On the Contribution of Mucosal Mast Cells to the Regulation of Mouse Intestinal Barrier Function

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On the Contribution of Mucosal Mast Cells to the Regulation of Mouse Intestinal Barrier Function

Over de bijdrage van mucosale mestcellen aan de regulatie van de intestinale mucosale barrière functie van de muis

(met een samenvatting in het Nederlands)

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Contents

Chapter 1

General introduction

The primary functions of the small intestine are the digestion and transport of luminal content and the absorption of nutrients. During these processes, however, the intestinal mucosa is exposed to various ingested and resident pathogens. The ability of the intestinal wall to prevent transmucosal passage of toxins or of harmful micro-organisms and their products is defined as the intestinal barrier function [1, 2]. The main component of the intestinal barrier is the continuous single-cell layer of epithelial cells in the mucosa. The transport of molecules across the epithelium can occur either through the cells (transcellular pathway) or between cells (paracellular pathway) [2]. The transcellular pathway requires active uptake of molecules by the epithelial cells whereas the paracellular pathway mediates the passive transport of solutes via tight junctions (TJ) which interconnect the epithelial cells [2]. The TJ act as a barrier and the regulation of their tightness determines the paracellular permeability of the intestinal epithelium [2, 3].

The intestinal mucosal barrier is under a strict control of the local gut immune system which samples luminal content to maintain tolerance to harmless antigens but not to invading pathogens [4]. The local immune system consists of many types of cells located in the submucosa, such as mast cells, dendritic cells, neutrophils,T- and B-cells and M-cells [2, 5]. The mucosal barrier is also under control of the nervous system, namely the enteric nervous system [6] and the afferent endings of sympathetic and parasympathetic nerve fibers [7]. The enteric nervous system is located in the wall of the gut and is considered as "the brain in the gut" because it can function independently and uses as input local information collected by intrinsic sensory neurons [6]. The afferent nerve fibers in the intestinal wall originate from sensory neurons with their cell bodies in the central nervous system [7]. These fibers are involved in collecting and sending information to the central nervous system, but also in local reflexes which affect properties of the intestinal barrier [8, 9]. Together, these complex regulatory mechanisms enable a rapid and coordinated response of the mucosal barrier to potential threats and allow for adaptations to changing circumstances in the gut [2, 10].

Defective intestinal barrier function plays a role in a number of disorders such as inflammatory bowel disease [1, 11], acute pancreatitis (AP) [12], parasite infections [13, 14] and chronic stress [2]. An impaired intestinal barrier function may result finally in bacterial translocation and sepsis [15]. The mechanisms underlying the regulation of the intestinal barrier under (patho)physiological conditions are not well known [2, 16]. For instance, the release of substances from primary sensory nerve terminals can result in inflammation which negatively affects the mucosal barrier function[17, 18]. This so called neurogenic inflammation is mediated partially by mast cell mediator release, but information about this process is scarce [18, 19]. The mucosal mast cells and their interactions with the nervous system make an important contribution to the regulation of neurogenic inflammation and are the focus of this thesis.

Mucosal mast cells and neuroimmune interactions in the regulation of the intestinal barrier function.

Although mast cell are predominantly known as effector cells of allergic inflammation reactions [20], their role in tissue defense and homeostasis is being increasingly recognized [21]. Mast cells originate from the bone marrow and migrate under influence of adhesion molecules to target tissues where they mature and differentiate under influence of tissue specific factors such as cytokines and growth factors [20].

The maturation in different target tissues gives rise to mast cells of heterogeneous phenotypes with specific functional characteristics. Rodent mast cells can be classified into connective tissue mast cells (CTMC), located in the peritoneal cavity and skin, and mucosal mast cells (MMC) located at mucosal surfaces such as the intestinal lamina propria [20]. Mast cells produce a large number of mediators which are either preformed and stored in secretory granules or synthesized *de-novo* upon activation [20]. Mast cell mediators include enzymes, biogenic amines, chemokines, cytokines, proteoglycans, polypeptides, phospholipid metabolites, and growth factors [22]. Upon activation of the mast cell these products are released. Mast degranulation is a massive secretory process characterized by the fusion of secretory granule membranes with the plasma membrane resulting in nonselective release of the various granule-associated mediators into the extracellular space [20]. Alternatively, differential mediator release can occur without opening of the secretory granules and allows for release of certain mediators only [23]. Under some conditions the release of mediators from mast cells is limited to small amounts; this type of release is called piecemeal release [24, 25].

The variety of mediators allows mast cells to regulate multiple functions in the resident tissues. In the intestinal wall, mucosal mast cells have been implicated in the control of for instance epithelial secretion, intestinal barrier function and blood flow [26]. There is mounting evidence indicating that mucosal mast cells are key regulators of the intestinal barrier function via the modulation of the TJ in the paracellular pathway. For instance, intestinal parasitic infection and inflammation are characterized by a decrease of the intestinal barrier integrity in several animal models [22, 26]. This impairment has been shown to be mediated by mediators released from mucosal mast cells resident in the tissue and by mast cells additionally recruited to the site of inflammation[22, 26] . One of the main mediators, the chymase mouse mast cell protease-1 (mMCP-1) is released in large amounts by mucosal mast cells during inflammation, and has been shown to directly affect TJ proteins, thereby increasing the intestinal permeability [13, 27]. Although their activity is most noticeable during inflammation, mast cells are also involved in regulation of the mucosal barrier function under physiological conditions [17, 21].

Mast cells can be activated by a variety of substances and express a multitude of membrane receptors and intracellular signaling pathways which are involved in mediator release [20]. Direct activation of mucosal mast cells by neuropeptides released from afferent

nerve fibers seems to be one of the important mechanisms leading to mucosal barrier impairment [28]. A crosstalk between mast cells and afferent nerve fibers has been implied by their intimate anatomical association in the intestinal mucosa [28]. In the rodent ileum, sensory sympathetic nerves expressing the neuropeptides Substance P (SP) and Calcitonin Gene Related Peptide (CGRP) are closely associated with mucosal mast cells. In the course of inflammation, the density of mast cells as well as SP- and CGRP-containing nerve fibers increases and also the number of their anatomical close contacts [29-31]. A functional connection between mucosal mast cells and the nervous system has been indicated in studies on co-cultures of these cells, demonstrating that nerve stimulation can result in an activation of the mast cells [32]. The communication between these two cell types appears to be bidirectional [28, 32-34].
In this thesis an investigation is described of the regulation of the mouse intestinal mucosal barrier function. In the first experimental chapter (Chpter 2), an investigation of the effects of probiotics on the mucosal barrier during acute pancreatitis (AP) is presented. In the following chapters the focus is on the role of mucosal mast cells and the mucosal mast cell mediator mMCP-1 in regulation and impairment of the intestinal mucosal barrier. The different experimental models, approaches and techniques used to answer specific research questions are described below.

Effects of acute pancreatitis (AP) and probiotics on the mucosal barrier function

Acute pancreatitis (AP) is a sudden inflammation of the pancreas caused by pancreatic enzymes autodigesting the organ. The most common cause of acute pancreatitis is heavy alcohol use and the presence of gallstones obstructing the common bile duct [35, 36]. The inflammatory response during AP is not limited to the pancreas but spreads to other sites such as the intestinal tract. This results in a decreased intestinal motility leading to bacterial overgrowth, a disturbed immune response and an impairment of the intestinal barrier function [12, 37]. These disturbances favor the passage of enteric bacteria and their toxins from the gut lumen into the damaged pancreas and other sites resulting in infectious complications and mortality [38, 39]. The maintenance of the intestinal barrier function seems thus of particular importance in the clinical treatment of AP. In the pathogenesis of human AP, pancreatic mast cells play an important role [40, 41]. Moreover, in models of AP in the rat, the effects of application of mast cell stabilizers [42] and mast cell activators [43, 44] show that mast cells are involved in the development of barrier impairment in the pancreas itself, but also in the intestine.
Probiotics**,** defined as living microorganisms that have beneficial effects on human health [45], have been shown to support the epithelial barrier integrity in several studies (see [46]). The beneficial effects of probiotics have been attributed to stimulation of mucosal defense

at the level of both immune and epithelial function. *In-vitro* experiments showed, for instance, the ability of probiotics to directly affect epithelial barrier function by modulation of the phosphorylation status of TJ-proteins [47]. Further, a preliminary study has indicated that probiotics can decrease mast cell release of proinflammatory mediators involved in stress-induced impairment of the intestinal barrier [48]. The positive effects of probiotics on the intestinal function have led to investigations of their applicability as a therapeutic agent for the treatment of infectious complications associated with AP [49, 50]. In general, however, limited information was available on the intestinal barrier function in the course of AP and on the effect of probiotics on this function.
The are many reasons why the results of investigations of the applicability of probiotics as a therapeutic agent on rodents are difficult to translate to the human condition [49-51]. Moreover, a serious limitation of the insight in this applicability was the fact that in all animal studies done so far, probiotics were applied well before the onset of AP [49, 50], whereas in the clinical setting probiotic treatment can only be started after AP is diagnosed. For this reason, we have investigated in an experimental mouse model of AP the effects on the intestinal barrier function of probiotics applied prophylactically (before AP induction) as well as therapeutically (following AP induction). For these experiments a model of mild (cerulein-induced) AP was chosen, which is not associated with mortality [52]. In Ussing chamber experiments on ileal segments, the electrical resistance and the transepithelial flux of a marker molecule (NaFl) were measured *in vitro*. These physical properties are mainly determined by the tightness of the TJ and therefore reflect the condition of the paracellular epithelial permeability [3]. The results of this investigation are described in **chapter 2.**

Role of the mucosal mast cell mediator mMCP-1 in impairment of the intestinal barrier in schistosomiasis

Schistosomiasis is an infection caused by one of several species of the freshwater parasite of the genus Schistosoma. Schistosomiasis affects over 200 million people in the developing countries and is a major source of mortality [53]. In *Schistosoma mansoni* infected hosts, the parasitic worms mature in the mesenteric veins where they produce large numbers of highly antigenic eggs. By largely unknown mechanisms, *S. mansoni* eggs cross the walls of the mesenteric blood vessels and the intestinal wall to be excreted in feces. This process elicits a potent inflammatory response of the intestinal wall which is a main cause of pathology associated with the infection [54].
In *S. mansoni* infected mice, egg deposition is accompanied by the recruitment of large numbers of intestinal mucosal mast cells [55] and by an increase in density of extrinsic sensory nerve fibres expressing the neuropeptides SP and CGRP in the lamina propria [29]. The number of anatomical associations between MMC and CGPR and SP expressing

nerves also increases significantly during egg deposition which suggests a functional interaction between both cell-types [29]. The murine model of schistosomiasis is therefore valuable in the study of neuroimmune interaction and the role of mucosal mast cells during intestinal inflammation.
Previously, in models of other parasitic infection it was shown that MMC can release large quantities of the mouse chymase mMCP-1 or the rat homologue (RMCP-2), which, by proteolytic modification of tight junction proteins increases the intestinal permeability [13, 56]. Presumably the decreased integrity facilitates the expulsion of the parasite from the intestinal lumen [13, 57]. Whether this is a common mechanism which also facilitates the transepithelial passage of schistosome eggs was not known. Therefore, in the present study it was investigated whether the mast cell chymase mMCP-1 induces changes in the mucosal barrier thereby facilitating passage of schistosome eggs across the intestinal wall. A schistosomiasis mouse model, developed at the University of Antwerp, was used in combination with a genetic deletion (knock-out) of the mMCP-1 gene. The intestinal barrier function of the ileal mucosa was investigated by measuring in Ussing chambers the electrical resistance of the tissue, the transmucosal passage of a marker molecule, and the secretory response. The structural integrity of the ileum was assessed from the distribution patterns of the TJ proteins Occludin, Claudin-3 and ZO-1 determined by immuncytochemistry. The functional consequences of the absence of mMCP-1 on egg passage were determined by quantifying schistosome egg deposition in the ileal wall and egg excretion into faecal matter. The results are presented in **chapter 3**.

Presence and role of the CGRP1 receptor on mouse mucosal mast cells in neuroimmune communication

A growing number of studies indicate a role for associations between mast cells and afferent nerve endings in a functional cross-talk between the nervous and immune system [18, 58, 59]. Within the rodent intestinal lamina propria, mucosal mast cells are in close apposition to terminals of afferent extrinsic sensory nerves expressing the neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) [29]. Intestinal inflammation results in a upregulation of these contacts implying a role for SP and CGRP in the activation of the mucosal mast cell [29]. The activation of MMC by neuropeptides is thought to contribute to the process of neurogenic inflammation via release of mediators which induce vasodilatation, leukocyte infiltration, and tissue remodeling in the intestinal wall [28, 60, 61]. MMC mediators can regulate the intestinal barrier function directly by affecting the epithelial integrity [2]. In this way, neuropeptides released from afferent nerve endings participate in the regulation of mucosal barrier function in the course of inflammation. Several neuropeptide receptors have been identified on mast cells [28, 61], including receptors for Neurotensin [62], Vasoactive Intestinal Polypeptide [63] and Somatostatin [64].

However for other neuropeptides which can induce mast cell degranulation *in vitro*, no receptors could be detected on the mast cell membrane [65, 66]. Therefore, these neuropeptides have been proposed to operate through a receptor-independent binding to G_i proteins on the inner surface of the plasma membrane [65, 67, 68]. More recently, it has been suggested that the mode of action of neuropeptides might be in binding to the nonselective, low-affinity Mrg receptor [69]. However, it has been also demonstrated that the expression of the SP receptor (NK1) on mast cells can be induced by factors in their environment, such as cytokines [70].This suggests that the expression of specific neuropeptide receptors by mast cells is dependent on certain inflammatory conditions [61].
For CGRP, no receptors have been identified yet on MMC, although it was shown that CGRP can induce mast cell mediator release [32]. Therefore, we investigated if MMC express the CGRP1 receptor and whether a receptor independent pathway of MMC activation is utilized by CGRP. Furthermore, we investigated the type of mediator release induced by stimulation of MC with CGRP. Classically, MC activation has been studied in the context of a allergic response, which is characterized by a anaphylactic degranulation releasing the total MC granule content [20]. However non-immunogenic activation of MC can induce a release of mediators in absence of degranulation [23]. This so called 'piecemeal' release enables MC to secrete only certain mediators which can exert their functions without the initiation of a allergic inflammatory response [23].
In the experiments presented in **chapter 4,** the responses of cultured bone marrow-derived mucosal mast cells (BMMC) to the neuropeptide CGRP were recorded by measuring changes of the intracellular calcium concentration. Calcium signaling is involved in the stimulus-secretion coupling [71] of mast cells and provides a sensitive measure of cellular activation. This approach was used to study also a part of the signal transduction route utilized by the CGRP1 receptor in BMMC and the type of mediator release involved.
The MC response to neuropeptides does not necessary consist of mediator release, but can also consist of an increase the responsiveness of the MC to a subsequent stimulus [72]. Such a modulation of MC responsiveness, called "priming", has been demonstrated so far only for the neuropeptide SP [72]. Whether CGRP can also act as a modulator of MC responsiveness was not known. We have therefore investigated if a pre-exposure of BMMC to a low concentration of CGRP affects the response to a subsequent application of CGRP. The results of this preliminary study are described in **chapter 5.**

The aim of the current thesis is to elucidate the contribution of mucosal mast cells to the regulation of the mucosal intestinal barrier. For this purpose, we have addressed the following major questions:

1 Is the effect of probiotics on mouse intestinal barrier function during acute pancreatitis (AP) dependent on the time of application?

2 Is mouse mast cell protease-1 (mMCP-1) involved in intestinal mucosal barrier impairment in the parasitic infection schistosomiasis*?*

3 What is the functional effect of CGRP on mouse mucosal mast cells and is this effect receptor-mediated?

4 Can mouse mucosal mast cells be primed by CGRP?

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Chapter 2

Pretreatment but not treatment with probiotics abolishes mouse intestinal barrier dysfunction in acute pancreatitis

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Abstract

Background: Intestinal barrier failure during acute pancreatitis (AP) is associated with translocation of luminal bacteria, resulting in infectious complications. We examined the effects of multispecies probiotics on the intestinal barrier impairment in a murine model of AP.

Methods: Mice were injected with cerulein to induce AP and were sacrificed 11 (early AP) or 72 hours (late AP) after start of induction. AP and associated systemic effects were confirmed by histology of pancreas and lung. Animals received daily probiotics starting 2 days prior to AP induction (pretreatment) or at the moment of AP induction (treatment). Mucosal barrier function of the distal ileum was assessed in Ussing chambers by measurement of the epithelial electrical resistance and the permeability to Na-fluorescein.

Results: Histological analysis revealed pancreatic injury in both phases of AP, and lung damage in the early phase. Epithelial resistance of the ileum was reduced and permeability increased in both phases of AP, indicating impairment of the intestinal barrier. Pretreatment had no effect on resistance or permeability in the early phase of AP. In the late phase of AP, pretreatment but not treatment abolished the AP induced resistance decrease and permeability increase. Administration of probiotics as such (i.e. without induction of AP) had no effect on intestinal barrier function.

Conclusions: Pretreatment with multispecies probiotics for two days abolishes intestinal barrier dysfunction in the late phase of AP, while treatment does not. The effectiveness of probiotics in this model depends on the timing of administration. Clinical trials with probiotics should seek conditions where treatment can be started prior to onset of disease or elective surgical intervention

Introduction

In patients with severe acute pancreatitis (AP), the intestinal mucosal barrier plays a pivotal role in the pathophysiology of systemic complications. Soon after the onset of AP, splanchnic hypoperfusion and neurohormonal changes result in a reduced small bowel motility, eventually leading to small bowel bacterial overgrowth.[1-4] Ischemia-reperfusion injury accompanied by radical production and inflammatory mediators leads to a breakdown of the mucosal barrier.[3-5] This allows for bacterial endotoxins to cross the gut wall and is thought to promote bacterial translocation and to aggravate the local intestinal inflammation.[1,5] Counter-regulatory immunological pathways leading to immunosuppression, small bowel bacterial overgrowth and mucosal barrier failure render the patient liable for further translocation of endotoxins and bacteria causing infectious complications associated with sepsis.[6-9] Infectious complications are a major cause for morbidity and mortality in patients with severe AP.[10-12]

Probiotics are live micro-organisms which when administered in adequate amounts confer a health benefit on the host.[13] There is now mounting evidence that probiotics can provide health benefits to their human hosts in various chronic gastrointestinal diseases.[14] Possible mechanisms of probiotic therapy include modulation of gut microbiota, intestinal barrier fortification, and modulation of the mucosal immune system.[15, 16] Most of the early studies on probiotics have focused on single probiotic strains, but more recently it has been advocated that probiotic effectiveness might be increased through combination of specifically selected strains.[17]

We and others have shown that in experimental animals, pretreatment with probiotics can reduce the infection of pancreatic necrosis.[18,19] One clinical study with probiotic treatment, confirmed these findings.[20] In the rat model,[19] pretreatment with probiotics did reduce bacterial translocation in the late phase of AP. Presumably, this reduction was the result of beneficial effects of the probiotics on the intestinal mucosal barrier.[19] There is, however a lack of information on the effects of AP on functional properties of the intestinal barrier, such as electrical resistance and permeability, and whether these properties can be influenced by probiotics.

