

Mucosal mast cell activation patterns in the rat following repeated feeding of antigen

M. W. TURNER, GABRIELLE E. BARNETT and S. STROBEL

Department of Immunology, Institute of Child Health, London, U.K.

Summary

Groups of rats previously sensitized systemically on day 0 with a low dose of ovalbumin (OVA) were gavaged daily with ovalbumin or bovine serum albumin or a mixture of both proteins from day 14 to 18. Blood samples were obtained pre- and post-challenge and serum levels of the rat mast cell protease II (RMCPII) determined by immunoassay. Release of this specific mucosal mast cell mediator was only observed in animals challenged with ovalbumin and the initial challenge released levels of RMCPII 15-fold higher than normal resting levels ($P < 0.001$). Subsequent daily challenges evoked the release of significantly lower levels of mediator ($P < 0.001$ relative to day 14), but with one exception each test group released significantly more RMCPII than the matched control group on each day ($P < 0.001$ – $P = 0.015$). An increased uptake of BSA 'bystander' protein was observed when OVA-sensitized animals were repeatedly gavage-challenged with OVA but there was no correlation with the release of RMCPII mediator. After a 9-day rest period the levels of RMCPII released 6 hr post-challenge on day 26 were still significantly lower ($P = 0.004$) than the levels of mediator released on day 14. In contrast, animals not previously challenged were still capable of releasing high levels of mediator at the time of first mucosal contact. The levels of RMCPII detected in the serum after enteral protein antigen challenge never exceeded 6000 ng/ml and were lower than those previously observed in parasitized rats following intravenous antigen challenge.

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Introduction

An animal model of food allergy would permit detailed investigations of the mechanisms underlying this ill-defined syndrome. Following earlier work in calves [1] and rats [2] we confirmed the observation that rats rendered hypersensitive to one food protein antigen would, when subsequently challenged locally in the gut, absorb increased amounts of an unrelated 'bystander' protein antigen [3]. The induction of local intestinal hypersensitivity was confirmed by measuring serum levels of rat mast cell protease II (RMCPII), a mediator released by mucosal mast cells but not by connective tissue mast cells [4].

A major disadvantage of the above model was the

requirement to induce hypersensitivity reactions by instillation of antigen into the duodenum at laparotomy. We have now developed a model in which challenge by gavage obviates the need for laparotomy and permits long-term studies of repeated antigen exposure and mediator release in anaphylactically sensitized animals. We believe that this model more closely mimics the clinical condition and is more relevant to the investigation of regulatory events in intestinal hypersensitivity states.

Materials and methods

Animals

Wistar rats obtained from the Moredun Research Institute (Edinburgh, U.K.) and established as an in-house breeding colony at the Institute of Child Health were used for all of the experiments. They were raised on a diet free of egg and milk proteins ('Labsure CRMS', Labsure, Manea, U.K.) and maintained on this feed throughout.

Correspondence: Dr M. W. Turner, Department of Immunology, Institute of Child Health, 30 Guilford Street, London WC1N 1EH, U.K.

Animals were used at 8–10 weeks of age, and with the exception of Experiment 1 (defining appropriate challenge doses), each experimental group comprised six to eight animals with equal numbers of males and females.

Experimental protocols

General procedure. The rats were sensitized on day 0 with an intraperitoneal injection of 100 µg ovalbumin (OVA) (Sigma Grade V, Poole, U.K.) in 1 ml alum. On day 14 all groups of rats received a gavage feed (2 ml) containing either 100 mg OVA and/or 700 mg BSA (Sigma fraction V), dissolved in bicarbonate buffer (0.15 M NaHCO₃–0.15 M NaCl, pH 8.2), or the buffer alone. The gavage feeds were introduced into the stomach using a stainless steel feeding tube (Fine Science Tools Inc., North Vancouver BC, Canada).

Blood samples were collected immediately before gavage and, according to the experiment, at various time intervals up to 6 hr post gavage. The samples were obtained either by tail vein bleeding (*c.* 300 µl) or terminal cardiac puncture (*c.* 10 ml). All blood collection was carried out whilst the animals were anaesthetized (nitrous oxide–halothane inhalation). Sera were stored at –20°C.

