA uptake into the serum remained unaltered. We conclude that there is no simple correlation between gut permeation of low-molecular weight sugars and the uptake of macromolecular proteins.

INTRODUCTION

The nature of the mechanisms underlying food allergy remains controversial, and an acceptable animal model would permit a range of investigations that are either difficult or unethical in man. Two published models suggest that animals rendered hypersensitive to one food protein antigen will, when challenged locally in the gut, absorb increased amounts of an unrelated 'bystander' antigen. Kilshaw & Slade (1980) showed that in calves made sensitive to soya flour there was an increased uptake of the protein β-lactoglobulin when the animals were fed milk and soya simultaneously. Similarly, Bloch & Walker (1981) demonstrated enhanced uptake of BSA from the gut when rats pre-sensitized to OVA were challenged with that antigen. Such observations provide a possible explanation for the development of multiple food protein hypersensitivities.

In man, an increased uptake of macromolecular protein antigens has been reported in food-allergic individuals (Paganelli *et al.*, 1979; Paganelli, Levinsky & Atherton, 1981; Paganelli, Atherton & Levinsky, 1983; Dannaeus *et al.*, 1979). Such individuals have frequently presented clinically with eczema, and an increased intestinal permeability has been reported in a proportion of patients with atopic eczema when PEG 4000 (Jackson *et al.*, 1981), lactulose/rhamnose (Pike *et al.*, 1986) and cellobiose/mannitol (Strobel, Brydon & Ferguson, 1984) were administered as inert probes. It is tempting to view such increased permeability as responsible for the increased uptake of macromolecular proteins. There is, however, no supportive data for such a direct link and the question needs to be addressed either directly in patients or with an appropriate experimental system.

In the present study we have investigated, simultaneously, bystander-protein uptake and changes in intestinal permeability to sugars in rats experiencing local hypersensitivity reactions. The presence of such reactions was confirmed by measuring the release of rat mast-cell protease (RMCPII), a specific marker for mucosal mast-cell secretion (Miller *et al.*, 1983). In a parallel investigation, non-allergic gut damage was induced in rats using the detergent cetrimide, and the uptake of administered sugars and proteins measured.

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MATERIALS AND METHODS

Experimental protocols

Comparison of bystander protein and sugar uptake and rat mast-cell protease release. The basic protocol was essentially as described by Bloch & Walker (1981), and is illustrated in Table 1.

Table 1. Experimental protocol for simultaneous investigation of bystander protein uptake, changes in sugar permeability and release of RMCPII. (Modified after Bloch & Walker, 1981)

Stage	Procedure
Immunize	100 μg OVA (i.p.) with alum
	↓ 14 days
Oral feed	1g BSA in 0·15 M $NaHCO_3$–0·15 M NaCl
	↓ 1 hr
Laparotomy	Instil into lumen of small intestine a mixture of:
	100 mg OVA
	140 mg lactulose
	20 mg rhamnose in 0·1 M K_2 HPO_4, pH 7·0
Blood collection*	1, 3, 6 hr
Urine collection†	0–6 hr

* Blood samples were assayed for maximum BSA levels (radioimmunoassay) and RMCPII levels (ELISA).

† Urine samples were analysed for lactulose/rhamnose ratios by thin-layer chromatography.

Sham-challenge group: identical protocol except that OVA omitted at laparotomy stage

Adult Sprague-Dawley rats were used.

Adult Sprague-Dawley rats (male and female) were obtained from Charles Rivers, U.K. (Margate, Kent). They had been raised on a diet free of egg and milk proteins and were maintained in our animal house on a similar diet ('Labsure CRMX', Labsure, Cambridge, Cambridgeshire). Sensitive immunoassays for β-lactoglobulin and BSA failed to reveal these proteins in saline extracts of the diet sampled at intervals over 3 years. After an initial equilibration period of 6 weeks, the rats were sensitized with a single i.p. injection of 100 μg OVA (Sigma Grade V, code no. A5503, Poole, Dorset) in 100 mg alum (total volume 1 ml). After 14 days 1 g BSA (Sigma Fraction V. code no. A4503) dissolved in 2 ml of 0·15 M $NaHCO_3$–0·15 M NaCl was introduced into the stomach using a plastic-coated metal cannula. One hour later a small midline abdominal incision was made under combined Sagital–Hypnorm anaesthesia. The pylorus was occluded with rubber-tipped forceps and 2 ml of a solution of OVA (100 mg), lactulose (140 mg) and rhamnose (20 mg) in 0·1 MK_2 HPO_4, pH 7·0, were introduced into the duodenum using a 25-gauge needle and the incision closed. Blood was obtained by tail-vein bleeding immediately before the BSA gavage stage, and further samples were obtained by cardiac puncture 1, 3 and 6 hr after intraduodenal challenge, supplementing the anaesthesia with Hypnorm as necessary. Urine samples were obtained over 6 hr following antigen challenge. In the case of female rats, the tissues surrounding the urethral meatus were clamped with a foam-padded spring-loaded clip. The penis of the male rats was occluded in similar fashion. The bladder of each rat was drained completely at the end of the experiment. Sham-challenge animals underwent an identical protocol except that OVA was omitted at the laparotomy stage.