Recently, a large multicenter randomized trial from our group showed that treatment with probiotics of patients with AP did not reduce the incidence of infectious complications.[21] A totally unexpected finding was the over two-fold higher mortality in the probiotics group compared to the placebo group. The cause of this elevated mortality remains elusive; therefore information on the effects of probiotics on intestinal mucosal barrier function in an animal model of AP is of special importance.

A striking difference between animal and clinical studies of the effects of probiotics on AP is that in animal studies [18, 19] the probiotics are usually applied well before the onset of AP, while in clinical trials [20,21] their application is restricted to treatment only. Thus investigating the role of timing of probiotic application might provide information on the

mechanism of probiotic action and on the differences in results obtained from animal studies and clinical trials.
In the present study we investigated the effects of probiotic pretreatment and treatment on intestinal barrier function in the well-established [22] mouse model of cerulein-induced AP. Advantage of the chosen mild and non-lethal AP model is that changes of mucosal barrier integrity can be measured in disease and during recovery so that the effects of timing of application of probiotics can be investigated. Aims of the study were 1) to quantify the impairment of the ileal mucosal barrier function in the early and late phase of AP and 2) to determine the effects of probiotic pretreatment and treatment on the AP-induced dysfunction of the intestinal barrier.

Methods

Animals
Male CD-1 mice, 25-35 grams (Harlan, Horst, The Netherlands) were kept under constant housing conditions (22°C, 60% relative humidity, 12-hour light/dark cycle) and had free access to water and food (RMH 1110, Hope Farms, Woerden, The Netherlands). The mice were allowed to adjust to these conditions for one week prior to the start of the experiments. The experimental design was approved by the institutional animal experiments committee of the University Medical Center, Utrecht, The Netherlands.

Probiotics
The probiotic mixture (Ecologic® 641, Winclove Bio Industries, Amsterdam, The Netherlands) consisted of viable, freeze-dried strains: *Lactobacillus acidophilus* (W70), *Lactobacillus casei* (W56), *Lactobacillus salivarius* (W24), *Lactococcus lactis* (W58), *Bifidobacterium bifidum* (W23) and *Bifidobacterium lactis* (W52). These bacterial strains had been selected for their immunomodulatory capacity, and antimicrobial activity in *in vitro* models.[23] The placebo consisted of carrier substance only (corn-starch and maltodextrins). Probiotics and placebo were packed in identical sachets and coded by the producer to guarantee blinding. Directly before administration, the products were reconstituted in sterile water for 15 minutes at 37°C. The single probiotics dose volume of 0.5 ml contained a total of 2.5 x 10^9 colony forming units (CFU) of bacteria.[19] Probiotics or placebo were administered intragastrically by oral gavage once daily.

Induction of AP and collection of tissue samples
AP was induced by 10 hourly intraperitoneal injections of 0.1 ml saline containing 50μg/kg cerulein (Sigma-Aldrich). Sham treated animals received injections with saline only. Mice were terminated by cervical dislocation. Ileal sections were collected and prepared for Ussing chamber measurements. Samples of lung and pancreas were collected

and fixated in 4% formaldehyde for histopathological analysis. Lung samples for myeloperoxidase assay (MPO) were snap frozen in liquid nitrogen.

Experimental groups

Mice were randomized among 14 experimental groups. Each group consisted of 14 animals. The time schedules of AP induction and probiotic application are shown in figure 1. To determine mucosal barrier function during AP, mice were terminated either immediately (i.e. 1 h after the last injection) after AP induction (group: early AP; fig 1) or 72 hours after AP induction (group: late AP; fig 1). This protocol was subsequently used to examine the effects of probiotics on intestinal barrier function. One group of animals received probiotics during five days starting two days prior to AP induction until termination three days after start of AP induction (group: late $AP_{p\&t}$; fig 1).

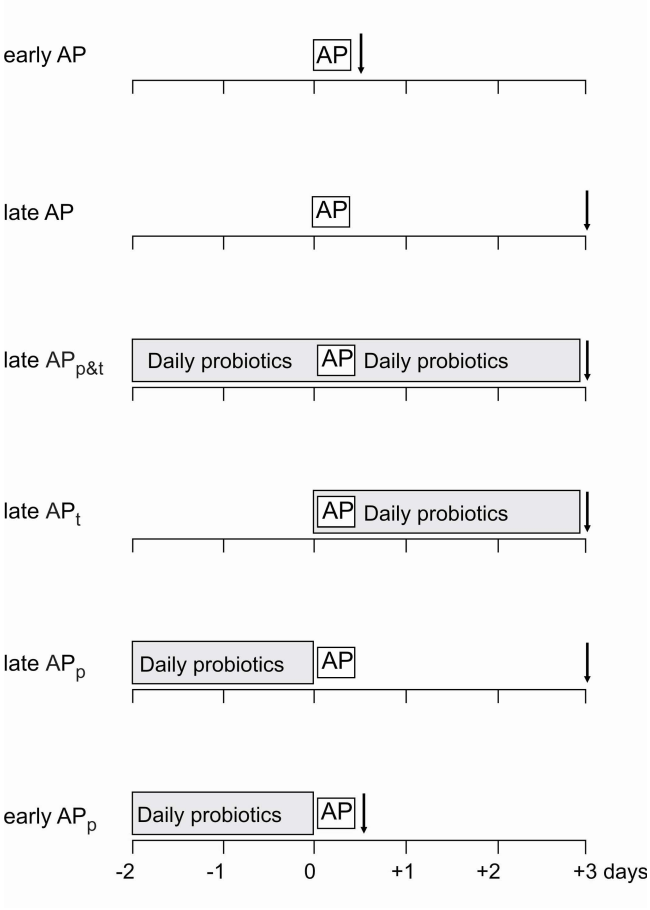

Figure 1
Schematic overview of 6 of the 14 experimental groups. Group names are given with corresponding time schedule of AP induction (AP), probiotic application (in gray) and termination (arrow). Early AP: Animals were terminated immediately after induction of AP. Late AP: Animals were terminated three days after induction of AP. Late $AP_{p\&t}$: Late AP group with combined, probiotic pretreatment ($_p$) and treatment ($_t$) from two days before until three days after AP. Late AP_t: Late AP group with probiotic treatment of three days starting at AP induction. Late AP_p: Late AP group with probiotic pretreatment of two days, starting two days before induction of AP. Early AP_p: Early AP group with probiotic pretreatment of two days, starting two days before induction of AP. Two sham AP groups (sham early AP and sham late AP), 4 placebo probiotic treatment groups (plac late $AP_{p\&t}$, plac late AP_t, plac late AP_p and plac early AP_p), the probiotics only group (no AP, treatment) and the control group (no AP, no treatment) are not depicted.

The effect of probiotic treatment in the late phase of AP was investigated by administration of probiotics only after start of AP induction for a period of 3 days (group: late AP_t; fig 1). To determine the effect of probiotic pretreatment in the late phase of AP, probiotics were administered only during two days prior to AP induction (group: late AP_p; fig 1). To investigate the effect of probiotic pretreatment in the early phase of AP, probiotics were administered during two days prior to AP induction (group: early AP_p; fig 1). The eight other experimental groups are not shown in Fig.1. Two sham AP groups were included (groups: sham early AP and sham late AP). Also, for each probiotic application a placebo group was included (plac late $AP_{p\&t}$, plac late AP_t, plac late AP_p and plac early AP_p, respectively). To determine baseline characteristics, healthy control animals (no AP, no treatment; group: control) were used. To establish the potential effect of probiotics on healthy ileum, mice received daily probiotics for five consecutive days (no AP, probiotic treatment; group: prob only).

Histopathological examination of AP

In order to confirm and grade the severity of the AP, pancreatic tissue specimen and both lungs were processed for histopathological examination. The samples were fixed in 4% formalin, embedded in paraffin and cut in serial sections of 4 μm following standard procedures. Haematoxilin & Eosin stainings were initially performed on all slides, followed by more specific stainings (PAS-diastase and Leder) in order to monitor the amount of extracellular edema and the composition of the inflammatory infiltrate. Histopathological evaluation was performed by a pathologist, experienced in gastro-intestinal and lung pathology, blinded for the groups and as described in literature.[24, 25] Pancreatic injury was assessed by three variables: edema (0 = none, 1 = focal interlobular, 2 = interacinar), inflammatory infiltrate (0 = none, 1 = periductal infiltrate, 2 = ≤ 50% parenchymal infiltrate, 3 = > 50% parenchymal infiltrate), necrosis (0 = none, 1 = < 5% periductal, 2 = 5-20% focal, 3 = > 20% diffuse). The severity of lung injury was assessed by four parameters: neutrophil infiltrate (score: 0 (none) – 4(severe)), alveolar edema (0 – 4), interstitial edema (0 – 4) and extravascular blood (0 – 4).

Myeloperoxidase assay

The degree of neutrophil sequestration in the lung was assessed by measurement of tissue myeloperoxidase (MPO) activity. Briefly, lung samples were homogenized in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide to provide a 10% homogenate (w/v). Samples were freeze-thawed and sonicated on ice. Suspensions were centrifuged at 14.000×g, for 15 minutes. The MPO activity in the supernatants was measured as described by Bradley *et al*.[26]

Measurement of epithelial electrical resistance and permeability in Ussing chambers
Immediately after termination, a 4 cm segment of the distal ileum was removed and placed on a pipette, gently stripped of the muscle layers and cut along the mesenteric border. Flat sheets of mucosa free of Peyer's patches were placed in teflon holders and mounted in Ussing chambers within 5 minutes after being cut-off from the blood supply. In each Ussing chamber experiment, six ileal samples (3/animal) were used. The exposed mucosal area was 0.2 cm^2. Both sides of the epithelium were in contact with 1.6 ml Krebs-Ringer's solution, stirred and gassed with humidified carbogen at 37 ˚C. The Krebs-Ringer's solution was composed of (in mmol/L) 117.5 NaCl, 5.7 KCl, 25 $NaHCO_3$, 1.2 NaH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgSO_4$ and 5 inosine, pH 7.4.
The transepithelial potential difference V_{te} (mV) was continuously monitored with Calomel electrodes (Fisher Scientific UK Ltd, Leicestershire, UK) connected to the chambers with agar bridges. The tips of these bridges were placed at less than 1 mm from the epithelium in the middle of the exposed area. Transepithelial electrical resistance R ($\Omega.cm^2$) was calculated according to Ohm's law from the voltage deflections induced by bipolar constant current pulses of 10 µA (every 30 seconds) applied through platinum wires at a distance of 2 cm from the epithelium. The potential and resistance data were stored on a PC using custom made software (Natural Simstrument, Amsterdam, The Netherlands). During off-line data-analysis (Origin 7.5; OriginLab Corporation, Northampton, USA), corrections were made for resistance of the solution in the chambers and for potential differences between Calomel electrodes, both measured before as well as after each experiment. The equivalent short-circuit current (I_{sc}) was calculated from the continuously monitored values of R and V_{te}. Reported values for the parameters V_{te}, R and I_{sc} were obtained at the end of a 15 to 20 min equilibration period. Generally, these values were stable during the subsequent 2 hour experiment. At the end of the experiment, viability of the tissue segments was confirmed by measuring their response to application of the secretagogue carbachol (10^{-4} M) in the serosal compartment.
Paracellular mucosal-to-serosal permeability was determined using Na-fluorescein (NaFl; Sigma-Aldrich) as a model molecule.[27] NaFl dissolved in Krebs-Ringer's solution was added to the mucosal compartment to a final concentration of 0.01 g/l. A sample was immediately collected to verify the initial concentration NaFl in the mucosal chamber. Throughout the 2 hour incubation period, 200 µl serosal samples were taken every 15 minutes and replaced by Krebs-Ringer. The concentration of NaFl in the serosal bath was determined by measuring fluorescence of the samples in a fluorometer (Polarstar Galaxy, BMG), with 485 nm and 530 nm as excitation and emission wavelengths, respectively. A calibration curve relating measured fluorescence intensity to the concentration of NaFl was prepared for each experiment. After application of NaFl to the mucosal compartment, the NaFl-flux increased linearly over a period of 45 to 60 min, after which it reached a steady level. The steady state NaFl-flux (fig 2) was quantified and expressed as $ng/cm^2.h$.

For each animal average values of electrophysiological parameters and NaFl-flux were calculated from simultaneous measurements of 3 ileal samples.

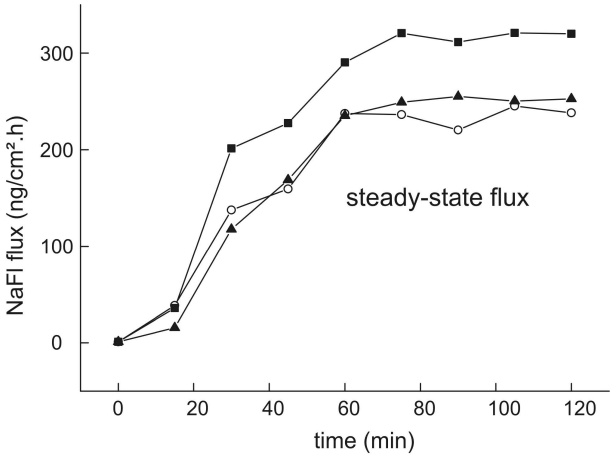

Figure 2
Example of the time course of the transepithelial flux of Na-fluorescein (NaFl) in three samples of one ileum. After addition (t=0) of NaFl (0,01 g/l) the NaFl-flux increased until after approximately 60 min a steady state level was reached.

Statistical analysis
All statistical analyses were performed using SPSS v.12.0 software. Parametric data are expressed as mean ± standard error of the mean (SEM). Statistical comparisons between multiple groups were performed by one-way analysis of variance (ANOVA) followed by post hoc analysis (Fisher's Least Significant Difference (LSD) analysis) when ANOVA yielded significance. To correct for multiple comparison a Bonferroni alpha was computed as 0.05 divided by the number of comparisons. Box and whisker graphs depict the median (line within the box), 25-75 percentiles (upper and lower border of the box) and 10-90 percentiles (whiskers). Non-parametric data (histology scores) are expressed as median (range) and comparisons between two or multiple groups were analyzed using the Mann-Whitney U or Kruskal-Wallis test, respectively. Statistical significance of non-parametric data was accepted for p values < 0.05. The n values represent the number of mice studied in each test group.

Results

Validation and severity of cerulein-induced AP
The severity of the cerulein-induced AP in the mouse model was assessed immediately (early AP) and three days after (late AP) AP induction. In early AP, the histopathological abnormalities of the pancreas were dominated by extracellular edema, localised around the

individual acini and in between the lobules. A slight inflammatory infiltrate of neutrophils and necrosis of a few clustered exocrine pancreatic cells was seen only in the peripheral segments of the pancreatic lobules. The late AP was characterized by an inflammatory infiltrate of lymphocytes, plasma cells, macrophages and neutrophils around clearly necrotic pancreatic acini and in the surrounding fatty tissue (table 1, fig 3). Ingrowth of fibroblastic and endothelial cells and formation of small capillaries was present. Scoring of the histopathological parameters of pancreatic injury did not reveal a difference between early and late AP (table 1 and fig 3).

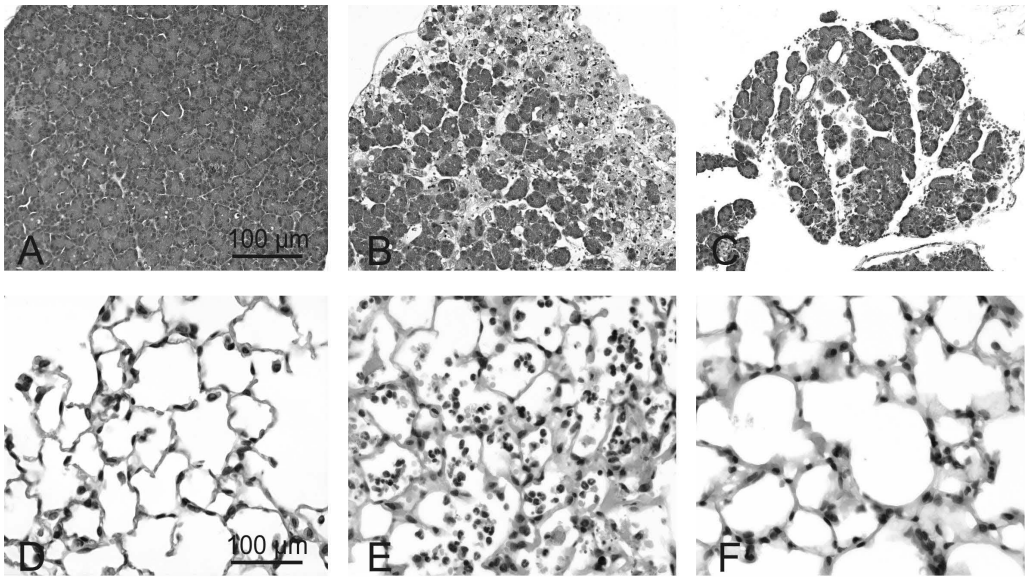

Figure 3
Morphological changes in pancreas (A-C) and lung (D-F) after cerulein-induced AP in hematoxylin-eosin stained sections. (A) Normal pancreas, (B) Pancreatic histopathology immediate after the induction of AP (early AP): Tissue shows diffuse interlobular edema, up to 20% of acini demonstrate necrosis, 50% of parenchyma contains inflammatory infiltrate. (C) Pancreatic histopathology three days after the induction of AP (late AP): edema, inflammatory infiltrate and necrosis are still pronounced. (D) Normal lung. (E) Pulmonary histopathology, immediate after the induction of AP (early AP): inflammatory infiltrate of mainly neutrophils, prominent alveolar and interstitial edema. (F) Three days after the start of AP induction (late AP): pulmonary histopathology closely resembles healthy tissue.

Lung injury in early AP was histopathological demonstrated by a slight intra-alveolar exudate, some neutrophils in the interstitial lung septa, migration of neutrophils in the alveolar space and extravasation of erythrocytes (table 1, fig 3). This increased amount of neutrophils in the lungs in early AP was reflected in the increased MPO activity in

pulmonary tissue (table 1). In the late phase of AP the lungs did not show any histopathological abnormalities (table 1, fig 3). This was confirmed by MPO analysis (table 1) and is in line with earlier reports.[28] Based on these findings, the literature data on this model,[29] and since mortality due to AP did not occur, the induced AP was considered to be mild.

Table 1. Characterization of AP. Histopathology scores of the pancreas and lung tissue, and the pulmonary myeloperoxidase (MPO) activity in healthy control animals, immediately (early AP) and three days after induction of AP (late AP).