Experiment 1

In order to determine the optimum OVA challenge dose for use in subsequent experiments we used rats presensitized to ovalbumin to investigate the effect of different doses of gavaged OVA on the levels of RMCPII released into the serum. Adult rats previously sensitized to ovalbumin on day 0 were challenged by gavage on day 14 with either 10 µg, 100 µg, 1 mg, 10 mg or 100 mg OVA dissolved in 2 ml of bicarbonate buffer. A control group received no OVA in the gavage. Blood was obtained by cardiac puncture 10 min, 1, 3 and 6 hr post-challenge and the sera assessed in a competitive enzyme-linked immunosorbent assay for levels of rat mast cell protease II.

Experiment 2

In order to investigate the kinetics of serum RMCPII release and bystander protein uptake in animals challenged repeatedly with OVA over a period of 5 days, four groups of animals (A–D) were sensitized to OVA on day 0 and were gavaged on days 14–18, as indicated below, with 2 ml bicarbonate buffer, with or without added OVA and/or BSA.

Group A—bicarbonate buffer alone; Group B—100 mg OVA; Group C—100 mg OVA + 700 mg BSA; Group D—700 mg BSA. Animals in a further group, E, were not sensitized to OVA but on days 14–18 were gavaged with 100 mg OVA + 700 mg BSA in 2 ml of bicarbonate buffer.

Table 1. Protocol for studying the kinetics of repeated gavage challenges in animals previously challenged and then rested

Group	OVA sensitization Day 0	OVA challenge		Samples and assays
		Days 14–16	Days 26–28	
F	+	+	–	Blood taken at 0 hr (pre-challenge) and 6 hr post-challenge for RMCPII assays
G	+	–	+	
H	+	+	+	
I	+	–	–	
J	–	+	–	

Blood was collected from all groups of animals 1, 3 and 6 hr post-gavage on day 14. On days 15–18 tail vein blood samples were taken (under anaesthesia) immediately prior to feeding and at 6 hr post-gavage. Serum samples were stored at –20°C before being assayed for levels of both BSA and RMCPII using the appropriate ELISA techniques. Note that 0 hr on consecutive days was approximately 16 hr after the 6 hr bleed on the previous day.

Experiment 3

This experimental protocol is summarized in Table 1. Adult rats sensitized to OVA on day 0 were challenged on days 14–16 and then rested for a 9-day period. Following this the animals were again challenged on days 26–28. Rats in groups F–I (sensitized to OVA) were gavaged as indicated below; animals in Group J were an unsensitized control group.

Days 14, 16: Group F—100 mg OVA; Group G—bicarbonate buffer only; Group H—100 mg OVA; Group I—bicarbonate buffer only; Group J—100 mg OVA. Following the 9-day rest period the animals in each group were challenged as follows on days 26–28: F—bicarbonate buffer only; G—100 mg OVA; H—100 mg OVA; I—bicarbonate buffer only; J—bicarbonate buffer only.

Blood for serum RMCPII measurements was obtained by tail vein bleeding immediately prior to the gavage stage (0 time sample) and 6 hr later.

Assays

Quantification of RMCPII in rat serum samples. Serum RMCPII levels were quantified in a competitive ELISA. Flat-bottomed 96-well Dynatech Immulon2 micro-ELISA plates (Dynatech Laboratories, Billingham, U.K.) were coated with 50 µl of RMCPI (1 µg/ml) diluted in carbonate buffer, pH 9.6, and incubated overnight at

4°C. In a separate plate, the standards and samples were incubated with an equal volume (50 µl/well) of affinity-purified sheep anti-RMCPII-horseradish peroxidase conjugate (diluted to 1/1000 in PBS containing 0.05% Tween-20 (PBS-T)). Standards were diluted from a stock solution at 1 µg/ml using PBS-T containing 4% normal rat serum to give a working range from 10 to 100 ng/ml. Samples were diluted (1/10, 1/20 or 1/100) in the same buffer.

Both samples and standards were set up in duplicate (total reaction volume 100 µl/well) and the plate incubated at 37°C for 1 hr.