Cannulation experiments for continuous monitoring of protein uptake. Adult male rats previously sensitized to OVA were anaesthetized with Sagital–Hypnorm supplemented with nitrous oxide–halothane inhalation. A surgical incision was made to expose the internal carotid artery and a plastic cannula (diameter 0·96 mm) inserted. One gram of BSA dissolved in 2 ml of 0·15 M $NaHCO_3$–0·15 M NaCl was introduced into the stomach as above, and blood removed through the cannula at intervals over the next 60 min. One hour after the BSA feed, the animals were challenged with 100 mg OVA (without sugars), instilled into the duodenum as described above. Control animals were subjected to a sham challenge. After closure of the abdominal incision, blood was removed through the cannula at frequent intervals. The patency of the cannula was maintained with heparin-saline.

Effect of cetrimide on uptake of protein and sugar from the rat gut. In this experiment, naive unimmunized adult Sprague-Dawley rats were given an oral gavage of lactulose (140 mg), rhamnose (20 mg) and BSA (1 g) in 2 ml of 0·15 M $NaHCO_3$–0·15 M NaCl. In addition some of the animals received 4 mg of the detergent cetrimide with the protein–sugar mixture. Control animals received no cetrimide. Blood samples were obtained by cardiac puncture under appropriate anaesthesia 1, 3 and 5 hr after gavage. Urine was collected as above.

Assays

Radioimmunoassay for immunoreactive BSA. Immunoreactive BSA in rat serum samples was measured using a competitive radioimmunoassay.

The following were mixed in a plastic tube (LP3, Luckham, Burgess Hill, West Sussex): 10 μl of serum or BSA standard (Sigma Fraction V); 100 μl of rabbit anti-BSA (Dako, Copenhagen, Denmark) diluted 1:15000; 100 μl of normal rabbit serum (diluted 1:100); 100 μl of ^{125}I-BSA (Sigma Fraction V, labelled by the Iodogen procedure, diluted to approximately 10,000 c.p.m. and containing approximately 100 pg of protein); 100 μl of PBS containing 0·1% Tween-20, 1% polyvinylpyrrolidone (average MW 360,000; Sigma); and 0·05% NaN_3.

The BSA standards (100 ng/ml–100 μg/ml) were dissolved in a pool of serum obtained from normal, fasting rats. All other dilutions were prepared in phosphate-buffered saline (PBS) containing 0·1% Tween-20. After overnight incubation at room temperature, 100 μl of donkey anti-rabbit precipitating serum (Wellcome, Beckenham, Kent) diluted 1:25 were added. After 4 hr at room temperature, the mixture was centrifuged at 1500 *g* for 45 min, the supernatant decanted and the radioactivity in the precipitate counted in an LKB Ultrogamma 1260 gamma counter (LKB Instruments, Croyden). The counter generated a standard curve using a spline function algorithm and automatically calculated the BSA content of the unknowns. The interassay coefficient of variation was 15%.

Determination of lactulose/rhamnose ratios in urine samples. The levels of the sugars lactulose and rhamnose in samples of rat urine were determined by quantitative thin-layer chromatography, using a modification of the technique of Menzies, Mount & Wheeler (1978).

Urine samples were mixed with internal sugar markers (palatinose and fucose) and the mixtures desalted using duolite MB 5113-mixed ion-exchange resin (BDH; Poole, Dorset). Desalted samples that were not to be analysed immediately were stored frozen at −20°.

