

A role for the hepatobiliary system in IgE-mediated intestinal inflammation in the rat

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Summary

Background For many years the central focus of research into gastrointestinal hypersensitivity reactions has been the mast cell population of the intestinal lamina propria. Since bile is known to deliver immunological mediators to the gastrointestinal tract, the possibility arises that extra-intestinal populations of mast cells may also contribute to IgE-mediated intestinal damage.

Objectives To characterize hepatic mast cells in the rat and to investigate the role of the hepatobiliary system in a model of IgE-mediated reactivity to dietary antigen.

Methods Wistar rats were passively sensitized with monoclonal antidinitrophenyl (DNP) IgE antibodies, and were later challenged orogastrically with DNP-HSA. Additional animals were sensitized, then bile duct-cannulated prior to antigen challenge. At various time points, liver and intestinal samples were collected for histological examination, and bile was collected and assayed for histamine and $\text{TNF}\alpha$.

Results Hepatic mast cells display a mucosal mast cell-like phenotype, and are closely associated with the vessels of the portal triads. Orogastric antigen challenge led to a rapid and significant decline ($P < 0.0001$) in detectable mast cells as a result of anaphylactic degranulation. The median number of granulated mast cells associated with each portal triad in liver sections declined from six per portal triad to one per portal triad post-antigen challenge. After 15 min, biliary histamine concentrations rose above background levels ($P < 0.01$). $\text{TNF}\alpha$ was also detectable in the majority (4/6) of bile samples within 15 min of challenge. Histological examination of the gastrointestinal mucosa revealed disruption to the villous epithelium ranging from oedematous changes to gross destruction. Such damage was not seen in animals in which bile had been externally drained.

Conclusion The data indicate that biliary products are major contributors to the gastrointestinal damage arising from IgE-mediated hypersensitivity reactions in the rat, and such hypersensitivity reactions may involve a population of mast cells which reside in the liver.

Keywords: hepatic mast cell, IgE, hypersensitivity, hepatobiliary, food allergy

Clinical and Experimental Allergy, Vol. 29, pp. 262–270. Submitted 1 August 1997; revised 21 November 1997; accepted 3 March 1998.

Introduction

Food hypersensitivity and food allergy are terms used to describe a diverse group of clinical conditions ranging from acute, life-threatening anaphylaxis to chronic, debilitating conditions such as food-induced eczema, eosinophilic

gastroenteritis, diarrhoeic-type irritable bowel disease and other food-induced gastroenteropathies [1]. Nausea, vomiting, abdominal pain and diarrhoea are symptoms associated with most of these conditions. Gastrointestinal manifestations are also a common feature of systemic anaphylaxis in humans [2] and in animal models [3], while allergic asthma may sometimes be triggered by food antigens [4]. Although the underlying pathophysiological basis for these symptoms remains unclear, Type 1 IgE-mediated immediate

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hypersensitivity reactions are clearly associated with much food sensitivity [5].

The development of animal models has been crucial to the advancement of our understanding of IgE-mediated gastrointestinal hypersensitivity over the last 20 years, with published models focusing on the actions of mast cells from the gastrointestinal lamina propria and submucosa [3,6,7]. While these cells can certainly be accorded a watchkeeping role at mucosal surfaces, key features of hypersensitivity reactions suggest that the emphasis on these cells might be misplaced. For example, studies of IgE-mediated antiparasite defences have shown that there is neither a change in the morphology nor in the numbers of mucosal mast cells of the intestine in response to luminal antigen [6]. The failure of *in vitro* and *ex vivo* studies to demonstrate key observations arising from *in vivo* studies has also been noted [8].

The gastrointestinal damage reported from *in vivo* models shares many features with that seen in a model developed by the authors to study the nature and function of biliary factors produced in response to challenge with bacterial lipopolysaccharide (LPS) [9]. The LPS model developed from our realization that changes to the gastrointestinal epithelium seen in a variety of clinical conditions point to the importance of factors operating within the gastrointestinal lumen. Recent research has established that high concentrations of cytokines such as TNF α can be delivered to the gut in the bile of rats [10] and humans [11]. Such cytokines have also been shown to remain active in that environment, where they are able, for example, to regulate the expression of MHC class II by the intestinal epithelium [12], and where they mediate tissue damage in our rat model of endotoxic shock [9]. Here evidence is presented suggesting that the hepatobiliary system plays an important role in IgE-mediated reactions to antigens originating from the gastrointestinal tract lumen, and that two mast cell products, histamine and TNF α , are present in the bile of sensitized and challenged animals.