		Pancreas-score		Lung-score		Lung MPO (nmol.min.mg)	
Group	**n**	**Median (range)**	***P* - value**	**Median (range)**	***P* - value**	**Mean ± SEM**	***P* - value**
Control	16	0 (0 – 0)		5 (5 – 6)		0.10±0.02	
Early AP	16	4 (0 – 8)	0.002	9 (4 – 10)	0.038	0.29 ±0.04	< 0.001
Late AP	13	3 (0 – 5)	0.004	6 (5 – 9)	0.284	0.17 ±0.03	0.144

Scores of pancreas and lung tissue are expressed as median with range, and were compared to control using the Mann-Whitney U or Kruskal-Wallis tests. The MPO activity is expressed as mean ± standard error of the mean (SEM) and was compared to control by one- way ANOVA

Mucosal barrier impairment in early and late AP

The effect of AP on intestinal barrier integrity of the ileum was assessed by measuring the electrical resistance and transepithelial flux of NaFl in Ussing chambers. The ileal resistance of control mice (R 35.8 ± 1.6 $\Omega.cm^2$) was comparable to that previously reported.[30] The values for transepithelial potential (V_{te} 1.00 ± 0.05 mV) and the equivalent short-circuit current (I_{sc} 30.5 ± 1.4 $\mu A.cm^{-2}$) demonstrated the good condition of the tissue samples,[30, 31] as did the relatively large increase in V_{te} (1.51 ± 0.18 mV) and I_{sc} (increase with 108 ± 9 % of basal I_{sc}) in response to the secretagogue carbachol. The steady state flux of NaFl in distal ileum preparations of healthy control mice (219 ± 16 $ng/cm^2.h$) was comparable to the mean flux reported for small intestine of guinea-pigs.[27]

Figure 1 shows the two protocols used to differentiate the effects of early AP and late AP on mucosal barrier integrity. Two sham AP groups (not shown in fig 1) were included (sham early AP and sham late AP). AP induction resulted in a reduction of the mucosal

resistance and an increase of the flux of NaFl when compared to sham AP (fig 4), indicating an impairment of the intestinal barrier function. The resistance and the NaFl-flux were equally affected in early AP and in late AP (R: p 0.34; flux: p 0.04, Bonferroni alpha 0.02), whereas the resistance and NaFl-flux of mice receiving sham AP were not different from those of healthy controls (R, sham early AP: p 0.49, R, sham late AP: p 0.89; flux, sham early AP: p 0.41, flux sham late AP: p 0.37). The basal transepithelial secretion (I_{sc}) and maximal secretory capacity (d_{Isc} in response to carbachol) were not affected in early AP (p 0.94 and p 0.81, respectively) but were both slightly increased in late AP (I_{sc} 38.4 ± 2.5 µA.cm^{-2}, p 0.001, d_{Isc} 176 ± 17 % of basal I_{sc}, p < 0.001).

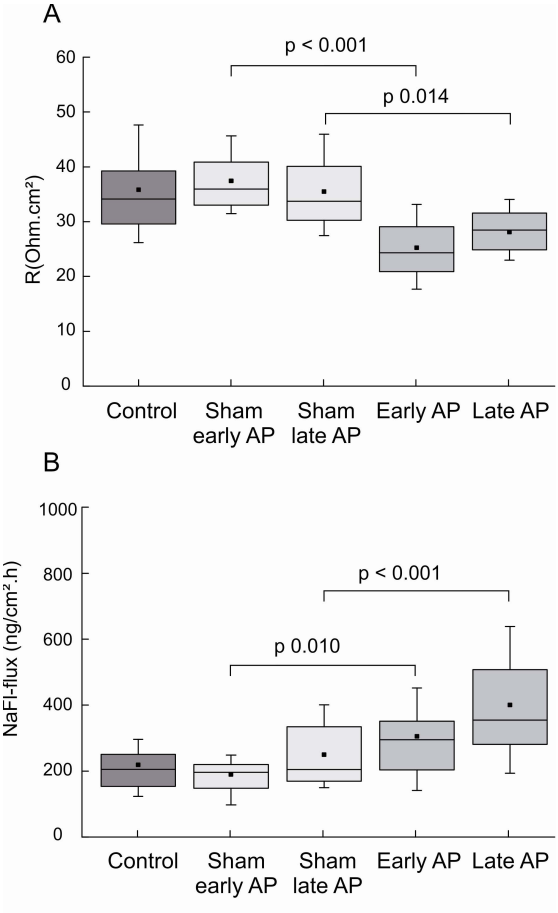

Figure 4
Effect of early and late AP on transepithelial electrical resistance (A) and flux of Na-fluorescein (NaFl) (B). Induction of AP resulted in a decrease of the resistance and an increase of NaFl-flux in early AP and in late AP in comparison to the corresponding sham treatments. Mucosal barrier function was equally impaired in early AP and in late AP (R: p 0.337; flux: p 0.039). Box and whisker plot show the mean (square within box), median (horizontal line), 25th and 75th percentiles (box), and 10th and 90th percentiles (whiskers) of 14 animals per group. The mean values were compared by one-way analysis of variance (ANOVA) followed by a post-hoc comparison (Fisher's LSD) with Bonferroni correction for multiple comparison. A p-value of < 0.017 (Bonferroni alpha = 0.05/3) was considered statistically significant.

Effect of probiotics on AP-induced mucosal barrier impairment

The four different application schedules of probiotics that were tested for their effect on the AP-induced impairment of the intestinal barrier function are shown in figure 1. For each application a placebo group (not shown in fig 1) was included. The combination of probiotic pretreatment and treatment which lasted from two days prior to AP induction until three days after AP induction (fig 1: late $AP_{p\&t}$) abolished the AP-induced changes in resistance and flux. Following this application, the resistance was increased and the flux decreased when compared to placebo (fig 5) and were not different from control values (R: p 0.71; flux: p 0.50). The probiotic treatment which started at the time of AP induction (fig 1: late AP_t) and had a duration of three days, did not affect the AP-induced changes in resistance and flux when compared to placebo (fig 5).

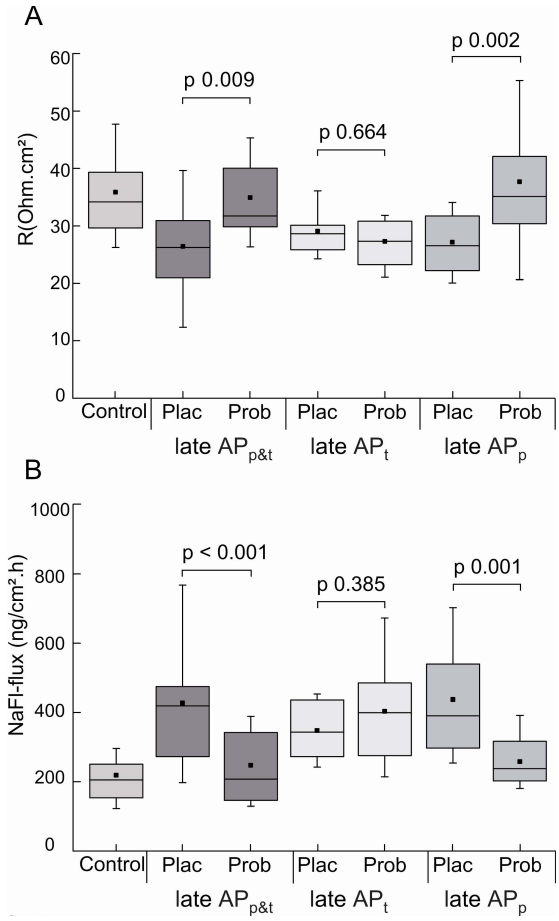

Figure 5
Effect of probiotics on late AP-induced impairment of the mucosal barrier function. Resistance (A) and NaFl-flux (B) were measured three days after AP induction (late AP) Probiotics (prob) or placebo (plac) was applied as described in figure 1. After the combined probiotic two day pretreatment and three day treatment (late $AP_{p\&t}$), the resistance and flux were different from those of the placebo treated animals and not different from controls (R: p 0.714; flux: p 0.500). The treatment of three days (late AP_t) did not result in a change in resistance or flux compared to the placebo. After the pretreatment of two days only (late AP_p), the resistance and flux were not different from control values (R: p 0.501; flux: p 0.378). Box and whisker plot showing the mean (square within box), median (horizontal line), 25th and 75th percentiles (box), and 10th and 90th percentiles (whiskers) of 14 animals per group. The mean values were compared by one-way analysis of variance (ANOVA) followed by a post-hoc comparison (Fisher's LSD) with Bonferroni correction for multiple comparison. A p-value of < 0.013 (Bonferroni alpha = 0.05/4) was considered statistically significant.

The pretreatment with probiotics during two days prior to AP induction (fig 1: late AP_p) abolished the AP induced changes in resistance and flux at late AP (fig 5) so that both returned to the level of healthy controls (R: p 0.50; flux: p 0.38). Remarkably, there was no difference between the effect of this two day pretreatment (fig 1: late AP_p) and the combined five day (pre)treatment (fig 1: late $AP_{p\&t}$) described above. These findings suggest that the pretreatment, by itself or in combination with treatment, abolishes AP induced barrier impairment in late AP, but that treatment by itself had no effect in late AP. We therefore next investigated whether the pretreatment had effect on the AP-induced impairment of the mucosal barrier in early AP (fig 1: early AP_p) and found that the two day pretreatment had no effect on intestinal resistance or flux values (fig 6).

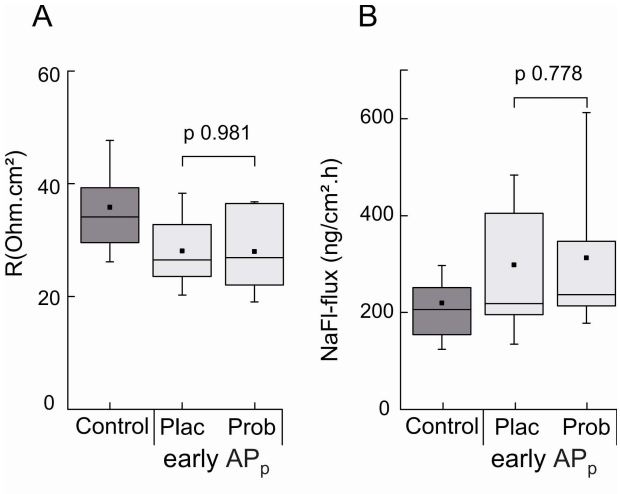

Figure 6
Probiotic pretreatment does not prevent impairment of the mucosal barrier function in early AP. Resistance (A) and NaFl-flux (B) were measured in the early phase of AP (early AP), immediately after AP induction. Probiotics (prob) or placebo (plac) was applied as described in figure 1 (early AP_p). The two day pretreatment did not prevent the decrease of resistance and increase in NaFl-flux induced by AP (R: p 0.009; flux: p 0.037; Control vs early AP_p). Box and whisker plot showing the mean (square within box), median (horizontal line), 25th and 75th percentiles (box), and 10th and 90th percentiles (whiskers) of 14 animals per group. The mean values were compared by one-way analysis of variance (ANOVA) followed by a post-hoc comparisons (Fisher's LSD). A p-value of < 0.05 was considered statistically significant.

Additional experiments showed that pretreatment or treatment of AP with the placebo did not affect the resistance or flux in any of the four application schedules (all: $p > 0.26$). In control mice, daily application of probiotics during five days did not change the resistance and flux (R 34 ± 2.0 Ω.cm^2, p 0.61; flux 267 ± 23 ng/cm^2/h, p 0.10, control vs. prob only). The transepithelial flux of NaFl and the resistance R of the 14 experimental groups were linearly correlated (fig 7).
The AP induced increase of basal secretion (I_{sc}) and maximal secretory capacity (d_{Isc} to carbachol) observed in late AP was not affected by any of the four probiotic application schedules (I_{sc}: p 0.21; d_{Isc}: p 0.52). This lack of effect of the probiotics on the late AP-

induced secretion might be due to the relatively small increase in our model in comparison to, for instance, the increase in secretion induced by chronic stress. [32]

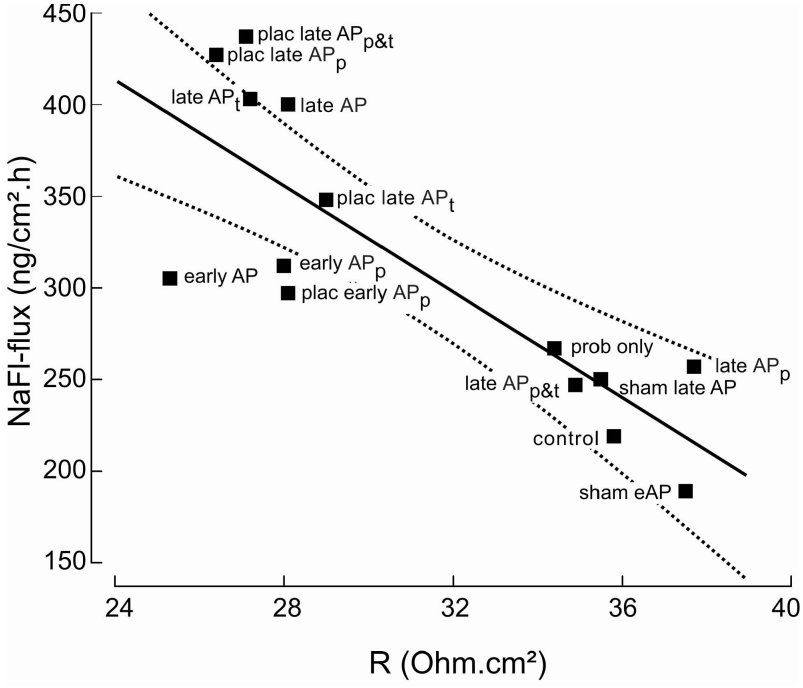

Figure 7
Linear correlation between the transepithelial flux of NaFl and the electrical resistance R of all experimental groups. Each circle represents the mean values (n = 14) of an experimental group. The calculated (Origin 7.5; OriginLab Corporation, Northampton, USA) linear ($p < 0.001$) correlation has a regression coefficient of 0.82 and the 95% confidence bands are indicated. Early AP: Animals were terminated immediately after induction of AP. Late AP: Animals were terminated three days after induction of AP. Late $AP_{p\&t}$: Late AP group with combined, probiotic pretreatment ($_p$) and treatment ($_t$) from two days before until three days after AP. Late AP_t: Late AP group with probiotic treatment of three days starting at AP induction. Late AP_p: Late AP group with probiotic pretreatment of two days, starting two days before induction of AP. Two sham AP groups: sham early AP and sham late AP. Four placebo probiotic groups: plac late $AP_{p\&t}$, plac late AP_t, plac late AP_p and plac early AP_p. Two other groups: prob only (no AP, probiotics) and control (no AP, no probiotics).

Discussion

In the mouse model of cerulein-induced AP we measured the integrity of the ileal paracellular mucosal pathway to determine the impairment of the intestinal barrier, which is thought to contribute to the endotoxin and bacterial translocation during AP.[6, 8] Our results demonstrate that a preservation of the integrity of the intestinal barrier in the late

phase of AP can be accomplished by pretreatment with a multispecies mixture of probiotics. Probiotic treatment starting immediately after AP induction, however, was found to be without any effect on intestinal barrier impairment. We conclude that in the mouse model, multispecies probiotics are effective only against AP-induced intestinal barrier impairment when administrated before induction of disease.

Impairment of the mucosal barrier in AP

The well-established mouse model of cerulein-induced AP is characterized by pancreatic neutrophil infiltration and acinar necrosis.[28] This model produces a mild AP with no mortality. The characteristic biphasic course of AP, as described in human,[6] and in animal models [22] was recognizable in our experiments. The histopathological examination revealed that damage of the pancreas was present in the early as well as in the late phase of AP, while damage to the lung was observed only in the early phase of AP (cf [28]). The AP-induced impairment of the mucosal barrier in mice, which is quantified here for the first time, was observed both in the early and the late phase of AP. This is in line with clinical observations in patients with AP.[4, 6] As shown in figure 7, the steady state flux of NaFl was linearly correlated to the electrical transepithelial resistance R of the 14 experimental groups. This figure also shows that the AP-induced enhancement of the permeability for NaFl was under all conditions accompanied by a decrease in resistance. Since the resistance is mainly determined by the tight junctions in the epithelium,[33] the correlation suggests that the AP-induced enhancement of the permeability for NaFl involves the opening of tight junctions of the epithelial paracellular pathway, rather then alterations in the transcellular pathway. We conclude that in the cerulein AP-model, the impairment of the mucosal integrity is mild and comparable to that induced by chronic stress.[32]

Pretreatment with probiotics abolishes AP-induced mucosal barrier impairment

The finding that pretreatment with probiotics did not counteract the damage to the mucosal barrier function in the early phase of AP is in agreement with the notion that the damage in the early phase of AP results mainly from the hemodynamic changes in the splanchnic circulation,[1, 4] which are not expected to be affected by the probiotics. In the late phase of AP, ongoing small bowel bacterial overgrowth, mucosal inflammation and release of endotoxins are considered to be responsible for the adverse changes in mucosal barrier function.[6, 34-36]

Our results demonstrate that pretreatment with multispecies probiotic Ecologic® 641 restores mucosal barrier function in the late phase of AP to the level of healthy control animals. This is in accordance with the findings in the rat model of severe AP, in which during the late phase small bowel bacterial overgrowth, bacterial translocation and mortality were significantly reduced after application of Ecologic® 641 probiotics.[19] This suggests that the reduction of the bacterial translocation in the late phase of AP observed in the rat study [19] may have been a direct result of the beneficial effects of the probiotic

pretreatment on the intestinal barrier. In the cerulean mouse model, the preservation of mucosal integrity in the late phase of AP was achieved by a pretreatment with probiotics for two days, while a three days probiotics treatment did not preserve the mucosal integrity in the late phase of AP. This remarkable difference in effectiveness leads to the conclusion that the timing of application of probiotics is an important factor determining the outcome of the treatment. Although pretreatment of AP in clinical practice is not feasible and probiotic treatment of AP increases mortality,[21] administration of probiotics might be relevant for other conditions where mucosal barrier dysfunction is associated with infectious complications.[37]

Timing and mode of action of probiotics

The complete elimination of the AP-induced impairment of the mucosal integrity as a result of the probiotic pretreatment is thought to indicate a considerable interaction between the probiotic bacteria and the host. In this context it is important to note that there was no effect of the probiotics on mucosal integrity of healthy control animals. Thus the beneficial effects of the probiotics cannot be explained by an improved mucosal barrier condition of the probiotic treated animals before AP induction. Instead, the probiotics appear to interfere with the pathophysiological condition induced by AP.

Not much is known about the relation between the timing of probiotic application and the effectiveness, whereas usually this timing differs between clinical trials and animal experiments. In clinical settings, treatment with probiotics has been shown to be beneficial in several chronic intestinal diseases,[14] suggesting that the action of the probiotics is in restoration of already existing damage. The alternative mechanism, namely prevention of damage, has not been investigated in clinical trials. The results of animal studies have so far not approached these presumed mechanisms since in these studies the application of probiotics generally started several days before damage was induced (pretreatment) and usually continued for many days afterwards (treatment).[18, 19, 32]

The present results show that in order to be effective the probiotics have to be present in the period before AP is induced and not necessarily in the period following AP induction (fig 5).

The results also show that a 2-day pretreatment with probiotics does not prevent AP-induced damage to the mucosal barrier (fig 6) but is able to completely restore the barrier function in the 3 days following induction of damage (fig 5). This finding strongly indicates that the main beneficial effect of probiotics on the mucosal barrier is to accelerate the repair of damage.