Each desalted urine sample was applied in multiple 5-μl volumes to a plastic-backed thin-layer chromatography plate (Schleicher and Schuell, F1500 TLC Ready-Foils, Anderman and Co., Kingston-upon-Thames, Surrey). The chromatograms were subjected to two initial developments using methanol (30

ml), – butanol-1-ol (30 ml) – water (30 ml) followed by three successive runs in the following solvent mixtures: (i) butanol-l-ol (60 ml) – ethanol (30 ml) – glacial acetic acid (10 ml) – water (10 ml); (ii) butanol-l-ol (5 ml) – ethyl acetate (70 ml) – pyridine (15 ml) – glacial acetic acid (10 ml) – water (10 ml); (iii) butanol-l-ol (60 ml) – ethanol (30 ml) – glacial acetic acid (10 ml) – water (10 ml).

Sugars were located on the plate using 4-aminobenzoic acid dissolved in methanol and orthophosphoric acid.

After heat treatment at 120°–125° for 10 min, the plates were scanned using a Chromoscan 3000 (Shandon Ltd, Runcorn, Cheshire). The height of each peak was measured using a blank test strip as a baseline, a graph was constructed of the peak heights for each of the sugar standards (internally corrected) and the concentrations of the sugars in the urines determined. The inter-assay coefficients of variation for the determination of lactulose and rhamnose were 7·4% and 10%, respectively. Full technical details of this methodology are to be published elsewhere.

Quantification of RMCPII in rat serum samples. Serum RMCPII levels were quantified in an ELISA assay. Ninety-six-well round-bottomed Cooke microtitre plates (Sterilin, Feltham) were coated with affinity-purified sheep anti-RMCPII diluted to 1 µg/ml in carbonate-bicarbonate buffer, pH 9·6. One-hundred microlitres of antibody solution were incubated in each well overnight at 4°. The solution was removed and residual binding sites blocked with 4% normal sheep serum, diluted in coating buffer. The plates were then washed six times with PBS containing 0·1% Tween-20 and used immediately.

Samples and standards for assay were diluted in PBS–Tween-20 containing sheep serum diluted 1 in 25. Standards were diluted from a stock solution of 1 µg/ml to give a working range from 0·5 ng/ml to 32 ng/ml.

One-hundred microlitre-volumes of diluted samples and standards were incubated in duplicate in the plate for 1·5 hr at 37°. The plate was then washed six times with PBS–Tween-20 before the addition of sheep anti-RMCPII–horseradish peroxidase conjugate (150 µl per well, diluted to 1 in 2000 in PBS–Tween). After further incubation at 37° for 1·5 hr, the plate was washed six times with PBS–Tween-20. One-hundred microlitre-volumes of ortho-phenylene diamine (40 mg per 100 ml H_2O_2-citric acid phosphate buffer, pH 5·0) were then added to each well to provide a substrate, and the reaction stopped by the addition of 20 µl of 0·4 M H_2SO_4 to each well. Absorbance values at 492 mm were read using a Titertek Multiscan Plate Reader (Titertek, Flow Laboratories, Irvine, Ayrshire).

Statistical analyses

Non-parametric Wilcoxon rank sum tests were used when comparing various groups. One- and two-tailed tests were used as appropriate.

Kendall's rank correlation method was used to determine rank correlation coefficients (τ).

RESULTS

The model of bystander protein uptake published by Bloch & Walker (1981) involved challenging the sensitized animals either by gavage or by intraduodenal laparotomy. In our hands the latter procedure was technically superior and all of the results to be reported were obtained with this method. Bloch & Walker

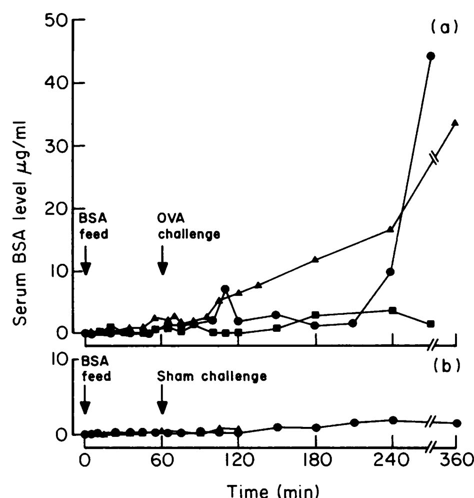

Figure 1. Serum BSA levels measured in cannulated rats. (a) Three rats, presensitized with OVA, received a BSA feed at Time 0 and an intraduodenal OVA challenge 60 min later. (b) Two presensitized rats received a BSA feed at Time 0 but no OVA challenge.