Materials and methods

Animals and experimental design

Inbred SPF Wistar rats (8–10 weeks) were obtained from Culas Ltd (Little Bay, NSW, Australia) and for the duration of the experiments were housed in the University of New South Wales School of Microbiology and Immunology animal house. Conventional outbred Wistar rats were bred and maintained in the School of Microbiology and Immunology animal house. Murine IgE antidinitrophenyl (DNP) antibodies were raised as ascites using the H-1 ϵ -DNP cell line, and IgE antiovalbumin (OVA) antibodies were raised as ascites using the OVA6B cell line. IgE antibody

concentrations were quantified by ELISA assay and by rat basophil leukaemia cell degranulation assay as previously described [13]. Groups of rats were sensitized by i.v. injection with 3 mg mouse monoclonal anti-DNP IgE or monoclonal anti-OVA IgE. Animals were returned to their cages with free access to food and water for 48 h, and were subsequently denied further access to food for 24 h, after which 1 mL of dinitrophenylated-human serum albumin (DNP₃₂-HSA: Sigma Chemical Co., St Louis, MO, USA) in PBS (5 mg/mL) was given by gavage.

Prior to antigen challenge, some groups of rats were deprived of intestinal bile by bile duct cannulation (BDC) and external drainage using the method of Lambert [14]. Briefly, rats were anaesthetized with ether, laparotomies were performed and cannuli (inner diameter 0.4 mm, outer diameter 0.8 mm) were inserted into the bile ducts. Tubing was secured in two places with suture cotton and passed out through the rats' flanks. Bile was collected externally over a 2-h period and then stored at -20°C until required. Control animals were either sham operated or left untreated. The rats were observed for up to 2 h post-challenge before being anaesthetized with ether and killed by cervical dislocation. Livers and intestines were removed, divided into portions and immediately fixed in either Carnoy's solution, for later mast cell staining, or in formaldehyde for H & E staining.

All surgical procedures were carried out under ether anaesthesia, and the experiments were approved by the University of New South Wales Animal Care and Ethics Committee.

Histology

Tissue sections which had been fixed in Carnoy's solution were blocked in 0.5 mol/L HCl for 10 min, then stained in toluidine blue (0.5% w/v in 0.5 mol/L HCl) overnight. Additional sections were stained in Alcian blue (0.5% w/v in 0.7 mol/L HCl) overnight and then counter stained with safranin (1% w/v in 0.125 mol/L HCl).

Toluidine blue-stained liver sections from four unsensitized controls and from four sensitized and challenged animals were scanned by phase contrast microscopy to locate portal triads. This technique allowed the portal triads to be located independently of the presence of mast cells as stained mast cells can not be identified under phase contrast. An eye-piece graticule was used to measure the long and short diameters of each elliptical portal vein. The same portal triad was then viewed under normal illumination and the surrounding mast cells counted and scored as mast cells per portal triad. Intestinal mast cells were counted as mast cells per villus crypt unit, where a villus crypt unit (VCU) was defined as one villus and the crypt immediately below that villus, extending to the smooth muscle of the intestine [15]. All mast cell counts were conducted blind.

Segments of duodenum, jejunum and ileum were collected from normal controls, sensitized and challenged animals, and sensitized animals which were bile duct cannulated prior to antigen challenge. Tissue was collected from four animals from each group. From each animal, two jejunal segments were sectioned and stained with haematoxylin and eosin, and the full length of each centimetre long section was assessed for intestinal damage. The extent of damage was judged on a graded scale; 0 (no visible damage), 1 (slight oedema of villi), 2 (Oedema with a few enterocytes breaking away from ends of villi), 3 (Clear oedema with almost all enterocytes lost from ends of villi). Four sections of intestine were viewed and graded per animal. The mean of the four grades was taken as the overall grade of intestinal damage for that animal. All evaluations of tissue were conducted blind.

Western immunoblots

Biliary TNF α was investigated by Western blotting. SDS-PAGE gels were first run using a mini gel system (BioRad) and 12% separating gels. Proteins were then transferred onto nitrocellulose membranes using a mini trans-blot cell (BioRad). The membranes were then blocked with 1.0% (w/v) BSA in PBS and washed in PBS-0.05% Tween 20. Rabbit antimouse TNF α antiserum (Genzyme, Cambridge, MA, USA), which is known to cross-react with rat TNF α [16], was added at a dilution of 1:100, and incubated for 1 h at room temperature. Membrane-bound antibodies were then identified by further incubation with a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Southern Biotechnology Associates, Birmingham, AL, USA), followed by incubation with the substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma).