Pathogenesis of mucosal barrier dysfunction in AP

Information about the effects of AP on gut mucosal barrier function in animal models is scarce,[4, 38, 39] and information on the effects of probiotics on AP-induced dysfunction of this barrier was non-existing. This is the first report of a differential effect of probiotics on

the mucosal barrier which depends on the timing of application in a mild AP-model. The observed beneficial effects of pretreatment with multispecies probiotics makes this a useful model for investigation of the pathogenesis of gut mucosal barrier dysfunction in AP. Although further studies are clearly needed to elucidate the underlying mechanisms of pathogenesis, the observations reported in this communication indicate a possible future clinical value of this line of investigation.

In summary, induction of AP by cerulein causes in the mouse model an evident impairment of the ileal mucosal barrier function. In the early phase of AP this impairment is not improved by a two day pretreatment with probiotics. Most interestingly, the intestinal barrier failure in the late phase of AP, where the barrier impairment and consequent endotoxins and bacterial translocation are matters of concern, can be abolished by administration of probiotics starting prior to onset of AP. Future clinical studies that use probiotics to prevent complications due to intestinal barrier impairment should seek conditions where treatment can be started prior to onset of disease or elective surgical intervention.

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Chapter 3

Impairment of intestinal barrier and secretory function as well as egg excretion during intestinal schistosomiasis occur independently of mouse mast cell protease-1

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Abstract

Deposition of *Schistosoma mansoni* eggs in the intestinal mucosa is associated with recruitment of mucosal mast cells (MMC) expressing mouse mast cell protease-1 (mMCP-1). We investigated the involvement of mMCP-1 in intestinal barrier disruption and egg excretion by examining BALB/c mice lacking mMCP-1 (Mcpt-1$^{-/-}$). Tissue and fecal egg counts from 6 until 12 weeks post-infection (w p.i.) revealed no differences between wild type (WT) and Mcpt-1$^{-/-}$ mice. Ussing chamber experiments on ileal tissue revealed that at 8w p.i. the epithelial barrier and secretory capacity were severely impaired, whereas no difference was found between WT and Mcpt-1$^{-/-}$ mice in this respect. However, a fragmented distribution of the tight junction (TJ) protein occludin, but not of claudin-3 or ZO-1, was observed in WT mice at 8w p.i., while no changes in TJ integrity were seen in Mcpt-1$^{-/-}$ mice. Therefore, we conclude that, in contrast to the situation in *Trichinella spiralis*-infected mice, in schistosomiasis mMCP-1 is not a key mediator in egg excretion or impairment of the intestinal barrier. The marked decrease in ileal secretory capacity during S. mansoni egg excretion suggests that the mechanisms facilitating the passage of schistosoma eggs through the gut wall are directed more particularly at the epithelial cells.

Introduction

In *Schistosoma mansoni*-infected mice, egg deposition in the intestinal wall, starting 5-6 weeks after infection, is associated with granuloma formation and transition from an initial TH1 response against the adult worms to a predominantly TH2-regulated allergic inflammation in the gut [1]. Recruitment of an intraepithelial population of mucosal mast cells (MMC), characterized by the expression of the enzyme mouse mast cell protease-1 (mMCP-1, gene name Mcpt-1), which is exclusively found in recruited MMC and not in the epithelial cells [2], occurs as from the 6^{th}-8^{th} week of infection [3-5]. Coinciding with MMC recruitment is an increased density of calcitonin gene-related peptide (CGRP)-expressing extrinsic primary afferent nerve fibers in the intestinal lamina propria [6]. It is suggested that MMC activation and degranulation occur as a direct response to CGRP-release from these extrinsic primary afferents, while extrinsic primary afferent neurites are activated by mediators released by MMC [7]. This bidirectional interplay between immune and neural compounds, as well as classical IgE-mediated activation, are all likely to be important in the development and regulation of tissue defences against helminth parasites.

The function of MMC in intestines harbouring schistosome eggs is at present unknown, nor is the manner in which the eggs cross the impermeable mucosal barrier into the gut lumen. Serine proteinases are major constituents of mast cell granules, and appear to affect the barrier and transport properties of the intestinal epithelium [8,9]. So, it has been indicated that the MMC granule β-chymase, mMCP-1 and the homologous rat mast cell protease-2 (rMCP-2) , are able to disrupt epithelial integrity [10,11] and thereby increase intestinal permeability [12,13]. In an Ussing chamber set-up , McDermott and co-workers [14] demonstrated that Mcpt-$1^{-/-}$ mice did not show any increase in intestinal permeability to mannitol during *Trichinella spiralis* infection, in contrast to WT mice, in which permeability was increased during infection. This observation indicated an important role of mMCP-1 in modulating intestinal barrier permeability during infection with the nematode *T. spiralis*. In other studies concerning infection with the intraepithelial nematode *T. spiralis*, it has been observed that worm expulsion is delayed and larval deposition is increased in the absence of mMCP-1, despite comparable recruitment of MMC [15,16]. These studies point to a role of mMCP-1 in the proteolytic modification of the tight junctions (TJ), maintaining the integrity of the mucosal barrier, as a plausible mechanism of facilitated transepithelial parasite expulsion [17,18].

However, no quantitative information on intestinal permeability and epithelial secretion was available to support the proposed role of mMCP-1 in the excretion of eggs deposited by *S.mansoni* [15] which considerably differs from *T. spiralis* with respect to life-cycle and niche within the host. Therefore, we investigated if mMCP-1 contributes to schistosomiasis-induced alterations in epithelial permeability and secretion and to egg excretion.

Material and methods

Animals
Adult male Mcpt-$1^{+/+}$ (wild type, WT) and Mcpt-$1^{-/-}$ BALB/c F_{10} mice were generated as previously described [19] and were bred at the University of Antwerp (Antwerp, Belgium) under specific pathogen-free conditions. The animals were given food and water *ad libitum* and were kept in a 12:12 h light/dark cycle. All experimental procedures were approved by the local ethics committee of the University of Antwerp.

Infection
Mice were infected according to the method of Smithers and Terry [20] at 6-8 weeks of age. Briefly, after shaving the anesthetized animals, a heavy metal ring was placed on the lower abdomen and 1.2 ml water containing 100 freshly shed cercariae of a Puerto Rico strain of *S. mansoni* was pipetted into this ring. The animals were exposed for 10 min, allowing the cercariae to penetrate transcutaneously. The cycle of *S. mansoni* was maintained in the laboratory by passage through *Biomphalaria glabrata* snails. To prevent variation due to the infection procedure, in each independent experiment WT as well as Mcpt-$1^{-/-}$ mice were infected. Mice, infected 6-12 weeks prior to investigation, and age-matched control mice, were killed by cervical dislocation followed by exsanguination. Of all infected animals used in the study, the liver was macroscopically evaluated for the presence of granulomas.

Worm burden counts
In dedicated experiments, adult worms were recovered from the hepatic portal system and the liver of infected WT (n=5) and Mcpt-$1^{-/-}$ mice (n=5) by cardiac perfusion with citrate saline (0.85 % sodium chloride, 1.5 % sodium citrate) after intraperitoneal injection with an overdose of Nembutal (150 mg/kg) [20]. The worms were counted immediately.

Fecal egg counts
Infected WT and Mcpt-$1^{-/-}$ mice (6-12 weeks post-infection (p.i.); n=7/time point) were allowed to defecate overnight. Single fecal pellets were placed in isotonic saline solution, disrupted by aspiration with a 10-ml syringe and filtered through a 320-µm metal sieve, as previously described [21]. Each filtrate was passed through a sheet of Whatman No.4 filter paper and the eggs were stained with saturated Ninhydrin solution [22]. Dried papers were examined in triplicate at 50x magnification by two independent observers. The results are expressed as the number of eggs/100 mg fecal matter.

Tissue egg counts
The ileum of infected WT and Mcpt-$1^{-/-}$ mice (6-12 weeks p.i.; n=7/time point) was removed and washed in Krebs solution (in mM: 117 NaCl, 5 KCl, 2.5 $CaCl_2.2H_2O$, 1.2

$MgSO_4.7H_2O$, 25 $NaHCO_3$, 1.2 $NaH_2PO_4.2H_2O$ and 10 glucose; pH 7.4). One gram (wet weight) of each ileum was digested in 5 ml of a 5 % potassium hydroxide solution at 37 °C for 16 h [23]. Fifty-µl aliquots of the digests were evaluated on microscope slides and the eggs counted at 25x magnification. Each digest was examined in triplicate by two independent observers and the mean results were expressed as eggs/g wet weight of tissue.

Tissue preparation for immunocytochemistry

The ileum, excised from both normal and 8-week-infected (representative of the acute phase of schistosomiasis [3]) WT (n=6) and Mcpt-$1^{-/-}$ mice (n=6), was washed in Krebs solution. Three 10-mm segments were removed at the distal end of each ileum. One segment was formalin-fixed followed by paraffin embedding and 5-µm-thick paraffin sections were stained with hematoxylin and eosin (HE). The second segment was processed for cryosectioning. Briefly, the segment was fixed for 2 h at room temperature in 4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.0). Subsequently, it was rinsed in 0.01 M phosphate-buffered saline (PBS; pH 7.4), transferred to PBS containing 20 % sucrose and stored overnight at 4 °C. Next, it was embedded in OCT-embedding medium (Pelko, Torrance, CA, USA), cryostat-sectioned at 12 µm and thaw-mounted on poly-L-lysine-coated slides. Sections were allowed to air-dry and were immediately used for mMCP-1 and mMCP-2 immunostaining. mMCP-2 staining was applied to identify and count MMC in Mcpt-$1^{-/-}$. The third segment was embedded in OCT-medium, frozen in liquid nitrogen-cooled isopentane and stored at –80 °C. Subsequently, 60-µm-thick tangential sections were made by cryostat sectioning, and allowed to air-dry and fixed for 10 min in ice-cold acetone followed by rehydration in 0.01 M PBS and finally used for immunostaining of the TJ proteins claudin-3, occludin and ZO-1.

Immunohistochemistry

All incubations were performed at room temperature. The primary and secondary antibodies (Table I) were diluted in PBS containing 10 % normal goat serum, 0.01 % bovine serum albumin, 0.05 % thimerosal and 0.01 % sodium azide (PBS*). The sections were pretreated for 30 min with PBS* containing 1 % Triton X-100. Next, they were incubated for 90 min with a primary antibody. Subsequently, after rinsing in PBS, they were incubated with an appropriate secondary antibody for 30 min. For negative controls, primary antisera were omitted in the protocol. The specificity of the primary antibodies was tested by performing immunoblotting and preabsorption tests.

Table I. List of primary and secondary antisera used for immunohistochemistry.

Primary antisera

Antigen	Host	Dilution	Source
mMCP-1	Rat	1/200	Prof. Dr. HRP Miller, Edinburgh, UK [2,3]
mMCP-2	Rat	1/100	Prof. Dr. HRP Miller [2,3]
Claudin-1/3	Rabbit	1/100	Zymed Laboratories, San Francisco, CA, USA (51-9000)
Occludin	Rabbit	1/100	Zymed (71-1500)
ZO-1	Rabbit	1/100	Zymed (61-7300)

Secondary antisera

	Dilution	Source
Cy3-conjugated goat anti rabbit	1/3000	Jackson Imm Res Lab, West Grove PA, USA
Cy3-conjugated donkey anti rat	1/500	Jackson Imm Res Lab, West Grove PA, USA

Ussing chamber experiments

The effect of *S. mansoni* infection on intestinal barrier integrity of the ileum was assessed by measuring the electrical resistance and transepithelial flux of NaFl in Ussing chambers. The electrical resistance is mainly determined by the tight junctions in the epithelium. Alterations in the resistance are thought to reflect opening (in case of reduced resistance) or closing (increased resistance) of tight junctions of the epithelial paracellular pathway, rather than an alteration in the transcellular pathway. Alterations in the transepithelial flux of NaFl indicate changes in the permeability of the epithelial barrier for small molecules [24]. Each of the four groups (non-infected WT and Mcpt-$1^{-/-}$ mice; 8-week-infected WT and Mcpt-$1^{-/-}$ mice) consisted of 7 animals. Immediately after termination, a 4-cm segment of the distal ileum was removed and rapidly cleaned in carbogenated Krebs-Ringer solution (in mM: 117.5 NaCl, 5.7 KCl, 25 $NaHCO_3$, 1.2 NaH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgSO_4$ and 5 inosine; pH 7.4). From each animal, three flat sheets of unstripped ileum free of Peyer's patches were placed in Teflon holders and mounted in Ussing chambers within 5 min after being cut off from blood supply. Both sides of the sample (exposed area 0.2 cm^2) were in contact with 1.6 ml Krebs-Ringer solution, stirred and gassed with humidified 95 % O_2 + 5 % CO_2 at 37 ˚C.

The transepithelial potential difference V_{te} (mV) was continuously monitored with Calomel electrodes connected to the chambers with Krebs-Ringer-agar bridges. Transepithelial electrical resistance R ($\Omega.cm^{-2}$) was calculated from the voltage deflections induced by bipolar current pulses of 10 µA (every 30 sec) applied through platinum wires. The potential and resistance data were stored on a PC using custom software (Natural Simstrument, Amsterdam, the Netherlands). During off-line data analysis, corrections were made for resistance of the solution and for potential differences between Calomel electrodes, measured both just before and immediately after each experiment. The equivalent short-circuit current I_{sc} ($\mu A.cm^{-2}$) was calculated from the continuously monitored values of R and V_{te}. Reported values for the parameters V_{te}, R and I_{sc} were obtained at the end of a 15- to 20-min equilibration period. Generally, these values were stable during the subsequent 1- or 2-h experiment. At the end of the experiment, the secretory capacity of the tissue segments was tested by measuring their response (V_{te} and I_{sc}) to application of the secretagogue carbachol in the serosal compartment (10^{-4} M). In the Ussing chamber experiments, the measured transepithelial potential (V_{te}) and equivalent short-circuit current (I_{sc}) are indicative of the basal epithelial secretion, while the increase in these parameters (dV_{te} and dI_{sc}) in response to the secretagogue carbachol reflects the maximal secretory capacity.
Paracellular mucosal-to-serosal permeability was determined using Na-fluorescein (NaFl; Sigma, Zwijndrecht, the Netherlands) as a model molecule [25]. After the equilibration period, NaFl was added to the mucosal compartment (0.01 g/l) and 200-µl serosal samples were taken every 7.5 min and replaced by Krebs-Ringer. The concentration of NaFl was determined in a fluorimeter (Polarstar Galaxy fluorescence multi-well plate reader, BMG LabTech GmbH, Jena, Germany), with 485 nm and 530 nm as excitation and emission wavelengths, respectively. Steady-state NaFl-flux was quantified and expressed as $ng.cm^{-2}.h^{-1}$. For each animal, average values of electrophysiological parameters and NaFl-flux were calculated from simultaneous measurements of 3 ileal samples.

Statistics
Statistic analysis were performed using SPSS v.12.0 software (SPSS Inc., Chicago, IL, U.S.A.). Values obtained from Ussing chamber measurements were compared by one-way analysis of variance (ANOVA) followed by a post-hoc analysis (Bonferroni-corrected Fisher's Least Significant Difference (LSD) analysis) when ANOVA yielded significance. Worm burden counts were compared by t-test. Fecal and tissue egg counts were compared using a two-way analysis of variance (ANOVA; with weeks p.i. as one factor and WT vs. Mcpt-$1^{-/-}$ mice as the second factor) followed by a student t-test (for groups with unequal variances). The linear correlations between tissue and fecal egg counts were determined using Origin 7.5 (OriginLab Corporation, Northampton, USA) and compaired by a F-test (Origin 7.5). A p value less than 0.05 was considered significant.

Results

Validation of the S. mansoni infection

At 8 weeks p.i., the adult worm burden did not differ between WT and Mcpt-1$^{-/-}$ mice (WT: 12.2 ± 2.5 worms/animal; Mcpt-1$^{-/-}$: 13 ± 1.4 worms/animal; mean ± SD; n = 5), indicating that deletion of Mcpt-1 had no effect on worm establishment and survival. Histological evaluation of HE-stained sections of 8-week-infected mouse ileum of WT and Mcpt1$^{-/-}$ animals revealed the presence and distribution of granulomas, thickening of the tunica muscularis, broadening of the intestinal villi and disturbance of the architectural structure of the myenteric plexus (data not shown). These observations are considered characteristic of this infection [3,26] and are consistent with the establishment of adult worm infection and egg deposition in the ileal wall.

Macroscopic evaluation of the liver and intestine of all infected animals consistently revealed the presence of a large number of granulomas distributed equally over the surface of the liver, whereas the ilea were oedematous and showed a loss of flexibility indicating fibrosis. Mortality was especially apparent at 12 weeks p.i.

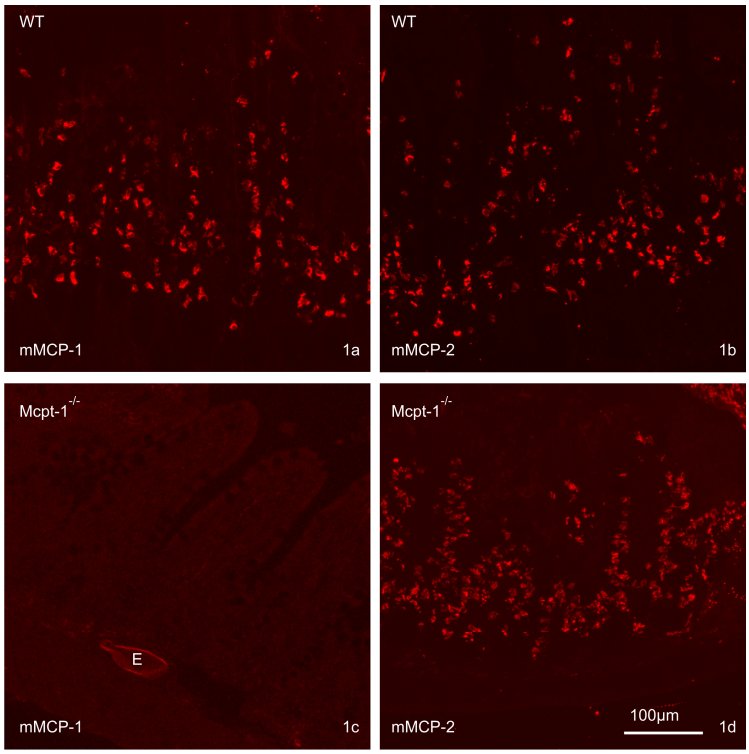

Figure 1.
Mastocytosis in WT and Mcpt-1$^{-/-}$ mice. mMCP-1- (a) and mMCP-2- (b) immunoreactive MMC (Cy3-epifluorescence) were observed intraepithelially and in the lamina propria of infected WT mice. As expected, only mMCP-2 (d), but not mMCP-1 staining (c) revealed MMC in infected Mcpt-1$^{-/-}$ mice. E: aspecific stained parasite egg. Scale bar = 100µm. The images shown are representative for observations on tissues from 4 mice in each of the two groups.