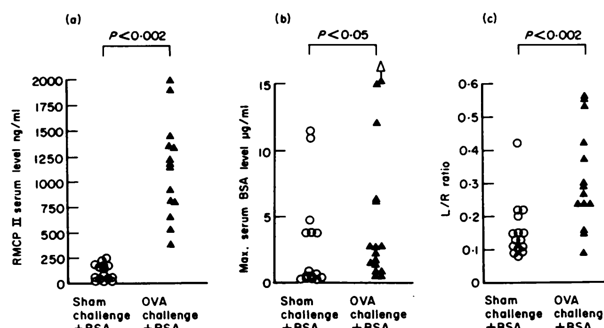

Figure 2. Serum RMCPII levels (a), maximum serum BSA levels (b) and lactulose/rhamnose ratios (L/R) (c) in presensitized rats following an intraduodenal OVA-challenge (▲) or a sham-challenge operation (○). The level of BSA in the off-scale sample shown in (b) was 100 µg ml.

(1981) obtained blood samples at 1, 3 and 6 hr after challenge, to account for possible variations in the rate of BSA uptake. In order to exclude the possibility of very early high uptake, we measured BSA levels in blood samples obtained at frequent intervals from cannulated animals (Fig. 1). Although only three challenged and two control animals were studied, it was clear that the rate of uptake was relatively slow and variable from animal to animal. In this pilot study, two of three test animals showed sharp rises in measurable BSA between 3 hr and 6 hr after the OVA challenge. In the experiments subsequently reported, we restricted blood sampling times to 1, 3 and 6 hr. The maximum level of BSA detected was then selected for all group comparisons. Frequently, though not invariably, this was present in the 6-hr sample. As expected, there was also some uptake of BSA in one of the two control animals, but subsequent work (see below) suggests that animals challenged with antigen absorbed significantly greater amounts of BSA than did control animals undergoing sham challenge.

In the definitive comparative study of protein and sugar uptake, all of the rats immunized by the protocol outlined in