Measurement of histamine content by radioimmunoassay

A commercial histamine radioimmunoassay (Immunotech, Marseille, France) was used for the quantitative determination of histamine levels in bile collected from five sensitized and challenged animals and from five unsensitized controls. Twenty five microlitres of acylation solution and 25 μ L of acylation buffer was added to 100 μ L of sample or standard solution, and the resulting solution vortexed immediately. To each antibody-coated tube, 50 μ L of acylated sample or standard and 500 μ L of iodinated tracer were added and incubated at 4 °C for 2 h. Test solutions were then aspirated and discarded, and the tubes counted in a gamma counter (Packard Instrument Co., Meriden, CT, USA). Sample concentrations were finally calculated with reference to a series of histamine standard solutions (0–10 ng/mL). Where activity was too high to allow quantification

against the standard curve, samples were reanalysed after appropriate dilution.

Statistical analysis

Data relating to mast cell numbers in the intestine was analysed using one way ANOVA, and where differences between groups were shown, further analysis was performed using the Bonferonii *T*-test. Western blot results were analysed by χ^2 test. All other data were analysed using the Mann–Whitney *U*-test. Significance was accepted at the 0.05 level.

Results

Enumeration of hepatic mast cells in untreated inbred and outbred Wistar rats was carried out on Alcian blue/safranin stained tissue sections. Three distinct mast cell staining patterns were observed. In addition to Alcian blue-staining and safranin-staining cells, a third population of double-staining cells, here designated as dichromatic mast cells (DMC), was seen. As shown in Table 1, this cell type accounted for a median of 3.1% (range: 1.6–4.9%) of hepatic mast cells in outbred animals. Virtually all hepatic mast cells seen in the inbred Wistar rats stained Alcian blue positive, but the median of 6.7% (range: 1.2–22.8%) of those counted in outbred animals were CTMC-like safranin positive cells.

Additional liver sections obtained from inbred rats were stained with toluidine blue, and examined to better visualize the spatial disposition of hepatic mast cells. Mast cells were seen to be closely associated with the portal triads, with

Table 1 Staining characteristics of rat hepatic mast cells

	Outbred Wistar	Inbred Wistar
Number of rats	8	7
Total number of portal triads	179	174
Total cells counted	1651	1299
% Alcian blue positive		
Median	90.5	99.6
Range	72.5–97.2	98.8–100
% Safranin positive		
Median	6.7	0
Range	1.2–22.8	0
% Alcian blue/Safranin double positive		
Median	3.1	0.4
Range	1.6–4.9	0–1.2

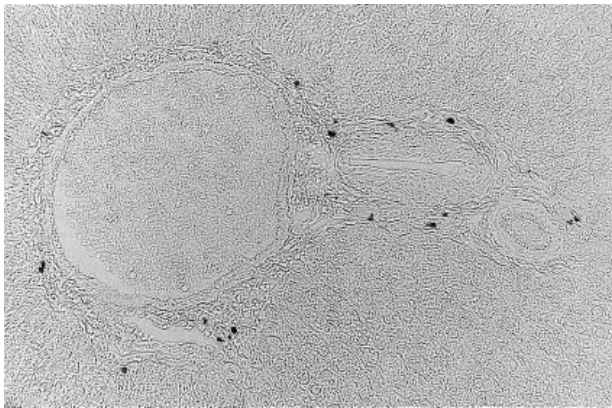


Fig. 1. Toluidine blue stained section of normal liver from an inbred Wistar rat showing the typical distribution of mast cells around a portal triad.

many mast cells clustered within the larger portal triads (Fig. 1). The number of mast cells per portal triad correlated well with the circumference of the portal vein ($r^2 = 0.694$) (Fig. 2a).

Liver sections obtained from IgE-sensitized and antigen-challenged animals showed a striking diminution in stainable mast cells. Within 30 min of antigen challenge, the numbers of identifiable mast cell declined dramatically ($P < 0.0001$). As shown in Fig. 2(b), the median number of mast cells declined from six per portal triad in unsensitized control animals ($n = 4$) to one per portal triad in sensitized and challenged animals ($n = 4$). Two sections containing at least 10 portal triads were examined per animal.