The RMCPII-coated plate was then washed six times with PBS-T and 50 µl aliquots of sample plus conjugated antibody, or standard plus conjugated antibody, were transferred from the non-coated to the RMCPII-coated plate. After a further incubation at 37°C for 1.5 hr, the plate was washed six times with PBS-T. Fifty microlitres *o*-phenylene diamine (40 mg per 100 ml of citric acid-phosphate buffer pH 5 with 40 µl H₂O₂) were added to each well as substrate, and the reaction stopped by the addition of 4 N H₂SO₄ (50 µl/well). Absorbance values at 492 nm were read using a Titertek Multiskan platereader (Titertek, Flow Laboratories, Irvine, U.K.).

Quantification of BSA in rat serum samples. Serum BSA levels were quantified in an ELISA. Flat-bottomed 96-well microtitre plates (Linbro, Rickmansworth, U.K.) were coated with affinity-purified rat IgG anti-BSA, diluted to 2 µg/ml in carbonate-bicarbonate buffer, pH 9.6. (This antibody was prepared in our own laboratory using the following procedures. Wistar rats were immunized subcutaneously in two sites with 100 µg BSA (Fraction V; Sigma Chemical Co.) using antigen emulsified in Freund's complete adjuvant (primary immunization) or Freund's incomplete adjuvant (booster immunizations). Anti-BSA antibodies in the serum were purified by sequential elution from a Sepharose 4B-BSA affinity column using (1) 0.1 M glycine-HCl pH 2.8, (2) 0.1 M glycine-HCl pH 2.5 and (3) 0.1 M glycine-HCl pH 2.2. These eluants were pooled and applied to a Protein-A column for purification of the IgG fraction.)

Volumes (100 µl) of diluted antibody were incubated in each well overnight at 4°C. The plates were then washed six times with PBS containing 0.1% Tween-20 and residual binding sites were blocked with 2% normal rat serum diluted in PBS-T (100 µl/well). After incubation for 2 hr at room temperature, the plates were washed once with PBS-T. Samples and standards, diluted in PBS-T containing 1% normal rat serum, were incubated in duplicate (100 µl/well) for 24 hr. The standard working range was 100 ng to 1 mg. The plates were then washed six times with PBS-T and 100-µl aliquots of affinity-purified

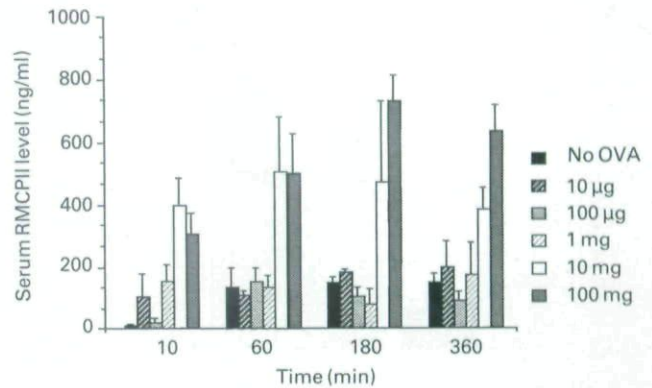


Fig. 1. Adult rats were sensitized to ovalbumin (OVA) on day 0 and then gavaged with various doses of OVA on day 14. Rat mast cell protease II (RMCPII) levels (mean + s.e.m.) were measured by ELISA in serum samples obtained at 10 min and 1, 3 and 6 hr after the challenge feeds (four animals per group at each time point).

rat IgG anti-BSA biotinylated antibody, diluted to 1 µg/ml in PBS-T containing 1% normal rat serum, were added to each well. The plates were incubated for 20 hr at 4°C and washed with PBS-T. Streptavidin peroxidase (Amersham International, Aylesbury, U.K.), diluted to 1/1000 in PBS-T, was then added (100 µl/well) and the plates incubated for a further hour at 37°C. The plates were washed six times with PBS-T, 100 µl of *o*-phenylene diamine substrate were added to each well and colour development evaluated as described above.

Statistical analyses

The statistical significance of differences in RMCPII levels and BSA levels between groups or between different times within the same group was analysed using the non-parametric Mann-Whitney *U*-test (two-tailed). Spearman rank correlations between BSA and RMCPII levels were sought using the STATVIEW 512+ software package from BrainPower Inc., Calabasas, U.S.A.