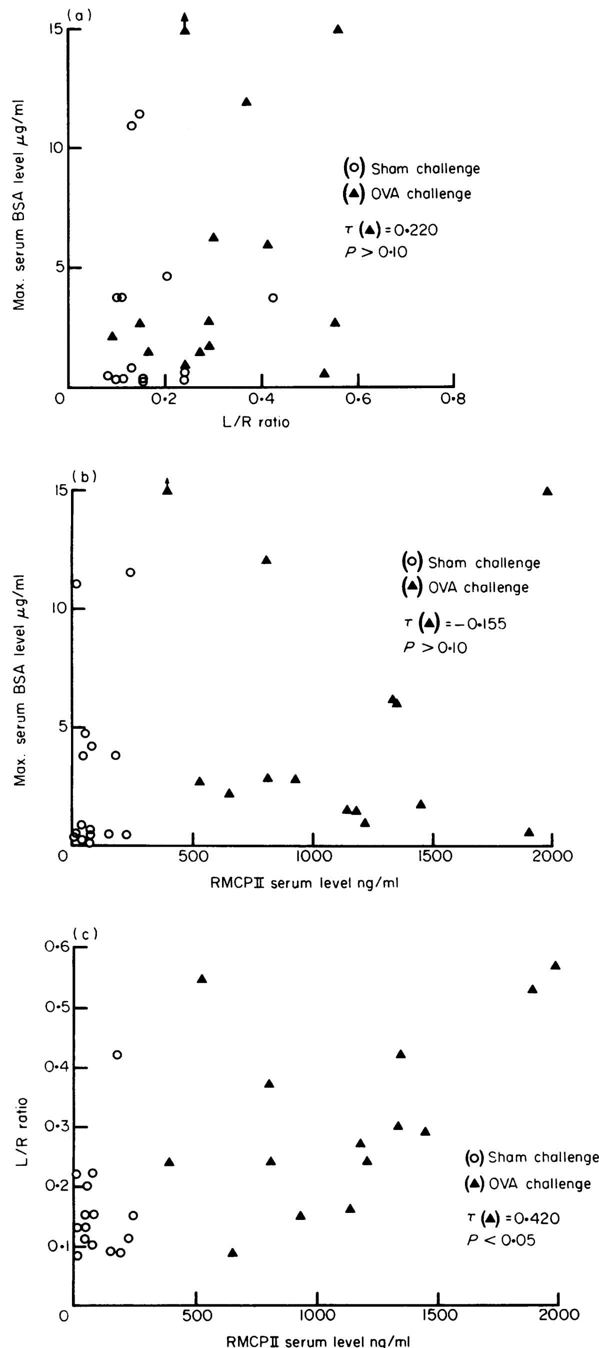

Figure 3. (a) Correlation between bystander protein uptake measured as maximum serum BSA levels and lactulose/rhamnose ratios in rats receiving an intraduodenal OVA-challenge (▲) or a sham-challenge operation (○). The off-scale BSA level was 100 μg/ml. (b) Correlation between bystander protein uptake and serum RMCPII levels in rats receiving an intraduodenal OVA-challenge (▲) or a sham-challenge operation (○). The off-scale BSA level was 100 μg/ml. (c) Correlation between lactulose/rhamnose ratios and serum RMCPII levels in rats receiving an intraduodenal OVA-challenge (▲) or a sham-challenge operation (○).

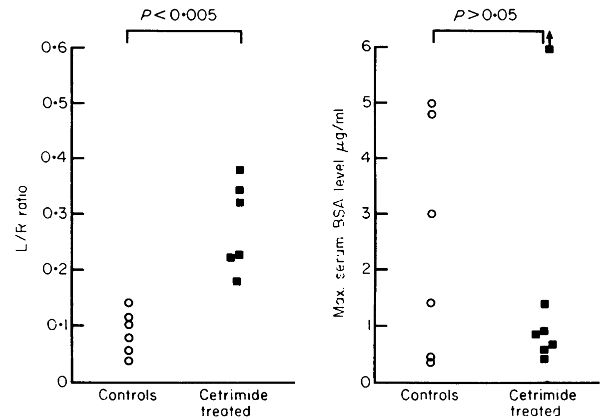

Figure 4. Lactulose/rhamnose ratios and maximum serum BSA levels in unsensitized rats gavaged with sugar–protein mixtures (○) or sugar–protein–cetrimide mixtures (■). The off-scale BSA level was 15 μg/ml.

Table 1 were shown by PCA testing to be systemically sensitized to OVA (titres ranged from 1 in 10 to 1 in 100). In order to obtain evidence of local sensitization, the serum samples obtained 1 hr after challenge were analysed for the presence of the enzyme RMCPII, a specific product of the mucosal mast-cell. There was unequivocal evidence of RMCPII release into the serum of all rats challenged intraduodenally with OVA, whereas the serum of sham-challenged animals contained very low levels of the enzyme (Fig. 2a). The difference between the groups was highly significant ($P < 0{\cdot}002$).

The levels of bystander BSA protein detected in the serum of the rats is shown in Fig. 2b. Although there was clearly a good deal of overlap between the positive-challenge and sham-challenge groups, the animals receiving the intraduodenal OVA challenge did have significantly higher BSA levels in the serum than the controls ($P < 0{\cdot}05$).

The addition of lactulose and rhamnose to the OVA challenge solution introduced at the laparotomy stage permitted simultaneous evaluation of protein and sugar uptake. As shown in Fig. 2c there was a markedly increased permeability to sugars in the positive-challenge animals as expressed by the lactulose/rhamnose ratios measured in urine samples ($P < 0{\cdot}002$).

In order to investigate whether or not there was a correlation between increased uptake of the BSA bystander protein and increased permeability to the low-molecular weight sugar probes, the data presented in Fig. 2 were replotted (Fig. 3a) and evaluated using Kendall's rank correlation method. Although there was a weak positive correlation between the two measurements when OVA-challenged animals were considered (0·220) this failed to reach statistical significance ($P < 0{\cdot}10$). Similarly, there was no correlation between bystander protein uptake and the serum RMCPII level of these animals (Fig. 3b). In contrast, however, there was a positive correlation (0·420) between the lactulose/rhamnose ratios and the serum RMCPII levels of the OVA-challenged group (Fig. 3c) and this was statistically significant ($P < 0{\cdot}05$).