Mast cell numbers in the intestine were also investigated and the results are presented in Fig. 3. Granulated mast cell numbers declined in the duodenum and jejunum, within 10 min of antigen challenge. Although the decline was statistically significant ($P < 0.05$), the magnitude of the change was relatively small compared with that seen in the liver. While the mean number of mast cells per VCU seen in the duodenum of normal animals was 13.4, this declined to 10.1 mast cells per VCU after antigen challenge. A significant decline in mast cell numbers was not seen in the ileum until 30 min post-antigen challenge. Mast cell degranulation and the subsequent decline in visible mast cell numbers were unaffected by bile duct cannulation.

Sections of duodenum were collected and stained for histological examination. The appearance of the villous epithelium from an unsensitized animal is shown in Fig. 4(a). Sections from animals sensitized with anti-OVA IgE antibodies and challenged with DNP-HSA all showed normal, undamaged villous epithelium. A typical section is shown as Fig. 4(b). In contrast, sections obtained from animals sensitized with anti-DNP IgE and challenged with DNP-HSA revealed increasing intestinal damage over time.

The damage ranged from minor oedematous changes in the villous epithelium over the first 15 min post-challenge, to total obliteration of the villous architecture which could be

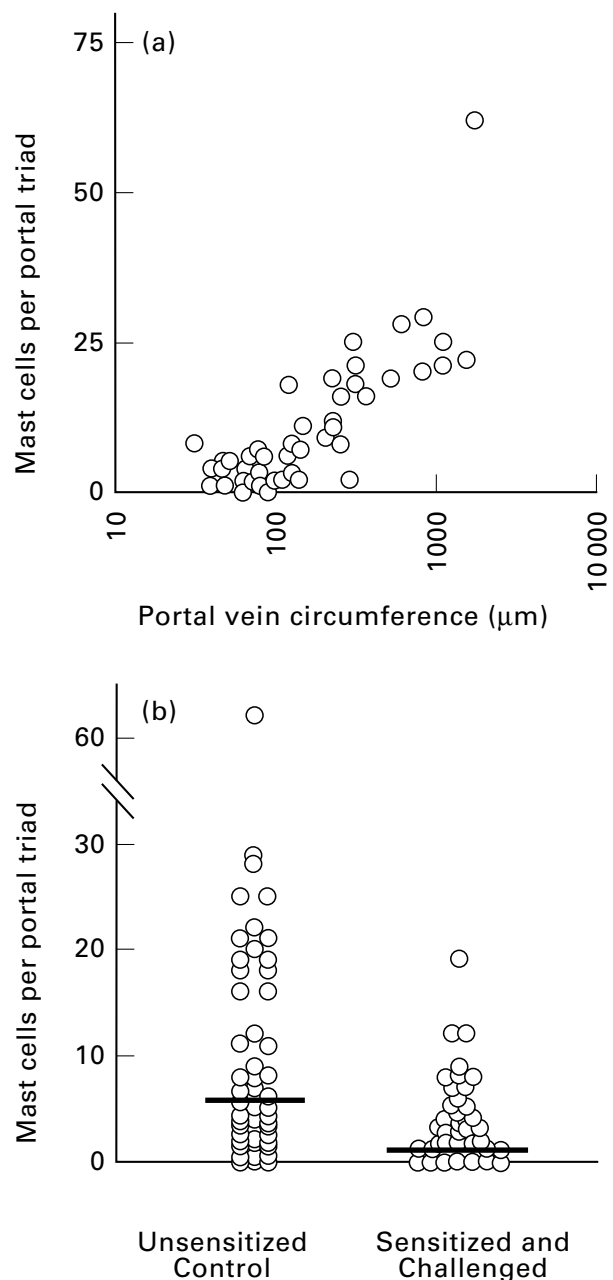


Fig. 2. Hepatic mast cell counts. Sections of liver from inbred rats were stained with toluidine blue. Portal triads were identified and the circumference of the portal veins were measured. The number of mast cells associated with each portal triad was then counted. (a) The relationship between portal vein circumference and mast cell numbers. (b) Mast cell counts in control animals ($n = 4$) and in IgE-sensitized animals ($n = 4$) 30 min post antigen challenge. Solid bars show the median values for each group. The difference between the two groups was highly significant ($P < 0.0001$).