Recruitment of MMC

We previously described a 30-fold increase in the density of mMCP-1-positive MMC in the mucosa of mice during the acute phase of *S. mansoni* infection [3]. In the present study, MMC ($116.10^3 \pm 13.10^3$ MMC/mm³ mucosa; n=5) expressing both mMCP-1 and mMCP-2 were found in infected WT mice at 8w p.i. (fig. 1a,b). In the absence of mMCP-1 (fig. 1c) comparable numbers of mMCP-2-immunoreactive MMC ($114.10^3 \pm 9.10^3$ MMC/mm³ mucosa; n=5) were detected in infected Mcpt-$1^{-/-}$ mice (fig. 1d).

Distribution pattern of TJ proteins

In uninfected WT and Mcpt-$1^{-/-}$ mice, the TJ proteins occludin (fig. 2a and 2d), claudin-3 (fig. 2b and 2e) and ZO-1 (fig. 2c and 2f) formed a continuous polygonal structure around the apices of the epithelial cells. At 8 weeks p.i., the polygonal architecture of the membrane structure containing occludin (fig. 2g) was distorted and disrupted in WT mice. In contrast, the distribution patterns of claudin-3, also an extracellular TJ protein, and ZO-1, an intracellular TJ protein, were unchanged in 8-week-infected WT mice (fig. 2h and 2i). The TJ change in the WT mice during egg deposition at 8 weeks p.i. contrasts with that in infected Mcpt-$1^{-/-}$ mice, which did not display any detectable change in TJ structure (fig. 2j-l). As was expected, no differences in the staining pattern of any of the TJ proteins were observed between uninfected WT and uninfected Mcpt-$1^{-/-}$ mice either.

Ussing chamber experiments

In non-inflamed WT mice, values for the ileal electrical transepithelial resistance (R; see Table 2) and steady-state flux of NaFl were comparable to those previously reported [24,25,27]. Basal epithelial secretion, as indicated by the transepithelial potential (V_{te}) and the equivalent short-circuit current (I_{sc}), and maximal secretory capacity (increase in I_{sc} in response to the secretagogue carbachol) also indicated the overall good condition of the tissue samples. In 8-week-infected WT mice, transepithelial resistance was markedly reduced (Table 2) and the flux of NaFl was increased (Table 2), pointing to a severe impairment of intestinal barrier function, quantified here for the first time. Moreover, the *S. mansoni* infection induced a severe reduction of the basal secretion (V_{te} and I_{sc}) and maximal secretory capacity (dI_{sc}). For non-infected Mcpt-$1^{-/-}$ mice, the values for the above mentioned parameters were not different from those of the WT mice (Table 2). Most remarkably, the data obtained from Mcpt-$1^{-/-}$ mice at 8w p.i. revealed impairment of the barrier function and secretory capacity that was not different from that observed in the infected WT mice.

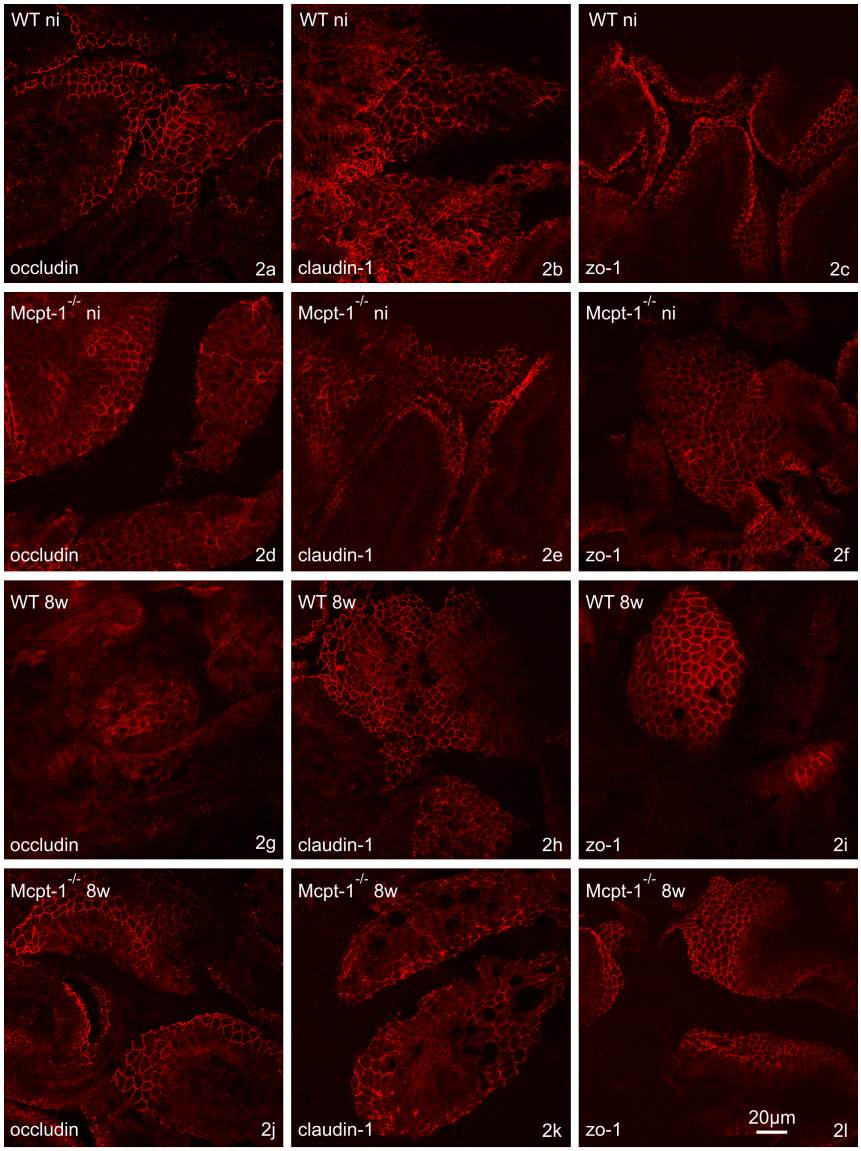

Figure 2.
Disrupted TJ in murine ileum. Immunohistochemical staining (Cy3-epifluorescence) for the TJ proteins occludin (a, d), claudin-3 (b, e) and ZO-1 (c, f) revealed polygonal structures around the apices of the epithelial cells of non-infected WT and Mcpt-1$^{-/-}$ mice (n=5). In 8w p.i. infected WT mice (n=5) this TJ network was totally disrupted for occludin (g),while no changes were observed for claudin-3 (h) or ZO-1 (i). In Mcpt-1$^{-/-}$ (n=5), infection with *S. mansoni* did not result in degradation of the network for any of the TJ proteins (j-l). Scale bar = 20µm. The images shown are representative for observations on tissues from 6 mice in each of the four groups.

Table II. Statistical comparison of parameters from Ussing chamber experiments.

	R ($\Omega.cm^{-2}$)	V_{te} (mV)	I_{sc} ($\mu A.cm^{-2}$)	carb dV_{te} (mV)	carb dI_{sc} ($\mu A.cm^{-2}$)	flux NaFl ($ng.cm^{-2}.h^{-1}$)
WT						
control	52 ± 16	1.6 ± 0.7	31.4 ± 11.7	2.1 ± 1.0	46.6 ± 36.3	209 ± 51
infected	15 ± 9	0.6 ± 0.4	12.2 ± 3.6	0.2 ± 0.3	5.3 ± 3.9	315 ± 143
p (contr vs inf)	< 0.0001	0.0029	0.0004	0.0003	0.0340	0.0520
Mcpt-1$^{-/-}$						
control	45 ± 13	1.5 ± 0.5	32.2 ± 6.0	1.8 ± 1.1	41.4 ± 34.7	228 ± 85
infected	10 ± 7	0.4 ± 0.2	11.7 ± 5.6	0.2 ± 0.3	8.7 ± 11.1	380 ± 100
p (contr vs inf)	< 0.0001	0.0012	0.0001	0.0030	0.0280	0.0300
p contr ($^{-/-}$ vs $^{+/+}$)	0.302	0.626	0.832	0.292	0.385	0.716
p inf ($^{-/-}$ vs $^{+/+}$)	0.495	0.595	0.939	0.910	0.759	0.287

Table II. Statistical comparison of 5 electrical parameters and NaFl flux (horizontal) of the distal ileum between 4 groups of mice: WT control and 8 weeks p.i., Mcpt-1$^{-/-}$ control and 8 weeks p.i. (vertical). The data were obtained in 7 independent experiments (one mice of each of the 4 groups per experiment) of which mean values (n=7) are given ± SD. Data of the 4 groups were compared by ANOVA (Bonferroni-corrected Fisher's LSD). The upper and middle parts of the table show that for both WT (upper) and Mcpt-1$^{-/-}$ mice (middle) infection with *S. mansoni* causes a reduction in all electrical parameters and an increase in NaFl flux. The lower part of the table shows that, there is no difference in any of the 6 parameters (hence p values are not Bonferroni-corrected) between WT and Mcpt-1$^{-/-}$, both under control and infected conditions. R: electrical resistance, V_{te}: transepithelial potential, I_{sc}: equivalent short-circuit current, carb dV_{te}: response of V_{te} to carbachol, carb dI_{sc}: response of I_{sc} to carbachol; flux NaFl: transepithelial steady-state flux of Na-fluorescein.

Fecal and tissue egg counts.

The number of *S. mansoni* eggs in the ileal tissue and the feces was determined each week from 6 until 12 weeks p.i. Tissue and faecal egg counts reached a peak at 10 weeks p.i. in both WT and Mcpt-1$^{-/-}$ mice (fig. 3). Tissue egg counts were higher in WT than in Mcpt-1$^{-/-}$ mice (p 0.003; two way ANOVA). A pairwise comparison by t-test revealed at 12 w p.i. in WT significantly more tissue eggs than in Mcpt-1$^{-/-}$ mice (p 0.020; fig 3a), but not in earlier weeks.

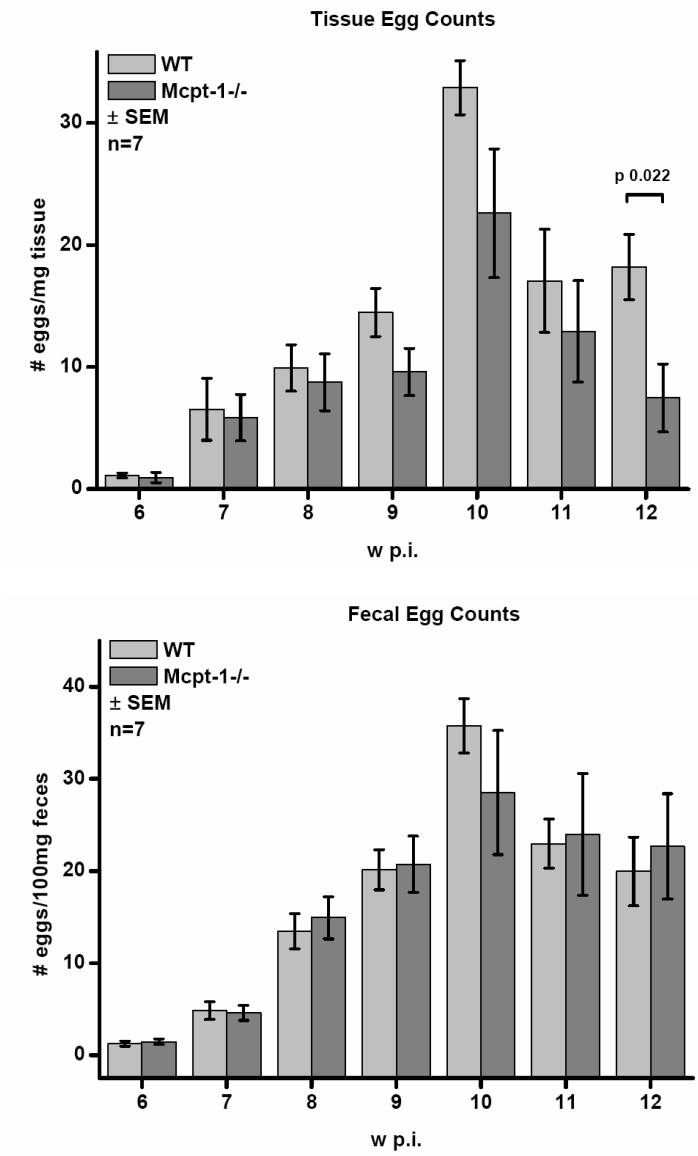

Figure 3.
Tissue and fecal egg counts at seven time points (6-12 w p.i.) of *S. mansoni* infection.
(a) Tissue egg counts. At week 12 w p.i. the number of eggs in the small intestinal wall was significantly higher ($p = 0.022$; ANOVA followed by t-test) in WT (n=7 per time point) than in Mcpt-1$^{-/-}$ mice (n=7 per time point). The vertical axis depicts the number of eggs per mg tissue (wet weight) of small intestine. (b) Fecal egg counts. No differences were seen at any time point upon comparison of fecal egg counts between mMCP-1-deficient (n=7 per time point) and WT animals (n=7 per time point) at the same infection stage. The vertical axis depicts the number of eggs per 100 mg feces. The data were obtained in 19 independent experiments (2 or 3 mice of each of the two groups per experiment).

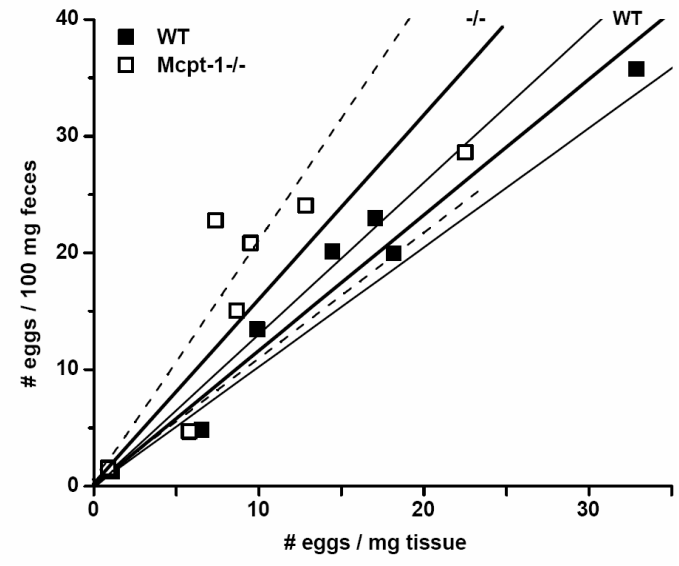

Figure 4.
Correlation between tissue egg counts and fecal egg counts. Linear correlations between tissue and fecal egg counts of WT and Mcpt-$1^{-/-}$ mice from 6 until 12 w p.i. The calculated linear correlations (Origin 7.5; OriginLab Corporation, Northampton, USA) (WT: $p < 0.0001$; Mcpt-$1^{-/-}$: $p = 0.02$) have regression coefficients of 0.976 (WT) and 0.829 (Mcpt-$1^{-/-}$) and similar slopes (WT $11.6 \times 10^{-4} \pm 0.6 \times 10^{-4}$; Mcpt-$1^{-/-}$ $15.8 \times 10^{-4} \pm 2.1 \times 10^{-4}$; F-test; p 1). The 95% confidence bands are indicated. The similarity of the slopes indicates that there is no difference in intestinal egg excretion between WT and Mcpt-$1^{-/-}$. The vertical axis depicts the number of eggs per 100 mg feces. The horizontal axis depicts the number of eggs per mg tissue.

No difference in egg excretion into the lumen was observed between infected WT and Mcpt-$1^{-/-}$ mice in the course of infection (p 0.901; two way ANOVA) (fig 3b).
The linear correlations between tissue and fecal egg counts did not differ between WT and Mcpt-$1^{-/-}$ mice (p 1; F-test), indicating that egg excretion was similar in both groups (fig. 4). These functional data on egg excretion and egg retention, combined with the results obtained from the Ussing experiments, showed that although mMCP-1 morphologically disturbs the distribution pattern of occludin, deletion of this β-chymase does not affect the impairment of the intestinal epithelial integrity and does not influence egg excretion into the gut lumen during intestinal schistosomiasis in the mouse.

Discussion

In accordance with earlier studies dealing with gastrointestinal nematodes [16,28], our results show that the numbers of mast cells recruited during infection with *S. mansoni* were similar in WT and Mcpt-1$^{-/-}$ mice. Our results further demonstrate that increased numbers of MMC lead to a disturbed pattern of the distribution of the TJ protein occludin in infected WT mice, but not in genetically modified mice that lack this chymase. The staining patterns of other TJ proteins, claudin-3 and ZO-1, were not altered in *S. mansoni*-infected mice, regardless of genotype. These findings are similar to results obtained in mice infected with *T. spiralis*, suggesting a major role for mMCP-1 mediated cleavage of occludin during infection [14]. The fact that occludin can be cleaved by cysteine and serine proteases [29,30] would imply that it can also be cleaved by mMCP-1, a serine protease [14,31]. The possibility that mMCP-2 is responsible for cleaving occludin can be ruled out since Mcpt-1$^{-/-}$ mice, though mMCP-2$^{+/+}$, did not display altered occludin patterns and Pemberton and coworkers [32] demonstrated that mMCP-2 does not show any proteolytic activity. Unfortunately, the finding that mMCP-1 influences the structure of the TJ by affecting occludin does not allow to extrapolate about functional consequences, because the function of occludin has not been defined to date [27,33].

The impairment of intestinal barrier function in *S. mansoni*-infected mice did not differ between WT and Mcpt-1$^{-/-}$ mice, indicating that during intestinal schistosomiasis mMCP-1 does not contribute to the decrease in epithelial integrity. The disturbed distribution pattern of occludin during infection in WT, but not in Mcpt-1$^{-/-}$ mice, does not conflict with these results, since occludin is not essential for TJ barrier function [27,33]. The observed intestinal barrier impairment could be due to changes in the epithelial regulatory processes of TJ permeability, such as second-messenger systems [34] or phosphorylation of the TJ proteins proper [35].

Our tissue and fecal egg counts in WT mice indicated a steady increase in egg production with a peak at 10 w p.i. Furthermore, the egg excretion through the gut wall always occurred in accordance with the number of eggs produced by the *S. mansoni* worms and without differences between infected WT and Mcpt-1$^{-/-}$ mice. Therefore, we conclude that mMCP-1 does not facilitate passage of *S. mansoni* eggs through the gut wall. Interestingly, at 12 w p.i. tissue egg counts were higher in the WT than in the Mcpt-1$^{-/-}$ mice indicating that at this stage of infection deletion of mMCP-1 results in a lower or a delayed deposition of schistosome eggs in the intestinal wall. Thus, although mMCP-1 does not facilitate schistosome egg excretion into the gut lumen, it may potentially facilite egg passage from the mesenteric blood vessels into the gut wall. This would be in line with the observation that mMCP-1 is a modulator of vascular permeability and possesses several tissue remodelling activities [31].