Results

Experiment 1

In order to establish the optimal challenge dose of ovalbumin for use in the gavage experiments, doses ranging from 10 µg to 100 mg were administered to pre-sensitized rats in 2 ml of buffer. Figure 1 shows the levels of mucosal mast cell mediator RMCPII subsequently detected in serum (expressed as ratios relative to background levels in normal rat serum).

There was an increased release of RMCPII with doses of 10 µg, 100 µg and 1 mg but at most time points this was

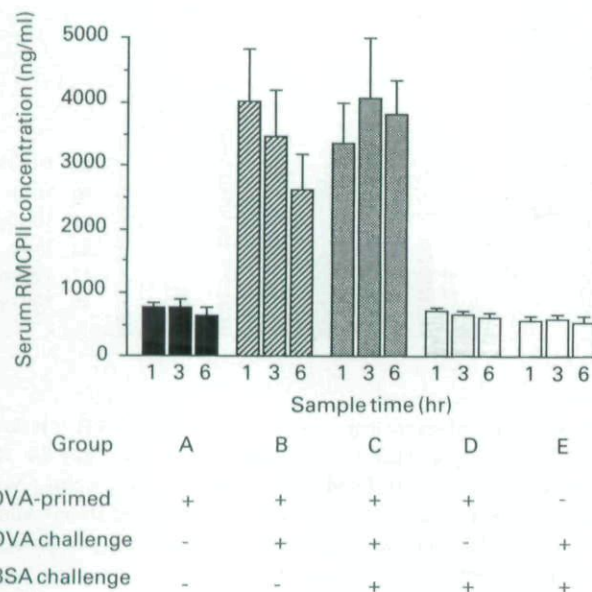


Fig. 2. RMCPII levels (mean $\pm$ s.e.m.) in the serum of various groups of rats primed and challenged by gavage as indicated. All challenges were performed 14 days after priming. Serum samples were withdrawn 1, 3 and 6 hr after gavage and assayed for RMCPII by ELISA.

similar to that induced by buffer alone. In contrast, 10 mg of OVA induced significantly more RMCPII release (peaking at 60 min) and 100 mg of OVA induced the highest levels of mediator, with a maximum at 180 min. The 100 mg dose was chosen for all subsequent challenge experiments.

Experiment 2

This experiment investigated both the kinetics and specificity of gavage challenges administered to rats over a period of 5 days. Animals in the various groups were challenged on day 14 and RMCPII levels measured 1, 3 and 6 hr later (Fig. 2).

Compared to unprimed controls (group E) or controls not challenged with OVA (groups A and D), markedly elevated levels of RMCPII were detected in the serum of animals pre-sensitized to ovalbumin and gavaged with this protein (groups B and C). These results show that the release of RMCPII was antigen specific and only occurred in animals both primed and gavaged with OVA.

The RMCPII levels of rats in groups A–D, repeatedly gavaged as appropriate from day 14 to 18 inclusive, are shown in Fig. 3. Animals in both group B (primed with OVA, gavaged with OVA) and in group C (primed with OVA, gavaged with OVA+BSA) showed 15-fold increases in RMCPII levels relative to normal rat serum when challenged on day 14. In both groups the RMCPII