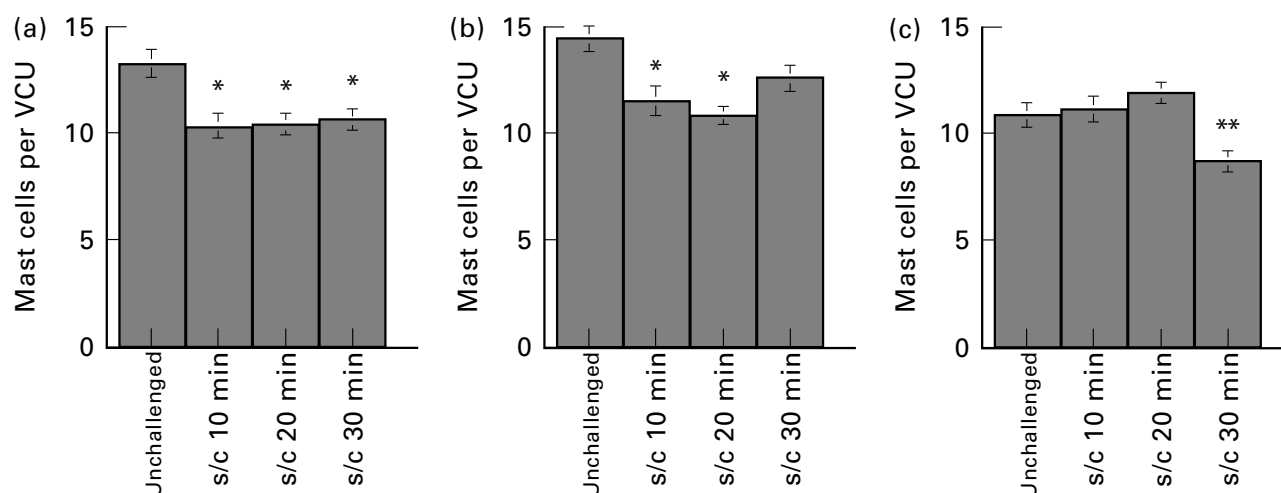


Fig. 3. Intestinal mast cell counts. (a) Inbred rat duodenum, (b) jejunum and (c) ileum sections were stained with toluidine blue, and the number of mast cells per villus crypt unit (VCU) was determined. Small intestine was collected from normal and sensitized/challenged (s/c) rats at 10, 20 and 30 min post-antigen challenge. ** and * indicate *p*-values for comparisons with the normal group of less than 0.01 and 0.001, respectively.

seen after 30 min. Typical epithelial damage seen at the later time point is shown in Fig. 4(c), which also shows dark-staining erythrocytes in areas of haemorrhage. To investigate a possible role for biliary factors in the changes seen within the gastrointestinal tract upon sensitization with IgE and challenge with antigen, animals were sensitized, then bile duct cannulated prior to antigen challenge. No damage occurred to the villi of bile duct cannulated animals (Fig. 4d).

Scores of intestinal damage seen in various animals groups are presented in Fig. 5. Intestinal damage was

particularly striking 30 min after antigen challenge of sensitized animals. All sections from this timepoint showed intestinal damage scores of at least two, and some sections from all animals showed damage scores of three. These scores were significantly different to those seen in normal unchallenged controls ($P < 0.05$). Bile duct cannulation of sensitized and challenged animals appeared to abrogate the intestinal damage. No significant difference was seen between these animals and normal controls.

Bile samples from five control animals and five sensitized and challenged animals were analysed for histamine con-