Since impairment of the intestinal barrier in *S. mansoni*-infected mice is similar for WT and Mcpt-1$^{-/-}$ mice and tissue and fecal egg counts revealed that egg excretion also takes

place independently of mMCP-1 we conclude that in *S. mansoni*-infected mice mMCP-1 is not a key factor in egg excretion or in the impairment of epithelial integrity. This conclusion is in contrast to observations made in Mcpt-1$^{-/-}$ mice that had been infected with *T. spiralis*, where intestinal epithelial permeability is decreased and parasite expulsion from the intestine is delayed in comparison with that in WT mice [14,15]. The expulsion of another intestinal nematode, *Nippostrongylus brasiliensis*, also occurs independently of mMCP-1 [15,36]. Our results hence confirm that, despite a number of common features in the host response to various gut parasites, differences in intestinal niches between parasites will bring along different excretion mechanisms [37,38]. For instance, expelling the adult (sub)epithelial *T. spiralis* or *N. brasiliensis* may be expected to depend on different mechanisms than the facilitation of egg passage through the intestinal wall in case of *S. mansoni*. Moreover, the maturing schistosome eggs actively release proteases [39] and several other proteins. Although the function of these proteins is largely unknown, it is likely that they modulate the host's immune response to promote egg excretion [40-43]. This may reduce the significance of mast cell-derived products, such as mMcp-1, in the process of egg excretion. Alternatively, mucosal mast cell mediators other than mMCP-1 may play a role in the *S. mansoni* egg excretion. For example, tumor necrosis factor (TNF)-α which is involved the pathology of schistostomiasis [44] is also released by mucosal mast cells [9,45]. In-vitro, TNF-α increases intestinal TJs permeability by modifying the distribution and the expression levels of ZO-1 [46] and by altering the lipid composition in membrane microdomains of TJs.[47,48] IL-1β, another cytokine released by MMC, [49,50] increases TJs permeability of Caco-2 monolayers which is accompanied by changes in the expression levels and distribution of occludin and claudin-1 [51]. Other mast cell mediators such as Il-4, Il-10 and IL-13 [52] also modulate TJs permeability, in vitro, via several specific mechanisms [53] and thus potentially participate in the impairment of the intestinal barrier observed in *S. mansoni*-infected mice.

The peak time of *S. mansoni* egg excretion was accompanied by a decrease in electrical resistance and secretory capacity of the ileal tissue, which are –as far as we are aware- quantified here for the first time. The ileal resistance was reduced to 25 % of control values, which is much lower than reported for infection with *Heligmosomoides polygyrus, N.brasiliensis or T spiralis* (to 55 %) [54] or, for instance, in response to chronic psychological stress (to 54 %) [55] and acute pancreatitis (to 75 %) [24]. The relatively large reduction of the mucosal resistance is in accordance with the increase of the flux of NaFl (to about 150 % of control) and might indicate a disturbed function also of the epithelial cells proper. This suggestion is strongly supported by our finding that spontaneous secretion of the tissues and also their maximal secretion capacity are 8 weeks p.i. reduced to 39% and 11% of control, respectively. This contrasts with the increase in secretion reported in other models of inflammation, such as experimental acute pancreatitis [24] or chronic stress [55]. The observed impairment in secretory capacity during schistosomiasis, which is evidently not induced by mMCP-1, might reflect epithelial pathology caused by

egg deposition within the intestinal wall. Indeed, morphological examination of the mucosa shows epithelial cells in various states of degradation in the vicinity of the schistosome egg [56]. Alternatively, the diminished secretion could result from adaptation of the ileal mucosa to the infection. Such an adaptive response has been described for *N.brasiliensis*-infected rats and is directed by a neurally-mediated mechanism possibly aimed at preventing excessive fluid loss [57]. Likewise, in *T.spiralis* infected ferrets, basal and stimulated jejunal secretion were attenuated during the enteric stage of infection [58]. In these models, the reduced secretion was accompanied by a shift from cholinergic to non-cholinergic regulation of secretion, which was associated with an increase in substance P immunoreactivity within the mucosa. Interestingly, in this context, *S. mansoni* infection in the mouse results in increased immunoreactiviy for the neuropeptide calcitonin gene related peptide (CGRP) in close apposition to mucosal mast cells within the ileum [6,7]. Although the role of CGRP in *S. mansoni* infection remains to be elucidated it is likely that CGRP is involved in neuro-immune interactions between local primary afferent nerve fibres and mast cells [7].

Extrapolation of these murine data to man involves a large number of uncertain assumptions, partly arising out of the lack of adequate human data (for reviews see [59,60]) but also since schistosome infection in mice differs in many respects from that in humans [60]. In both human and murine, however, the majority of pathology develops at the sites of maximal accumulation of eggs: the intestine and the liver [59,60]. Gastrointestinal schistosomiasis is characterized by chronic abdominal pain and discomfort, loss of appetite and diarrhoea that commonly contains occult blood [60]. The present results show that in mice, in addition to the previously described impairment of sugar and fluid transport [61], the basal secretion and also the maximal secretory capacity of the ileal epithelium are severely reduced 8 weeks after schistosome infection. If and how this finding relates to the patient symptoms can not be inferred at present, but a derangement of fluid transport may explain some of these. The reported impairment of the mucosal barrier in the murine ileum suggests that translocation of bacteria from the gut lumen to extra-intestinal sites [62] might be increased during schistosomiasis. At present, only limited information is available about the effects of schistosomiasis on murine intestinal function [63]. The present results suggest, however, that use of murine models may be of importance for the dissection of the intestinal pathologies.

In summary, in *S. mansoni*-infected mice, the intestinal barrier is severely impaired both in WT and in Mcpt-1$^{-/-}$ mice and egg excretion takes place independently of mMCP-1. Therefore, mMCP-1 is not a key factor in egg excretion or in the impairment of epithelial integrity in schistosomiasis. This finding contrasts with observations in another parasitic infection model [14,15], suggesting an important role for the β-chymase mMCP-1 in impairing intestinal permeability [19]. Most likely, this contrast is due to differences in excretion mechanisms between the different species of parasites, related to their specific niches. Furthermore, the reported severe reduction in the secretory capacity of the

epithelium during schistosomiasis shows that the mechanisms facilitating passage of schistosoma eggs through the mouse gut wall are directed not only at the TJs, but most particularly at the epithelial cells proper.

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Chapter 4

CGRP1 receptor activation induces piecemeal release of protease-1 from mouse bone marrow-derived mucosal mast cells

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Abstract

Background The parasitized or inflamed gastrointestinal mucosa shows an increase in the number of mucosal mast cells and the density of extrinsic primary afferent nerve fibers containing the neuropeptide CGRP. Currently, the mode of action of CGRP on mucosal mast cells is unknown. **Methods** The effects of CGRP on mouse bone marrow-derived mucosal mast cells (BMMC) were investigated by measurements of $[Ca^{2+}]_i$ and release of mMCP-1. **Key results** BMMC responded to application of CGRP with a single transient rise in $[Ca^{2+}]_i$. The proportion of responding cells increased concentration-dependently to a maximum of 19 ± 4% at 10^{-5} M (mean ± SEM; C48/80 100%; EC_{50} 10^{-8} M). Pre-incubation with the CGRP receptor antagonist BIBN4096BS (10^{-5} M) completely inhibited BMMC activation by CGRP (range 10^{-5} to 10^{-11} M; ANOVA $p < 0.001$), while pre-incubation with $LaCl_3$ to block Ca^{2+} entry did not affect the response ($p = 0.18$). The presence of the CGRP1 receptor on BMMC was confirmed by simultaneous immunofluorescent detection of RAMP1 or CRLR, the two components of the CGRP1 receptor, and mMCP-1. Application of CGRP for 1 h evoked a concentration-dependent release of mMCP-1 (at EC_{50} 10% of content) but not of β-hexosaminidase and alterations in granular density indicative of piecemeal release. **Conclusions & Inferences** We demonstrate that BMMC express functional CGRP1 receptors and that their activation causes mobilization of Ca^{2+} from intracellular stores and piecemeal release of mMCP-1. These findings support the hypothesis that the CGRP signaling from afferent nerves to MMC in the gastrointestinal wall is receptor-mediated.

Introduction

Mast cells and the terminals of afferent extrinsic sensory nerves form intimate contacts within several organ-systems such as the intestine [1-4], the skin [5] and the dura mater [6]. Neuropeptides released from the afferent nerve endings can cause functional alterations and release of mediators from mast cells [4,7]. In the rodent intestinal mucosa the neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) are expressed by the sensory sympathetic nerves associated with mucosal mast cells (MMC) [8]. Inflammation results in an increase in the numbers of both MMC and SP- and CGRP-immunoreactive nerve fibers [1,8-10] and in patients with ulcerative colitis the levels of SP and CGRP in plasma and in the colonic mucosa are elevated [11]. SP and CGRP released from sensory nerves are the major initiators of neurogenic inflammation [4,12,13] and it has been indicated that the protective effect of CGRP on epithelial cells is partially mediated by MMC [14]. SP and CGRP are thus likely to mediate the nerve-to-MMC communication in the intestinal wall.

Generally, non-immunological stimuli such as neuropeptides and Compound 48/80 (C 48/80) are thought to activate mast cells through receptor-independent [15] binding to pertussis toxin (PTX)-sensitive G_i proteins on the inner surface of the plasma membrane [16-18]. It has been shown recently, however, that several mast cell types can express mRNA for the SP receptor (neurokinin-1 NK-1) [19-21] and that SP can induce mast cell activation also via a receptor-mediated mechanism [9,19,22]. Likewise, the presence of functional receptors for the neuropeptide corticotropin-releasing hormone (CRH), involved in regulation of mucosal barrier function, has been recently demonstrated on mucosal mast cells in human colon [23] and rat ileum [24]. Further, the expression of neuropeptide receptors on mast cells can increase during pathophysiological or inflammatory conditions [9,19], due to the presence of cytokines such as IL-3, IL-4 and IL-10, in the environment. Presumably, such enhanced expression functions to more efficiently propagate and enhance neurogenic inflammatory responses [13]. Information about the effects of CGRP on the MMC type found in close proximity to the network of afferent nerve fibers in the ileum, which differ considerably from connective tissue mast cells (CTMC) [25], is scarce [20,21,26].

Bone marrow-derived mast cells (BMMC) abundantly contain the granule chymases mMCP-1 and mMCP-2 [27], which are uniquely present in the MMC population and are not expressed in CTMC [28,29]. The major constituent of mouse mucosal mast cell granules mMCP-1 can modulate the barrier and transport properties of the intestinal epithelium [30,31]. Moreover, the BMMC express the integrin $\alpha_E\beta_7$ [32] which is typical of cells predominantly located in the mucosal epithelium [32,33]. Using this BMMC model, it has previously been shown that application of CGRP evokes a transient rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) [26]. It is not known if this signaling is receptor-mediated and whether an anaphylactic-type of degranulation or selective release of mediators [13,34,35] is evoked. The aim of the present investigation was therefore to determine whether functional CGRP1

receptors are expressed by BMMC and whether CGRP signaling is exclusively mediated by these receptors. In addition, release of mMCP-1 and β-hexosaminidase from BMMC was measured to characterize the functional effects of BMMC activation by CGRP.

Material and Methods

Animals

Adult male BALB/c mice (Harlan, Horst, The Netherlands) were kept under constant housing conditions (22°C, 60% relative humidity, 12-hour light/dark cycle) and had free access to water and food. The mice were allowed to adjust to these conditions for one week prior to the start of the experiments. The experimental design was approved by the institutional animal experiments committee of the University Medical Center, Utrecht, The Netherlands.

Cell culture

Bone marrow-derived mast cell cultures were made as previously described [26,32]. Briefly, mice at the age of 10-12 wk were euthanized by cervical dislocation, and their femurs were removed under sterile conditions. Bone marrow was washed from the femurs using a 23-gauge needle and a 5-ml syringe filled with DMEM (Invitrogen, Breda, The Netherlands) containing 10% FCS, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 2.5 µg ml^{-1} fungizone, 2 mM L-glutamine, and 1 mM sodium pyruvate (Invitrogen) (DMEM/FCS). Cells were suspended by passing them three times through a 19-gauge needle. Cells were then centrifuged three times at 230 *g* for 7 min at room temperature (RT). After resuspension in 10 ml DMEM/FCS, viable cells were counted in a Bürker-Türk haemocytometer using trypan blue staining. Cells were cultured in a humid 5% CO_2 incubator at 37°C using 75cm^2 flat-bottomed flasks (BD Biosciences, Erembodegem, Belgium) at 5 x 10^5 cells ml^{-1} in DEMEM/FCS containing 50 ng ml^{-1} SCF, 5 ng ml^{-1} recombinant mouse IL (rmIL)-9, 1 ng ml^{-1} rmIL-3 (R&D Systems, Abingdon, UK), and 1 ng ml^{-1} recombinant human transforming growth factor (TGF)-β_1 (Sigma-Aldrich, St. Louis, MO, USA). This combination of cytokines is referred to as TI3S and is known to result in high BMMC viability (93%) and mMCP-1 expression [26,32]. At 2- to 3-day intervals non-adherent cells were transferred into a new flask and half of the existing medium was replaced with fresh medium. Cultures were maintained for 9-10 days.

Immunocytochemistry

BMMC were washed three times and resuspended in 0.01 M phosphate-buffered saline (PBS; pH 7.4) before being loaded onto Marienfeld adhesion slides (Paul Marienfeld, Lauda-Königshofen, Germany) according to the manufacturer's guidelines. Following fixation and permeabilization of bound cells in absolute methanol for 10 min at RT, the

cells were rinsed and stored in PBS at 4°C until further processing for immunocytochemistry. Single and double immunolabeling experiments were performed at RT. The primary and secondary antibodies (Table 1) were diluted in PBS containing 10% normal horse serum, 0.01% bovine serum albumin, 0.05% thimerosal, and 0.01% sodium azide (PBS*). To block non-specific immunoglobulin interactions and to enhance permeability, the fixed cells were immersed in the supplemented PBS* solution to which 1% Triton X-100 was added. Next, they were incubated for 16 h with a rabbit antibody directed against RAMP1 or CRLR in single immunostainings, or with one of the rabbit primary antibodies and a sheep antibody directed against mMCP-1 in double immunostainings. Subsequently, after being rinsed in PBS, the cells were incubated for 1 h with biotinylated swine anti-rabbit IgG in single immunostainings, or with Cy3-conjugated donkey anti-sheep IgG and biotinylated swine anti-rabbit IgG in double immunostainings. Following rinsing in PBS, the cells were incubated in all protocols with FITC-conjugated streptavidin diluted in PBS. The single immunostained cells were counterstained with propidium iodide (PI; Sigma-Aldrich) diluted 1:1000 in PBS. Finally, cells were evaluated with confocal microscopy. For interference control stainings, one of the primary antisera was omitted in the double-staining protocol, and for negative control stainings, pre-absorbed antibodies were used.

Table 1. List of antisera used for immunocytochemistry.

Primary antisera

Antigen	Host	Dilution	Source
mMCP-1	sheep	1/5000	Moredun Scientific Ltd., Penicuik, Scotland, UK (MS-RM7)
RAMP1	rabbit	1/50	Santa Cruz Biotechnology, Santa Cruz, CA, USA (sc-11379)
CRLR	rabbit	1/100	SIGMA-ALDRICH, St. Louis, MO, USA (C3866)

Secondary antisera and streptavidin complexes

	Dilution	Source
biotinylated swine anti-rabbit IgG	1/400	Dako A/S, Glostrup, Denmark
Cy3-conjugated donkey anti-sheep IgG	1/500	Jackson ImmunoResearch Laboratories, West Grove, PA, USA
FITC-conjugated streptavidin	1/1000	Jackson ImmunoResearch Laboratories

Electron microscopy

Ten-day-old BMMC in 10ml of culture medium were stimulated by incubation in CGRP 10^{-7} M or in the mast cell activator Compound 48/80 (C48/80; Sigma-Aldrich; 100 µg ml^{-1}) for 1h at 37°C and centrifuged at 250 g for 7 min. The supernatants were removed, and the pellet was fixed with 0.1 M cacodylate buffered 2.5% glutaraldehyde (pH 7.4). Subsequently, the pellet was broken into small pieces and postfixed in 0.1 M cacodylate buffered 1% OsO_4, followed by dehydration in acetone before embedding in Durcupan. Fifty-nm-thin sections were cut and contrasted with 2% uranylacetate and Reynolds solution for 10 min each. Sections were analyzed at a primary magnification of x 6,600 in a transmission electron microscope (CM10; Philips, Eindhoven, The Netherlands).

β-Hexosaminidase and mMCP-1 assay

BMMC cultured for 9 days were washed twice with DMEM F-12 (Invitrogen). $3\text{-}4\times10^4$ cells/well were aliquoted in a 96-well plate in triplicate in a total volume of 100 µl DMEM F-12/well and incubated with CGRP or C48/80 (100 µg ml^{-1}). Total β-hexosaminidase and mMCP-1 content of BMMC was established by lysing the cells with 1% Triton-X 100 (Sigma-Aldrich). Plates were incubated for 60 min at 37°C and centrifuged (5 min, 1000 RCF). Supernatant was removed and stored at -80°C.

For determination of β-hexosaminidase, 60 µl of supernatant was added in duplicate to a fresh 96-well plate and mixed with an equal volume of 7.5 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma-Aldrich) diluted in 0.2 M citrate buffer (pH 4.5). The plate was then incubated for 60 min at 37°C and the reaction was stopped by addition of 120 µl of 0.2 M glycine (pH 10.7) to each well. Absorbance at 360 nm and 460 nm was determined using a plate reader (Polarstar Galaxy, BMG). MMCP-1 in the supernatant was measured using a commercially available ELISA kit (Moredun Scientific Ltd, Midlothian, Scotland) according to the manufacturer's instructions. The percentage of β-hexosaminidase and mMCP-1 release was calculated as: $\{(a-b)/(t-b)\}\times100$, where a is the amount of release from stimulated cells, b is that released from unstimulated cells and t is the total cellular content.

Ca^{2+} imaging

BMMC (day 9-10) were centrifuged at 150 g for 5 min at RT and resuspended twice in DMEM F-12 (Invitrogen) before being seeded ($\sim10^5$ cells in 100 µl DMEM F-12) onto the poly-d-lysine-coated glass bottom (diameter 10 mm) of a 35-mm-diameter culture dish (MatTek, Ashland, USA). Cells were loaded with the Ca^{2+}-sensitive fluorescent dye fura-2 acetoxymethyl ester (Fura-2 AM; Invitrogen; 5 µM) for 20 min. Cells were rinsed twice with DMEM F-12 and incubated for an additional 40 min in DMEM F-12. All incubations were performed at 37°C in a 5% CO_2 humidified atmosphere. Dishes were transferred to an inverted microscope (Observer A1, Zeiss, Göttingen, Germany) equipped with a 40x oil objective (NA 1.3) and coupled to a TILL Photonics Polychrome IV (TILL Photonics

GmBH, Gräfelfing, Germany) light source and a Image SensiCam digital camera (TILL Photonics GmBH). Imaging software TILLvisION, v 4.01 was used for data collection and processing. Fluorescence was alternately excited with 340 and 380 nm light, and pairs of images were recorded at a frequency of 1 s^{-1} using a 510 nm emission filter with 1 ms exposure time for each wavelength. Regions of interest (whole cell) were defined on subconfluent areas of the dishes and the 340/380 ratio of fluorescence intensities corrected for background signal was calculated using custom-made MS-Excel macros. Prior to each experiment the baseline fluorescence of each cell was recorded during 30 s and normalized to 1. The relative fluorescence (RF) values of each region of interest, representing $[Ca^{2+}]_i$, were plotted as a function of time.