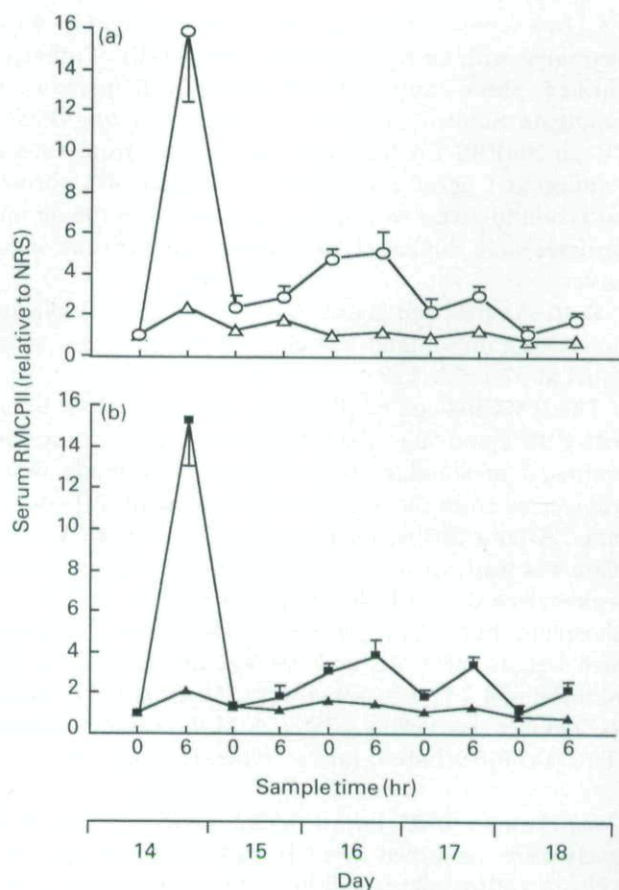


Fig. 3. Mean ($\pm$ s.e.m.) serum levels of RMCPII in groups of rats gavaged consecutively for 5 days commencing 14 days after priming with ovalbumin. (a) Group A (Δ , $n=8$), primed with OVA and challenged with bicarbonate buffer; Group B ($\circ$, $n=6$), primed with OVA and challenged with OVA. (b) Group C ($\blacksquare$, $n=8$), primed with OVA and challenged with OVA+BSA; Group D ($\blacktriangle$, $n=8$) primed with OVA and challenged with BSA. Results are expressed as the ratio of the mean RMCPII in the test sample relative to the background level in a pool of normal rat serum. For the sake of clarity, s.e.m. error bars are shown for the positive challenge groups B and C only.

level 6 hr post-gavage was highly significantly elevated compared to the zero time sample on day 14 ($P < 0.001$ for both comparisons). In contrast, the control groups A and D showed only a twofold increase relative to normal rat serum. Following gavage over days 15–18, relatively more RMCPII continued to be released into the serum of animals in groups B and C compared to groups A and D, but the peaks were much lower and appeared to be decreasing in amplitude with time. The differences in RMCPII levels between groups A and B and between groups C and D 6 hr post-challenge were statistically significant in every case except between groups A and B

Table 2. Statistically significant differences in RMCPII release of rats in groups A and B (Fig. 3a) and of rats in groups C and D (Fig. 3b)

6-hr sample	Day				
	14	15	16	17	18
Group A vs. Group B	B > A $P < 0.001^*$	B > A n.s.	B > A $P < 0.001$	B > A $P = 0.002$	B > A $P = 0.001$
Group C vs. Group D	C > D $P < 0.001$	C > D $P = 0.015$	C > D $P = 0.002$	C > D $P = 0.002$	C > D $P = 0.002$

The protease was measured in blood taken 6 hr after a gavage challenge at time zero on days 14–18 post-sensitization

* All comparisons by two-tailed Mann-Whitney rank sum test.

n.s. = not significant ($P > 0.05$).

on day 15 (Table 2). The level of mediator detected 6 hr post-challenge on day 14 in groups B and C was significantly higher than that measured at the same time on days 15–18 in the same groups ($P < 0.001$ for all comparisons).

Animals in groups C and D received BSA in the gavage and this bystander protein was assayed in the serum of these animals over the time course of the experiment. Figure 4 shows the mean BSA level at each time point in the two groups. In group C animals the serum levels of immunoreactive BSA were always higher at the 6 hr sampling time than at zero time and overall, with the exception of the 6 hr sampling time on day 14, the levels detected significantly exceeded those measured in the group D control animals ($P < 0.0001$ – $P = 0.01$).

In order to investigate whether BSA bystander protein uptake was related to the RMCPII profile of the animals (i.e. whether high RMCPII release resulted in a high BSA uptake), correlations were sought in the 6 hr blood samples obtained on days 14–18. On day 15 only was there a weakly significant positive correlation ($r = 0.704$; $P < 0.05$). As there was no discernible consistent pattern of correlation we believe this single result, though statistically significant, is probably not biologically relevant.