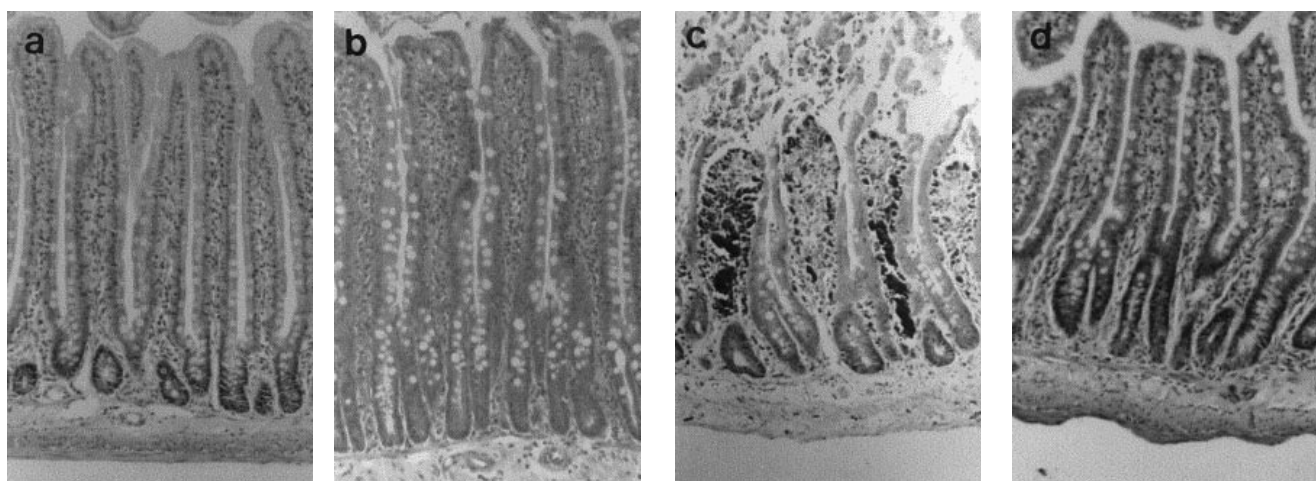


Fig. 4. H & E stained jejunum from (a) normal rat, (b) rat sensitized with monoclonal α -OVA IgE then orally challenged with DNP-HSA, (c) rat sensitized with monoclonal α -DNP IgE then orally challenged with DNP-HSA and (d) rat sensitized with monoclonal α -DNP IgE, bile duct cannulated, then orally challenged with DNP-HSA. Jejunum was collected 2 h post-antigen challenge in each case.

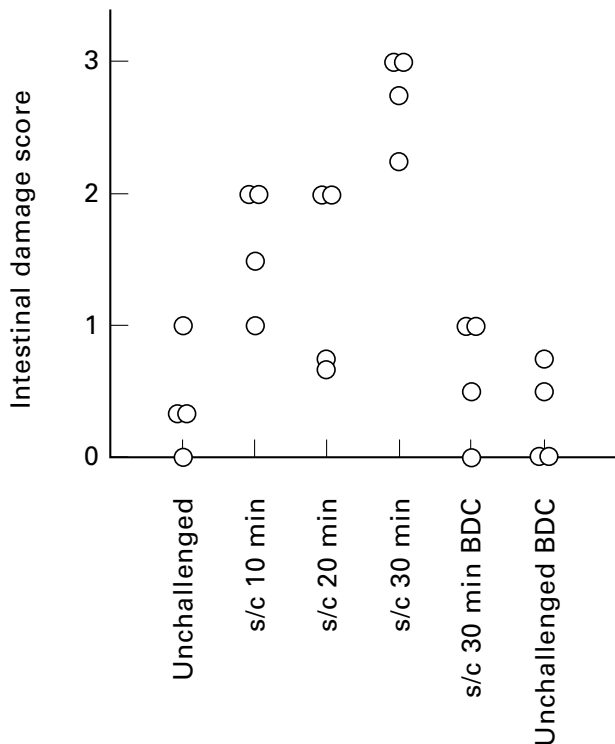


Fig. 5. Intestinal damage scores. Inbred Wistar rats were sensitized with monoclonal α -DNP IgE then orally challenged with DNP-HSA. Jejunal segments were collected 10, 20 and 30 min after antigen challenge, with tissue from four rats collected at each timepoint. Segments were also collected 30 min after antigen challenge of sensitized animals which had been bile duct cannulated (BDC) prior to antigen challenge. Additional segments were collected from normal control animals and from BDC controls. H & E stained jejunal sections were scored for intestinal damage as follows: 0 (undamaged normal jejunum), 1 (slight odema of villi), 2 (clear odema with some loss of enterocytes from tip of villi), 3 (complete loss of all enterocytes from tips of villi). The final score for each rat is given as the mean score from four sections examined from each animal. Damage at 30 min was significantly different to unchallenged and bile duct cannulated groups ($P < 0.05$).

tent, and the results are shown as Fig. 6. Samples were collected from sensitized animals 15 min after antigen challenge, and showed a significant increase over the levels seen in control rats ($P < 0.01$).