Cellular activation

To measure cellular Ca^{2+} responses to a single application of CGRP, SP or C48/80, the drugs were dissolved in DMEM-F12 and applied directly in the bath as a small drop (10 µl) from a micropipette. The location of the pipette tip was at least 500 µm from the nearest cells to avoid pressure-induced stretching of BMMC cell membranes. At 120 s following addition of CGRP or SP, C48/80 (100 µg ml^{-1}) was added to the cells. The Ca^{2+} response to C48/80 was used to determine the viability and maximal response of each cell. Cells unresponsive to C48/80 (~ 7%) were excluded from further analysis. The amplitude and lag of each CGRP- or SP-induced transient increase in $[Ca^{2+}]_i$ with an amplitude > 1.1 RF (threshold) were calculated. The amplitude was defined as the highest RF value of the response and was expressed as % of the RF obtained by application of C48/80. The lag (expressed in s) was defined as the time between drug application and the first noticeable change in the RF. The effects of the CGRP receptor antagonist BIBN4096BS (Boehringer Ingelheim Pharma, Germany) on the BMMC responses were studied following incubation of the cells for 1 h (10^{-5} M, 37°C). BIBN4096BS is a competitive and high-affinity [36,37] antagonist of the GCRP receptor, of which the selectivity is due to a specific interaction with the extracellular region of the RAMP1 subunit of the receptor [38]. In control experiments, it was verified that application of $LaCl_3$, used to block the cellular influx of extracellular Ca^{2+} [39], abolished the C48/80-induced Ca^{2+} responses of BMMC within 40 s (data not shown).

Statistical analysis

Data of all measured cells in a single dish were pooled and the percentage of responding cells and the mean values for the lag and amplitude of the responses were calculated. To establish the CGRP stimulus-response relationship (Fig. 4), values obtained on ~9 dishes per concentration were used. Values are presented as mean ± SEM of *n* dishes. The stimulus-response relationship in Fig. 4A was fitted with a Boltzmann curve using Origin 7.5 (Origin Lab, Northampton, USA). Statistical comparisons were performed by an

unpaired Student's t-test or analysis of variance (ANOVA) using SPSS v.12.0 software. P values < 0.05 were considered significant.

Results

Validation of BMMC

BMMC cultures were maintained for 9-10 days in TI3S supplemented medium [32] which selectively promotes BMMC survival and proliferation. Cell viability, measured by trypan-blue staining, was decreased to 78% by day 2 and to 85% by day 4 and returned to 98% at day 8, which is in line with previous studies [26,32]. Likewise, the total number of cells declined during the first 4 days of culture followed by an increase from the 6th day of culture onwards. After 9 days in culture, the cell suspension consisted mainly of non-adhering, uniformly sized (diameter ~10 μm) cells (Fig. 1A) containing, within their cytoplasm, a large number of basophilic granules as visualized by Leishman's staining (Fig. 1B). The expression of the MMC marker mMCP-1 [25] within these granules was confirmed by immunocytochemical staining (Fig. 1C). mMCP-1 in culture medium was detected (4 cultures) from day 4 (15 ± 2 ng ml^{-1}) and reached 839 ± 168 ng ml^{-1} at day 8 of culture (cf [32]). Stimulation of the BMMC with the mast cell secretagogue C48/80 (100 μg ml^{-1}, 1h, 37°C) resulted in a rise of $[Ca^{2+}]_i$ in 93% of the cells and in the release of 84% of their β-hexosaminidase content (see below) by degranulation, as observed by EM (Fig. 1D-E). Thus the characteristics of the BMMC are in good agreement with previous observations and confirm the MMC phenotype [26,32].

Detection of the CGRP1-receptor by immunocytochemistry

Immunocytochemical staining of BMMC (day 9 of culture) for the two subunits of the CGRP1 receptor [40] demonstrated the presence of RAMP1 and CRLR immunoreactivity (Fig. 2) on the majority of the BMMC (on 4 Marienfeld adhesion slides with BMMC from 4 cultures). RAMP1 and CRLR immunoreactivity was predominantly present on the cell membrane. Simultaneous detection of RAMP1 with mMCP-1 (Fig. 2A-C) revealed that the majority of BMMC expressed only RAMP1 and that a smaller subpopulation expressed RAMP1 and mMCP-1. Similar observations were made with simultaneous detection of CRLR and mMCP-1 (Fig. 2D-F). Omission of primary antibodies (Fig. 2G-I) or pre-absorption by the appropriate antigens did not yield immunoreactivity. All BMMC in the single immunostaining protocols expressed RAMP1 or CRLR immunoreactivity (Fig. 2J-K).

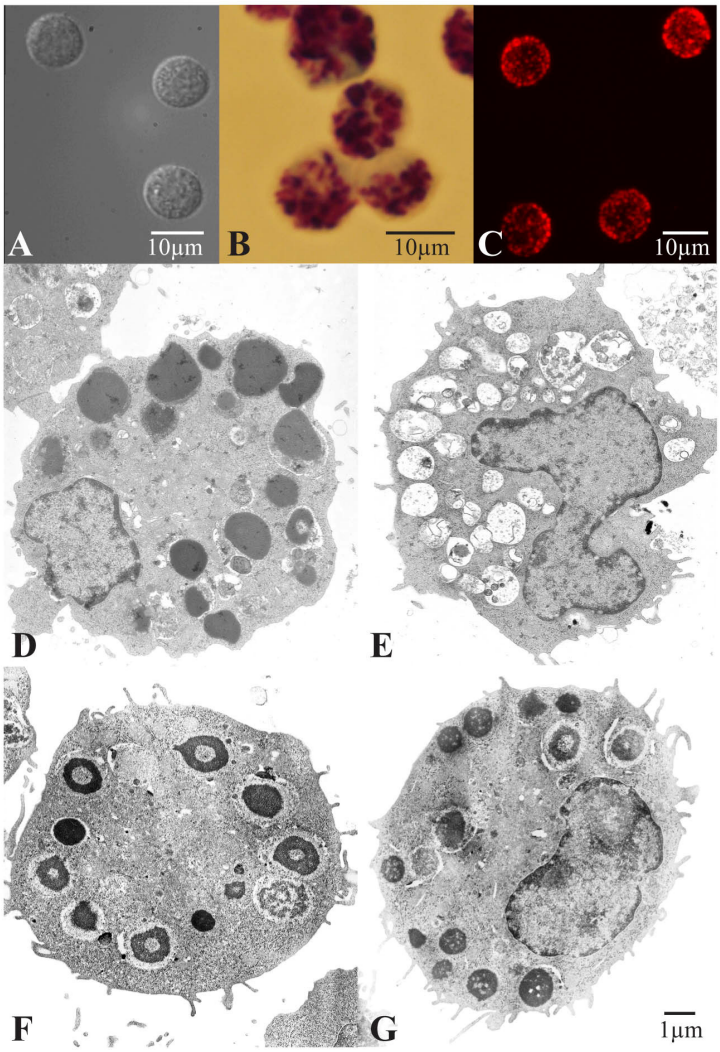

Fig. 1. Morphological characterization of BMMC
A: Differential interference contrast (DIC) image of BMMC (day 9 of culture) showing round, uniformly sized cells. **B:** Leishmann staining showing the presence of acidic cytoplasmic granules. **C:** Immunocytochemical detection of mMCP-1 (Cy-3 epifluorescence), abundantly present within the cytoplasmic granules in the vast majority of BMMC. **D:** Transmission electron microscopy image of BMMC, showing electron-dense granules. **E:** after 1 h stimulation with C48/80 (100 µg ml^{-1}), the granules had lost their content, indicative of degranulation. **F-G**: representative examples of BMMC after 1 h stimulation with CGRP (10^{-7} M), showing focal loss of density in some granules, while other granules retain their normal density. This observation is indicative of piecemeal release [35,46]

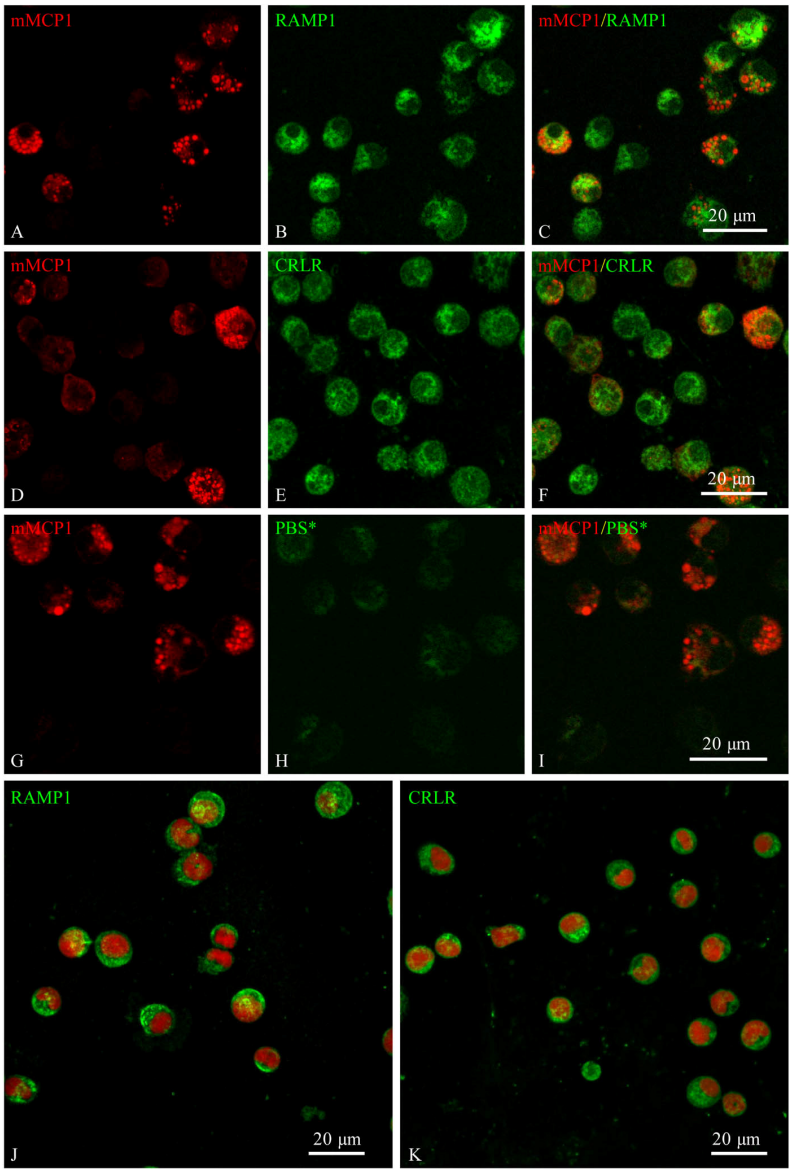

Fig. 2. Immunocytochemistry for CGRP receptor on BMMC
Double immunolabeling with antibodies directed against RAMP1 or CRLR and mMCP-1 on BMMC at day 9 of culture (A: mMCP-1, B: RAMP1, C merged image, D: mMCP1, E: CRLR, F: merged image), demonstrating co-expression of RAMP1 or CRCL with mMCP-1 Omission of one of the primary antibodies against the CGRP receptor subunits led to elimination of immunostaining (G: mMCP-1, H: PBS*, I: merged image). Single immunolabeling of BMMC counterstained with PI, showing all cells immunoreactive for RAMP1 (J) or CRLR (K).

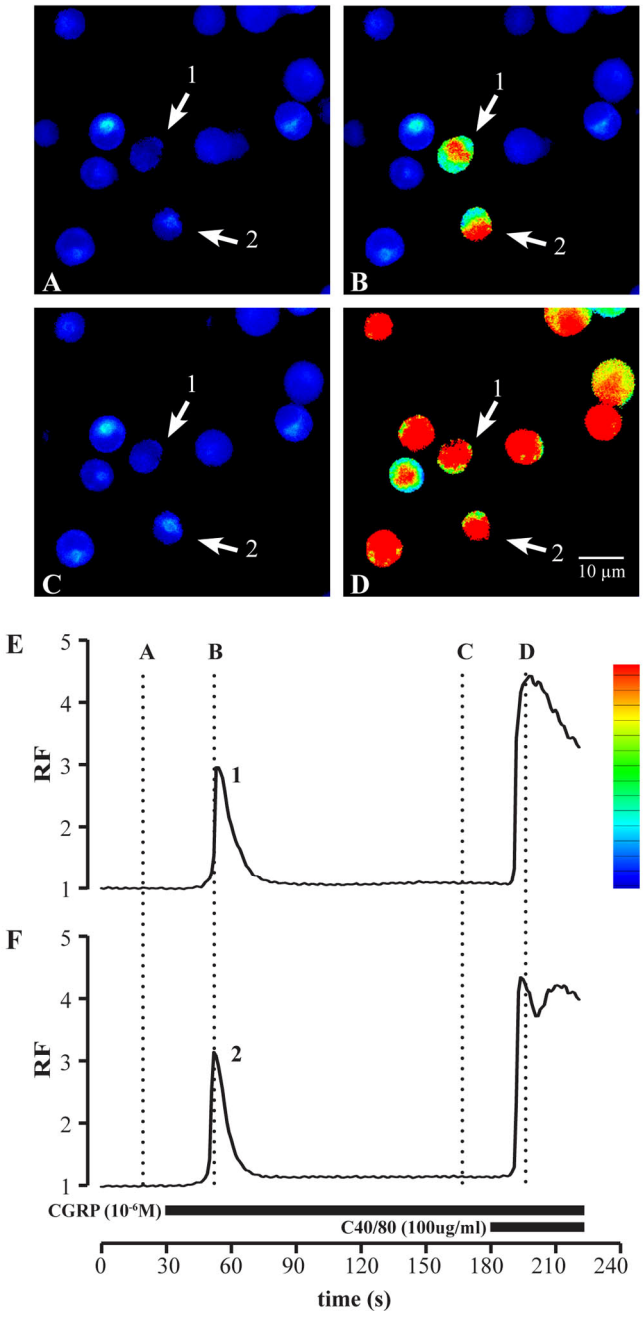

Fig. 3. $[Ca^{2+}]_i$ responses of BMMC to CGRP and C48/80
Microphotographs in the upper panel (A-D) show time lapse series of Fura-2 images of BMMC in a culture dish to which CGRP (10^{-6} M) and C48/80 (100 µg ml^{-1}) were applied. Arrows 1 & 2 point to BMMC that showed a $[Ca^{2+}]_i$ response to CGRP. Each microphotograph in the upper panel corresponds to the indicated time-point in the lower panel (dotted lines A-D). The lower panel (E-F) shows the relative Fura-2 fluorescence (RF) of the two BMMC indicated by the arrows as a function of time. The Fura-2 signal shows that application of CGRP (at 30 s) resulted, after a lag, in a rapid increase in $[Ca^{2+}]_i$ followed by a slow exponential decrease towards the baseline. Application of C48/80 (at 180 s) resulted, after a lag, in an increase in $[Ca^{2+}]_i$, which settled to a temporarily sustained level and subsequently decreased slowly. Of the BMMC in this particular dish, 20% responded to CGRP and 93% to C48/80.

$[Ca^{2+}]_i$ response of BMMC to CGRP

The response of individual BMMC (day 9 of culture) to CGRP (10^{-5} M to 10^{-11} M) was characterized by optical imaging of $[Ca^{2+}]_i$ with Fura-2. To verify the responsiveness of the BMMC, at the end of each 2-min recording period, cells were stimulated by application of C48/80 (100 μg ml^{-1}), as illustrated in Fig 3.

C48/80 induced a rise in $[Ca^{2+}]_i$ in 93 ± 10% of the cells (127 dishes; 4583 cells from 20 cultures) with a lag of 8 ± 1 s and an amplitude (RF) of 2.0 ± 0.1, The response to C48/80 consisted typically of a transient rise followed by a sustained elevation and/or a slow (sometimes oscillatory) decrease (Fig. 3), in line with earlier studies and typical of a degranulatory response of mast cells [26,41]. BMMC responded with a transient rise in $[Ca^{2+}]_i$ to the application of CGRP. A typical response, of which two representative examples are shown in Fig 3, consisted of a single steep $[Ca^{2+}]_i$ increase followed by a slower exponential decay to the basal level. The proportion of BMMC responding to CGRP, for each dish expressed as the percentage of C48/80-responding cells, increased from 10^{-11} M concentration-dependently to a maximum of 19 ± 4% at 10^{-5} M CGRP (Fig 4A). A Boltzmann curve fit (R^2: 0.93) revealed an EC_{50} of 10^{-8} M. In control experiments, in which only buffer was applied, 4 ± 1% of the BMMC (10 dishes; 295 cells) showed $[Ca^{2+}]$ transients (Fig. 4A), which presumably resulted from agitation of cells during the experimental procedures, as has been described for measurements of mast cell degranulation [42] and histamine release [39,43]. These transients could on basis of their shape and amplitude not be distinguished from the $[Ca^{2+}]_i$ responses to CGRP and therefore induced a relative high variability to the measured proportion of responding cells (Fig. 4A). The lag of the CGRP response decreased from 63 ± 9 s at 10^{-11} M with increasing CGRP concentration to a minimum of 18 ± 2 s at 10^{-5} M CGRP (Fig. 4B), and as such is comparable to that observed previously for responses of BMMC to CGRP (33 s; 10^{-7} M; [26]). The amplitude of the response to CGRP reached 81 ± 1% of the C48/80 response amplitude and did not correlate to the CGRP concentration (Fig. 4C). A series of dedicated experiments (306 cells in 23 dishes), in which $[Ca^{2+}]_i$ was recorded during an 1 h period following application of CGRP, showed that the majority (> 98%) of BMMC responded with a single Ca^{2+} transient within the first 2 min, without subsequent transients or oscillations of $[Ca^{2+}]_i$.

The CGRP-receptor antagonist BIBN4096BS blocks the BMMC response to CGRP

To determine if the response to CGRP was mediated by the membrane CGRP1 receptor, BMMC were incubated (10^{-5} M, 1 h, 37°C) with the selective CGRP receptor antagonist BIBN4096BS [36,37] prior to CGRP stimulation. In the presence of BIBN4096BS, application of CGRP failed to induce a change in $[Ca^{2+}]_i$ of the BMMC. A representative example of the inhibitory effect of BIBN4096BS is shown in Fig. 5A-B. This lack of response was observed at all tested concentrations (10^{-5} M to 10^{-11} M) of CGRP (Fig. 4A).