Experiment 3

This experiment studied the kinetics of RMCPII release in animals that received gavage challenges, were rested for 9 days and were then re-gavaged. As shown in Fig. 5, the animals continued to release RMCPII when gavaged with OVA on days 26, 27 and 28. Thus in group H animals the 6 hr sample on day 26 contained significantly more RMCPII than did the zero time sample on this day ($P < 0.002$) but this level was itself significantly lower than

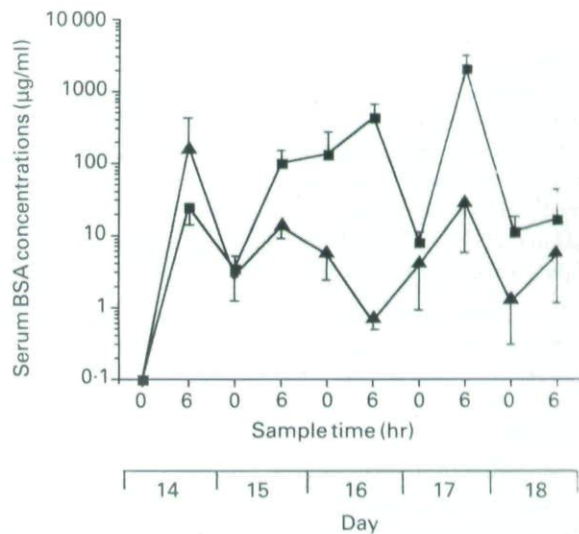


Fig. 4. Serum levels (mean $\pm$ s.e.m.) of the bystander protein BSA in groups of rats gavaged consecutively for 5 days commencing 14 days after priming with ovalbumin. Groups C (■) and D (▲) are as defined in the legend to Fig. 3b. Blood samples were obtained pre-challenge (0) and 6 hr later (6) on each day.

the 6 hr release of group G animals on day 26 ($P = 0.002$) (see below) or the 6 hr release of group H animals on day 14 ($P = 0.004$). There was no evidence that the mucosal mast cells could recover their original capacity to release RMCPII. This phenomenon was not related to the time interval between immunization and challenge because a group of animals which were not gavaged on days 14 and 16 but received their first challenge on day 26 (group G) showed a pattern of RMCPII release similar to that of group B in Fig. 3a. Thus the 6 hr sample from group G animals on day 26 contained significantly more RMCPII than did the zero time sample of those animals ($P < 0.002$)

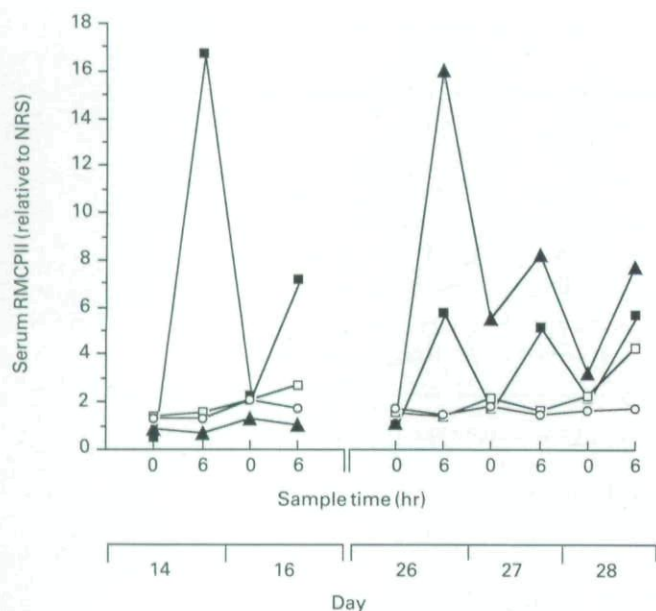


Fig. 5. Mean serum levels of RMCPII in groups of rats treated as follows: Group G (▲, $n=7$) primed with OVA on Day 0, gavaged with buffer on days 14, 16 and with OVA on days 26, 27 and 28; Group H (■, $n=8$) primed with OVA on day 0, gavaged with OVA on days 14, 16, 26, 27 and 28; Group I (□, $n=6$) primed with OVA on day 0, gavaged with buffer on days 14, 16, 26, 27 and 28; and Group J (○, $n=6$) non-primed rats, gavaged with OVA on days 14 and 16.

but it did not differ significantly from the 6 hr sample obtained from group H animals on day 14 ($P>0.05$).