Analysis of biliary proteins by SDS-PAGE showed a rapid change in the protein profiles of challenged animals. Within 15 min of challenge, a range of new low molecular weight proteins appeared in bile (results not shown). Western blotting of gels showed one such protein to be TNF α . TNF α was detected in one of six unsensitized control samples and in none of six samples collected from sensitized animals challenged with HSA. However, a significant

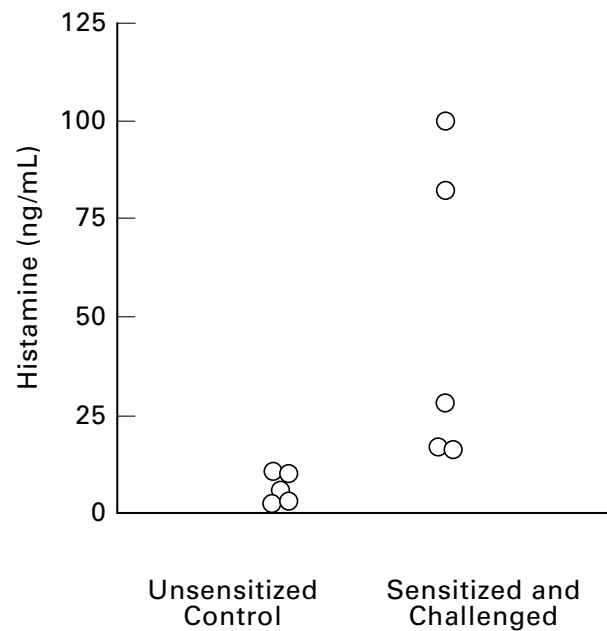


Fig. 6. Histamine concentrations in bile of five normal and five IgE-sensitized and antigen-challenged inbred rats. Bile was collected 15 min post-challenge. The difference between the two groups was significant ($P < 0.01$).

upregulation of TNF α expression was seen in animals sensitized and challenged with DNP-HSA, where TNF α was detected in four of six bile samples collected within 15 min of antigen challenge ($P < 0.05$). A representative blot showing the time course for the appearance of TNF α is

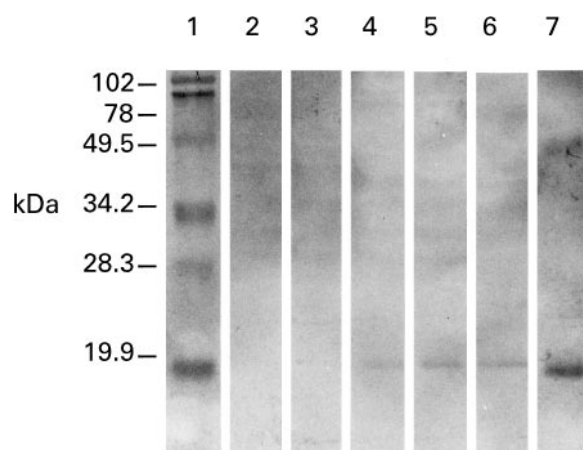


Fig. 7. Representative Western blot of TNF α in bile collected from an IgE-sensitized, antigen-challenged inbred rat. Bile was collected for 1 h post-antigen challenge. Lane 1 — MW markers; lane 2 — pre-challenge bile; lane 3 — 0–15 min; lane 4 — 15–30 min; lane 5 — 30–45 min; lane 6 — 45–60 min; lane 7 — recombinant rat TNF α .

shown as Fig. 7. Biliary $\text{TNF}\alpha$ appeared as a band with a molecular weight of around 20 kDa, slightly higher than that of the recombinant $\text{TNF}\alpha$ control.

Discussion

A simple model was developed to investigate whether the hepatobiliary system could be involved in IgE-mediated reactions to antigens originating from the lumen of the gastrointestinal tract. Both the macroscopic and microscopic features seen were in accordance with previous reports of animal models of allergic reactions to dietary antigens [3,17]. Sensitization of rats with monoclonal IgE anti-DNP antibodies, and later challenge with the appropriate antigen, led to a rapid accumulation of fluid in the gut and inflammatory changes suggestive of tissue destruction. Histological examination of intestinal segments confirmed the presence of gross destruction of the villous epithelia of test animals.

To establish whether biliary factors could be involved in the changes seen, IgE-sensitized animals were bile duct cannulated prior to antigen challenge. Strikingly, both the macroscopic and microscopic appearance of the intestine remained unchanged in these animals, and all animals appeared well.