We also induced gut damage in naive, unsensitized animals with the mild ionic detergent cetrimide. Both BSA uptake and changes in sugar permeability were measured and these results are presented in Fig. 4. Using a dose of 4 mg of cetrimide there was no evidence of increased BSA uptake compared to the

controls. In contrast, the lactulose/rhamnose ratios of cetrimide-treated rats were significantly elevated compared to the ratios observed in control animals ($P < 0.002$).

DISCUSSION

Low levels of protein antigens have been detected in the serum of healthy individuals following the ingestion of egg or milk (Paganelli *et al.*, 1979, 1981, 1983; Husby, Jensenius & Svehag, 1985) and similar studies in patients with atopic eczema have also shown increased amounts of such proteins (Paganelli *et al.*, 1979, 1981, 1983). One possible explanation for the latter observation invokes an increased permeability to food antigens in the gut of the eczematous individual and several groups have attempted to measure gastrointestinal absorption of various inert molecules in order to investigate this further. For clinical studies in man we have chosen to use the sugars L-rhamnose (a monosaccharide of 164 MW) and lactulose (a disaccharide of 342 MW), both of which are uncharged, metabolically inert molecules of poor lipid solubility (Dahlqvist & Gryboski, 1965; Fordtran *et al.*, 1965). L-rhamnose passes across the gut wall more readily than lactulose (Menzies, 1983), and any increase in the excretion ratio is believed to reflect a greater leakiness at intercellular junctions or at areas of enterocyte loss.

We have recently shown that a proportion of children with atopic eczema have an increased lactulose/rhamnose excretion ratio (Pike *et al.*, 1986), with the younger children having the highest lactulose/rhamnose ratios. Although the underlying aetiology remains unclear, these findings provide no direct evidence for enhanced antigen uptake in eczematous patients since the mechanism for increased lactulose absorption may not be the same as that for food antigens. Identifying a protein probe which is totally unrecognized by the human immune system, thereby avoiding problems of immune clearance through antigen–antibody complex formation, is a major difficulty with human subjects and justifies the use of appropriate animal models.

Bloch & Walker's (1981) original observations of an increased uptake of bystander protein in pre-sensitized rats undergoing local antigen challenge were confirmed in this study, but the difference between antigen-challenged and sham-challenged animals was not striking. There was considerable overlap between the groups, presumably reflecting, in part, the tendency of the normal gut to take up small amounts of intact protein. Byars & Ferraresi (1976), using a different rat model, suggested that maximal gut permeability occurred 45 min after inducing a local hypersensitivity reaction, but Bloch & Walker (1981) found, when sampling at 1, 3 and 6 hr, that maximal BSA levels could occur at any of these times, and our cannulation experiments also suggest that responses are not uniform.

Measuring serum levels of RMCPII provided unequivocal evidence of local intestinal hypersensitivity reactions in those animals receiving local OVA challenges. The lowest value in an animal challenged with OVA was higher than the maximum 'resting' value of a sham-challenged animal, suggesting that the experimental protocol using systemic immunization also gave rise to a local sensitization.

The significant positive correlation between serum RMCPII levels and the increased lactulose/rhamnose excretion ratios of animals experiencing a local hypersensitivity reaction contrasted with the lack of any correlation between bystander protein uptake and either sugar permeability or RMCPII release. This suggests that the two processes (i.e. protein and sugar uptake) should probably be considered as quite distinct.

Various chemical agents, such as alcohol and detergents, have been used previously in both man and experimental animals to induce gut damage and an altered gastrointestinal permeability. For example, Cobden, Rothwell & Axon (1981) reported an increased lactulose uptake in rats receiving the detergent cetrimide, and we have confirmed that effect. Similarly, in man, Maxton *et al.* (1984) were able to demonstrate increased permeability to a range of probe molecules after the administration of cetrimide. In the present study we also searched for evidence of increased protein uptake in the animals receiving cetrimide. Our failure to observe any effect on protein absorption may be a true reflection of events in the gut or merely the result of abnormal handling of protein molecules after exposure to detergent. In contrast, Tagesson *et al.* (1985) reported increased permeability of the rat ileum to BSA in the presence of the naturally occurring detergent lysophosphatidylcholine, but jejunoileal differences could invalidate comparisons of absorptive studies, at differing sites in the small bowel.

We conclude from the various studies reported here that changes in intestinal permeability to sugars and large proteins are not correlated in any simple fashion in the rat. It remains to be established whether or not these data can be extrapolated to man.

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