Discussion

We have previously described a rat model of intestinal hypersensitivity which involved intraduodenal antigen challenge following gavage with an unrelated bystander protein. Using this model we showed that the enhanced uptake of the bystander protein observed following antigen challenge did not correlate directly with an increased lactulose/rhamnose excretion ratio or with release of the mucosal mast cell protease (RMCPII) which occurred concomitantly [3]. These results suggest that gut permeability to macromolecular proteins and low molecular weight sugars are not related in any simple fashion and that the extent of mucosal mast cell activation does not directly determine the degree of gut leakiness to proteins. In further studies using the same basic protocol we were able to modulate the intestinal hypersensitivity reactions by the administration of various anti-allergic drugs [5; Turner *et al*, unpublished observations].

The present model in which antigen challenges were administered by the much less invasive gavage procedure permitted repeated challenges in the same animal. We

believe that such chronic exposure to antigen is more akin to the human gastrointestinal hypersensitivity states when an unsuspected food allergen may be repeatedly ingested.

The patterns of RMCPII mediator release following repeated antigen challenge were similar in three separate groups of animals. A high initial release was followed by significantly smaller peaks on subsequent days. Even a 9-day rest period did not restore the initial responsiveness and low levels of mediator continued to be released as animals were subjected to further challenges.

Overall, the levels of RMCPII detected in the systemic circulation following the initial protein antigen challenges were in the range of 1000–6000 ng/ml. These levels are lower than those reported in primed rats challenged i.v. with extracts of the worm *Nippostrongylus brasiliensis* [6] where levels up to 400 μ g/ml were recorded. Even in primary nematode infections with *Trichinella spiralis*, *Eimeria nieschulzi* and *N. brasiliensis* 1–4 μ g/ml of RMCPII can be detected systemically in the first 2 weeks of infection [7]. These results suggest that in the present challenge model the capacity of the animals to synthesize and release RMCPII is not exhausted, even though the numbers of mucosal mast cells in the non-parasitized rat are significantly lower and absolute levels of RMCPII will also be correspondingly lower. Why, after a period of rest, the initial high response is not replicated following re-challenge with antigen remains unclear, but candidate mechanisms such as Fc-receptor blockade or down-regulation need to be considered.

RMCPII release also occurred in control animals, i.e. over and above the 'resting' background level measured in normal rat serum (see Fig. 2). This presumably reflected a stress response in animals subjected to repeated handling.

The incorporation of BSA bystander protein into the gavage mixture permitted the question of protein uptake and mediator release to be re-examined with the present model. The levels of gavaged bystander protein measured in the serum of rats after an intraduodenal challenge are known to overlap with the levels of protein detected in the serum of sham-challenged control animals [3,5]. Nevertheless, a statistically significant increase in uptake has been repeatedly demonstrated. In the chronic challenge gavage model the effect was again observed and with the exception of day 14 the mean uptake of BSA as measured at 6 hr was always significantly higher in the test group than in the controls. There was also a trend for the 6 hr BSA levels to increase with each challenge suggesting increasing 'leakiness' of the gut. However, with the exception of day 15 there was no correlation between the 6 hr BSA and RMCPII levels which further supports our

earlier conclusion that mucosal mast cell activation and increased gut permeability to protein antigens are not related in any simple fashion [3].

We believe that the present model closely mimics the chronic antigen challenge which typifies many human intestinal hypersensitivity states and should provide a basis for further studies on underlying mechanisms and possible therapeutic strategies. The restricted distribution of rat mucosal mast cells is particularly advantageous for such studies and is not paralleled in other species. For example, the distribution of human mast cell types is much more complex. Although two types with distinct neutral protease compositions have been described [8], the type most analogous to the mucosal mast cell of rodents (the so-called T mast cell containing mostly tryptase proteinase) has a wider tissue distribution. It accounts for 98% of mucosal mast cells but is also strongly represented in the alveolar (93%) and bronchial (77%) mast cell populations, a finding which is compatible with the existence of a common mucosal mast cell population. A consequence of this more widespread distribution is that at present there is no known human mucosal mast cell mediator which is equivalent to RMCPII, i.e. the synthesis of which is restricted to the gut. Despite the limitations of a rodent model in studying human disease, it is likely to remain an important focus of experimental work on mucosal hypersensitivity to food proteins. In addition to controlled comparative studies of various pharmacological agents, the measurement of other newly synthesized mediators of the cyclo-oxygenase and/or lipoxygenase pathways, which are known to be released into the gastrointestinal tracts of parasitized rats [9], would also be of interest in relation to food protein hypersensitivity.

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