Large numbers of mast cells are resident in the liver and they would seem a likely source of any inflammatory mediators released into bile in an IgE-dependent fashion. Although their presence in the liver has been recognized for some time [18] little is known of the nature or function of hepatic mast cells. The second aim of this investigation was, therefore, to better characterize rat hepatic mast cells, which like human hepatic mast cells [19] are found almost exclusively in association with portal triads.

Two apparently distinct subpopulations of mast cells have long been recognized in the rat: mucosal mast cells (MMC) and connective tissue mast cells (CTMC). These subpopulations have been most easily distinguished by the fact that MMC can be stained with Alcian blue, while CTMC can be stained with safranin [20,21]. In this study, the livers of inbred Wistar rats were shown to be almost entirely populated by Alcian blue positive cells. A minor population of dichromatic mast cells (DCM) was also seen. Cells with these same staining characteristics have been reported previously from the dermis of Wistar rats [22]. Enerback similarly reported that cells in the dermis of Sprague-Dawley rats that contained both blue and red granules after staining with astra blue: safranin [20]. The DMC population of the liver may represent a distinct and stable cell type, or may perhaps be a population of immature cells.

Interestingly, in additional experiments conducted using conventionally housed outbred animals, such DCM were

found at higher numbers. CTMC-like safranin positive cells accounted for almost 10% of the hepatic mast cells in these animals. These observations lend some credence to the notion that hepatic mast cells may be capable of phenotypic differentiation, perhaps under the influence of the intestinal microbiota.

Hepatic mast cells belonging to the CTMC phenotype has often been assumed. The Alcian blue/safranin staining properties observed challenge this view. Additional studies will be required to confirm the phenotype of the alcian blue-staining cells seen. However, a number of recent reports of MMC-specific chymase activity in the liver support this view [23–25].

Toluidine blue staining was used to monitor changes in hepatic mast cells after IgE-sensitization and antigen challenge. The toluidine blue stain is specific for mast cell granules [26], and therefore a completely degranulated yet intact cell will not be seen in toluidine blue-stained tissue sections. Although mast cell degranulation may often be incomplete or 'piecemeal', complete or 'anaphylactic' degranulation has been reported in the human intestinal tract [27]. Similar anaphylactic degranulation has been inferred from the decline in granulated intestinal mast cell numbers reported from animal models after antigen challenge [28]. A similarly slight but significant reduction in mast cell numbers per villous crypt unit was also noted in stained sections of the intestine of sensitized and antigen-challenged animals in this study. In contrast, a dramatic decline in granulated hepatic mast cells was seen. Degranulating mast cells were also a conspicuous feature of histological sections of test animal livers, while intestinal mast cells from these rats were not seen to degranulate, as has previously been reported [28].

To see whether mast cells are able to deliver inflammatory mediators to the gut via the hepatobiliary system, bile was analysed for the presence of mast cell products. Mast cells release a range of preformed and newly formed mediators upon activation [29]. Histamine and $\text{TNF}\alpha$ are two such mediators which have been widely studied in the context of allergic disease, and serve as good indicators of mast cell activation. Although $\text{TNF}\alpha$ is produced by many cell types, it is only known to be stored in mast cells [30]. Its early detection can therefore be taken as indirect evidence of mast cell degranulation. Analysis of bile for these mediators was therefore included in this study.

Biliary histamine levels rise within 15 min of antigen challenge of sensitized animals. $\text{TNF}\alpha$ was also identified in bile by Western blot, appearing as a band with an apparent molecular weight of around 20 kDa. This is in line with previous reports which have shown rat $\text{TNF}\alpha$ bands ranging from 17 kDa to 22 kDa [16]. While the authors present no evidence that $\text{TNF}\alpha$ is responsible for the intestinal damage

seen in the proposed model, the possibility can not be discounted. Some cytokines, including $\text{TNF}\alpha$, can retain much of their biological activity within the harsh environment of the gastrointestinal tract [9,31], and the authors have recently observed similar intestinal damage in animals infused intraduodenally with $\text{TNF}\alpha$ (manuscript in preparation).

The present model highlights the hepatobiliary system as a conduit for the delivery of mast cell products to the gastrointestinal tract of the rat. Whether or not the human hepatobiliary system is similarly involved in gastrointestinal hypersensitivity reactions awaits further investigation.

Acknowledgements

This study was supported, in part, by a grant from the National Health and Medical Research Council.

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