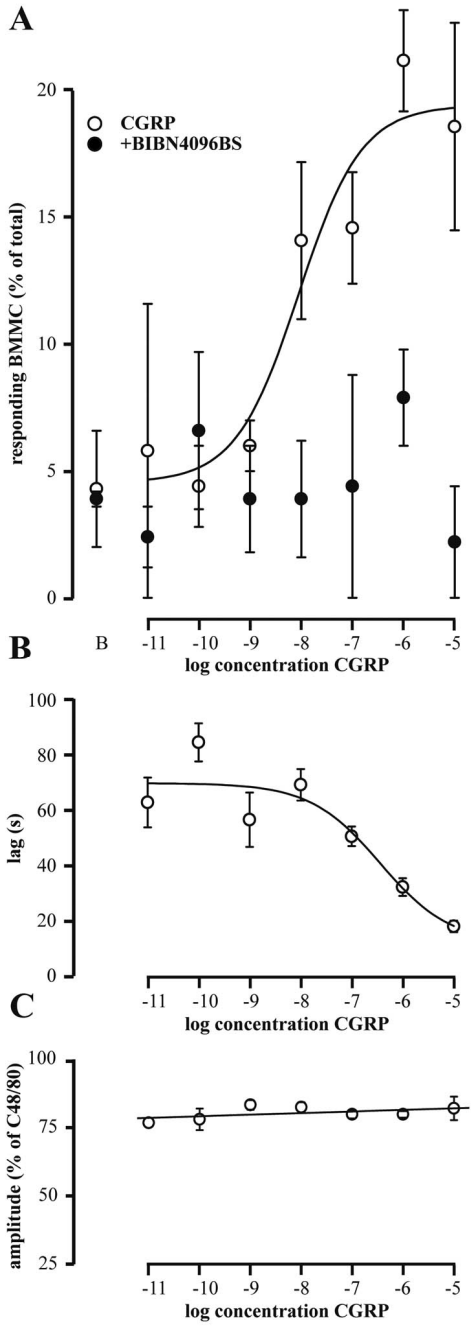

Fig. 4. Concentration-response relation for the effect of CGRP on BMMC

A: Concentration-response curve (-○-) showing that application of CGRP evokes a concentration-dependent increase in the proportion of BMMC (re C48/80 = 100%) responding with a transient increase in $[Ca^{2+}]_i$. A fit of the data (Boltzmann; R^2 0.93) revealed an EC_{50} of 10^{-8} M and a maximum response of 19%. In the presence of the CGRP receptor antagonist BIBN4096BS (10^{-5} M; 1h; -●-) the response to CGRP was completely blocked over the entire CGRP concentration range used ($p < 0.001$; ANOVA). Each data point in the graphs depicts the mean value (± SEM) obtained from approximately 270 BMMC in 9 dishes, from in total 20 cultures. On the left, the B on the x-axis indicates that in control experiments, in which only buffer was applied, about 4% of the BMMC showed $[Ca^{2+}]_i$ transients which on the basis of their shape and amplitude could not be distinguished from the $[Ca^{2+}]_i$ responses to CGRP. These spontaneous signals induced a relative high variability in the concentration-response curves. **B:** The lag of each response to CGRP was calculated as the time between CGRP application and the first noticeable rise in RF. The concentration-response curve shows that the lag (mean ± SEM) of the CGRP response decreased from 63 ± 9 s at 10^{-11} M with increasing CGRP concentration to 18 ± 2 s at 10^{-5} M CGRP. **C:** The amplitude of the CGRP response was expressed as a fraction of the amplitude of the maximal response elicited by application of C48/80 (100 µg ml^{-1}). The amplitude of the response to CGRP was 81 ± 1% (mean ± SEM; 76 dishes) of that of the C48/80 response and was not dependent on the CGRP concentration.

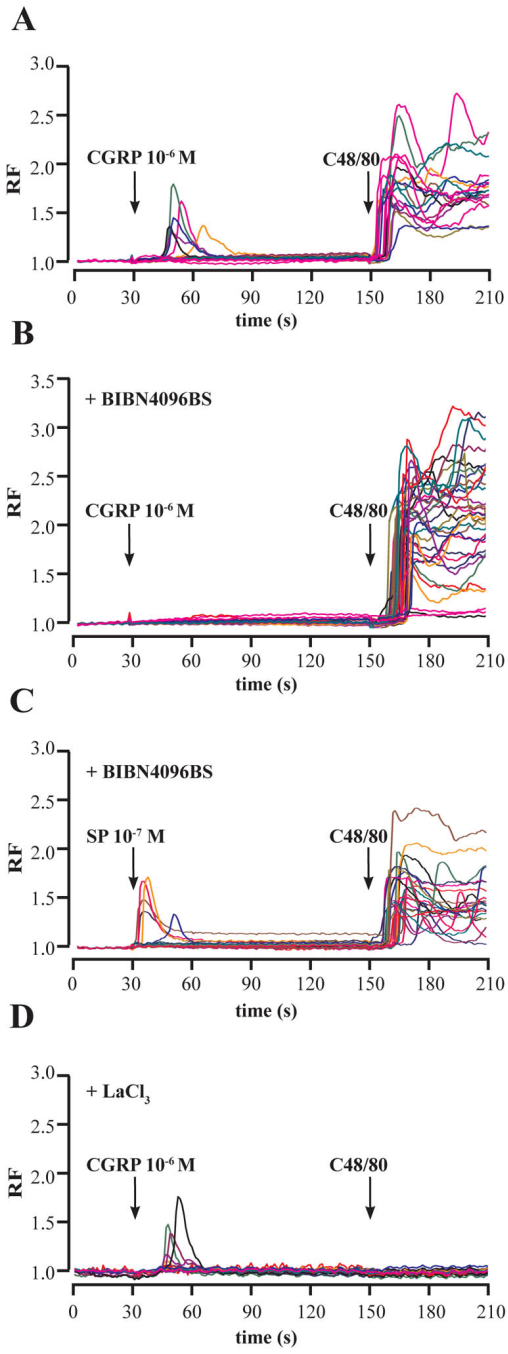

Fig. 5. CGRP receptor antagonist BIBN4096BS blocks the BMMC response to CGRP but not to SP

Recordings of relative Fura-2 fluorescence (RF) as a function of time. CGRP or SP was applied to the BMMC at 30 s and C48/80 at 180 s. **A:** Recording of transient Ca^{2+} responses in 6 out of 17 BMMC (35%) to application of CGRP (10^{-6} M). **B** and **C:** The response to CGRP (10^{-6} M), but not to SP (10^{-7} M), is completely blocked in the presence of the CGRP receptor antagonist BIBN4096BS (10^{-5} M, 1h), demonstrating that the response of BMMC to CGRP is receptor-mediated. **D;** Preincubation (40 s) of the BMMC with $LaCl_3$ (0.5 mM), which blocks the entrance of extracellular Ca^{2+} into the cell, does not affect the response of BMMC to CGRP but eliminates the response to C48/80, demonstrating that the CGRP-induced $[Ca^{2+}]_i$ responses are generated by mobilization of Ca^{2+} from intracellular stores.

Incubation of the BMMC with BIBN4096BS did not affect the properties of the response to subsequent application of C48/80 ($p > 0.3$). To ensure that BIBN4096BS did not have a general inhibitory action on the BMMC, the effect of BIBN4096BS was also investigated on the $[Ca^{2+}]_i$ responses to SP, which are thought to be mediated via the NK-1 receptor [19]. Application of SP to BMMC (10^{-7} M; 6 dishes, 147 cells) caused a transient increase in $[Ca^{2+}]_i$ (cf [26]), comparable to the responses induced by CGRP.
The SP-induced response occurred in 9 ± 5% of BMMC, had a lag of 40 ± 19 s and an amplitude of 66 ± 8% (re C48/80). A 1h preincubation with BIBN4096BS did not affect the BMMC response to SP (Fig. 5C) (response 14 ± 4% of BMMC, lag 64 ± 12 s, amplitude 77 ± 29%, all $p > 0.2$; 191 cells in 8 dishes).

CGRP induces release of Ca^{2+} from intracellular stores

To determine the source of the CGRP-induced $[Ca^{2+}]_i$ transients in BMMC, the cells were incubated with $LaCl_3$ (0.5 mM), which is known to block the influx of extracellular Ca^{2+} through membrane Ca^{2+} channels [44,45]. Other approaches to determine the $[Ca^{2+}]_i$ source, such as lowering extracellular $[Ca^{2+}]$ or pre-incubation with thapsigargin were not applicable because these caused alterations in $[Ca^{2+}]_i$ or rapid degranulation of the BMMC (cf [42]). After a 40-s incubation of BMMC with La^{3+}, the response to C48/80, which requires influx of extracellular Ca^{2+}, was abolished (responding cells: control 96 ± 4%; La^{3+} 2 ± 5%; $p < 0.001$, 10 dishes, t-test). An example is shown in Fig 5D. In contrast, the proportion of BMMC responding to CGRP (10^{-6} M) was not affected by La^{3+} (control 16 ± 6%; La^{3+} 12 ± 9%; $p = 0.18$, 10 dishes). The amplitude of the CGRP responses was not affected by La^{3+} either (RF; control 1.6 ± 0.3; La^{3+} 1.7 ± 0.3; p 0.32, 9 dishes). This lack of effect of La^{3+} on the Ca^{2+} responses to CGRP demonstrates that these are generated by mobilization of Ca^{2+} from intracellular stores, rather than by an influx of extracellular Ca^{2+} through membrane ion channels.

CGRP induces release of mMCP-1 but not of β-hexosaminidase

A 1 h incubation with C48/80 (100 μg ml^{-1}) induced the release of 90 ± 5% (*n*=12 experiments) of the total cellular β-hexosaminidase and 86 ± 9% (*n*=5) of the mMCP-1 content of BMMC, which is in line with the morphological changes of BMMC (Fig 1 D-E). A 1 h incubation of BMMC with CGRP (*n*=12) failed to induce the release of β-hexosaminidase, except at the highest concentration of 10^{-5} M (4 ± 2%), as shown in Fig 6. In contrast, CGRP induced concentration-dependently the release of mMCP-1 (*n*=5), up to 45 ± 7% of the total content at 10^{-5} M (Fig 6). Application of CGRP for 1 h at EC_{50} (10^{-8}M) evoked the release of about 10% of the total cellular content of mMCP-1. The release of mMCP-1 was linearly correlated ($p = 0.01$; correlation coefficient 0.87) to the percentage of BMMC responding to CGRP with a Ca^{2+} transient. These observations and the properties of the Ca^{2+} responses to CGRP indicated that mMCP-1 was released from BMMC not by degranulation but by piecemeal release [34,35]. Therefore, the changes in

BMMC granular density induced by incubation with CGRP 10^{-7}M were determined by transmission electron microscopy. BMMC incubated with CGRP (10^{-7} M) exhibit focal loss of granular density in absence of intergranular fusion or granule to plasma membrane fusion (Fig 1 F-G). The CGRP-induced alterations in granular density were in good agreement with those described for piecemeal release [35,46].

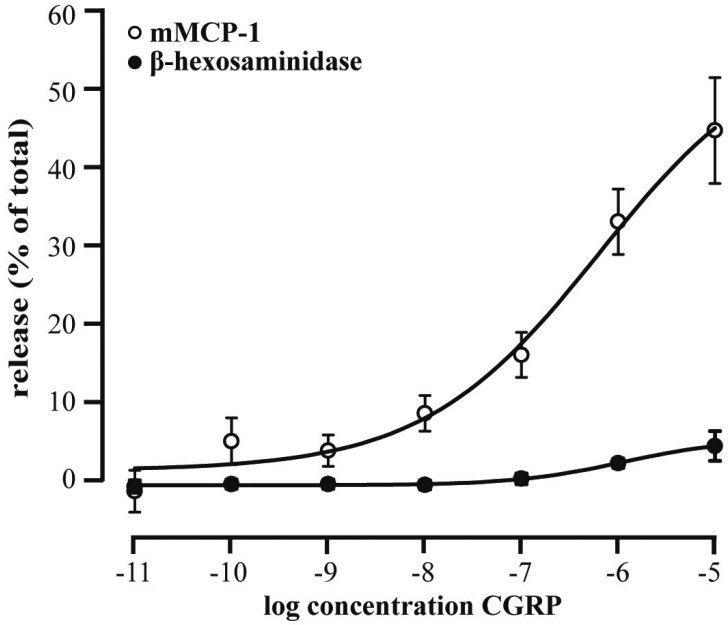

Fig. 6. CGRP induces the release of mMCP-1 but not of β-hexosaminidase from BMMC
A 1 h incubation of BMMC with CGRP evokes the concentration-dependent release of mMCP-1 (-○-; n = 5) but not of β-hexosaminidase (-●-; n = 12). A fit of the data (Boltzmann; R^2 0.99) revealed an EC_{50} of 10^{-6}M. Release is expressed as mean fraction ± SEM of the total cellular content.

Discussion

The present results demonstrate the presence of functional CGRP1 receptors on BMMC. Activation of this receptor (EC_{50} 10^{-8} M) results in the release of Ca^{2+} from intracellular stores and in differential [35], piecemeal [34] release of mMCP-1 from BMMC, in absence of degranulation. These findings suggest that under conditions of parasite infection [26] or inflammation [4,9,47], the CGRP signaling from afferent nerves to MMC in the gastrointestinal wall is receptor-mediated and has a selective stimulatory action on the release of mMCP-1 from MMC.

Activation of BMMC by CGRP is receptor mediated

The CGRP-induced activation of BMMC was blocked by the competitive, high-affinity CGRP receptor antagonist BIBN4096BS [36,48], at a concentration (10^{-5} M) that ensures complete receptor blocking [36,37]. The selectivity of BIBN4096BS for the CGRP receptor [36] is due to a specific interaction with the extracellular region of the RAMP1 subunit of the receptor [49]. Incubation of BMMC with BIBN4096BS completely abolished the CGRP-induced Ca^{2+} transients up to the highest tested CGRP concentration of 10^{-5} M. This finding is remarkable, since it has been suggested previously [19,43] that neuropeptides may act via membrane receptors at low concentrations, but also directly activate G_i-proteins at higher concentrations. Our results, however, indicate that CGRP acts on BMMC exclusively via the CGRP1 membrane receptor. The difference between the lag of the BMMC Ca^{2+} responses to CGRP, which increased from 18 s (10^{-5} M) to 63 s (10^{-11} M), and that of the response to C48/80 (8 s), is in accordance with this conclusion because C48/80 directly activates the G_i-protein [17]. The expression of the CGRP1 receptor subunits RAMP1 and CRLR [40] on the majority of the BMMC allows us to assume that on most of these cells both subunits are co-expressed forming a functional receptor. Both subunits were detected on cells expressing mMCP-1, but also on those negative for this chymase, suggesting that the CGRP1 receptor is expressed prior to mMCP-1.

The detection of both CGRP receptor subunits on BMMC, together with the observed blockade of the Ca^{2+} responses to CGRP by the CGRP receptor antagonist BIBN4096BS demonstrates that the BMMC response to CGRP is receptor-mediated. This conclusion is in good accordance with the low optimal CGRP concentrations reported for the chemo-attractive role [1] of CGRP in recruitment of lymphocytes (5.10^{-10} M; [50]) and for the mast cell-mediated modulatory effects of CGRP on epithelial function (10^{-9} to 10^{-12} M; [14]).

An elegant immunocytochemical study has recently demonstrated the presence of the CGRP receptor on CTMC in the trigeminovascular system of the rat [51]. The expression of mRNA of CGRP receptors has been reported for cultured human MC [20], but so far not for mouse MMC. Rat basophilic leukemia (RBL-2H3) cells were reported to be negative for mRNA of RAMP1, but positive for mRNA of CRLR and also for that of RDC-1, which is also capable of binding CGRP [52]. So far, extensive attempts to immunohistochemically detect CRLR and/or RAMP1 on MMC in cryosections and whole mounts of the small intestine of normal and *S. mansoni*-infected mice have failed (L. van Nassauw, unpublished observations). The immunostaining revealed expression of RAMP1 and CRLR on enteric nerve fibers, similar to the distribution of both subunits in the rat intestine [53]. We cannot exclude that differences in microenvironmental factors [19] between mucosal mast cells *in vivo* and the cultured bone marrow-derived mast cells used in our experiments may account for the observed difference in receptor expression. Alternatively, differences in the degree of maturity of the mast cells [32] may play a role in this. Thus, more research is needed to answer the question if CGRP receptors are present on MMC under *in vivo* conditions.

CGRP receptor-associated signal transduction pathway in BMMC

The CGRP-induced Ca^{2+} transients were not affected by La^{3+}, leading us to conclude that the latter are produced by Ca^{2+} release from intracellular stores. The amplitudes of the CGRP-induced whole-cell Ca^{2+} responses were remarkably high (81% of C48/80) and constant for all tested CGRP concentrations, suggesting that Ca^{2+} was released in a nearly all-or-none manner from stores within the cell. This indicates the presence of an intracellular threshold for induction of a Ca^{2+} transient in the BMMC, most likely reflecting the stringent regulation of Ca^{2+} release at the level of the ER [54,55]. The CGRP-induced responses of BMMC are mediated by activation of a PTX-sensitive G_i-protein [26], like the basic secretagogue pathway [15,17,56]. This suggests that, following activation of the CGRP1 receptor and subsequently the G_i-protein, phospholipase C activation, generation of IP3 [15,17] and binding of IP3 to ryanodine receptors on the ER induces the Ca^{2+} release from the ER.

CGRP induces differential release of mMCP-1 and β-hexosaminidase from BMMC

The $[Ca^{2+}]_i$ responses of BMMC to application of CGRP consisted in all cases of a single transient without repetitions or signs of $[Ca^{2+}]_i$ oscillations during the subsequent 60 min, which suggested that degranulation did not occur. In accordance with this finding, incubation of BMMC for 1 h with 10^{-8} M CGRP (EC_{50}) induced the release of about 10% of the cellular content of mMCP-1 without any release of the granule-associated mediator β-hexosaminidase. This differential release [35] shows that mMCP-1 can be released from BMMC independently of degranulation.

The release of relatively small amounts of mMCP-1 indicates that CGRP evokes piecemeal release [57], which has been described for several other mast cell mediators and is considered to be the main secretory mechanism of mucosal mast cells during inflammatory states [34,35]. The characteristics of the observed changes in granular density of the BMMC provide further evidence for the occurrence of piecemeal release [35,46] in response to application of CGRP. In contrast to degranulation, piecemeal release does not depend on influx of extracellular Ca^{2+} [35]. The conclusion that CGRP induces piecemeal release of BMMC is also supported by our observation that the CGRP-induced $[Ca^{2+}]_i$ responses were not affected by blockade of extracellular Ca^{2+} influx. Interestingly, in a recent morphological study, stress-induced piecemeal release in the intestinal mucosa was observed primarily in mast cells which were in close proximity to nerves [58]. Our results emphasize a neural control of differential mast cell release.

The present immunocytochemical and pharmacological data provide the first direct evidence for the presence of functional CGRP1 receptors on mouse BMMC. Moreover, the results show that the CGRP1 receptor on MMC is most likely involved in the binding of CGRP released from primary afferent sensory nerve terminals, thereby mediating a nerve-to-MMC signaling, which subsequently induces piecemeal release of mMCP-1.

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Author contributions

JR, LMA, RW and AK designed the research; JR, LvN, JPT and AK performed the research and analyzed data; JR, JPT and AK wrote the paper.

Competing interests

The authors have no competing interests.

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Chapter 5

Preliminary investigation of CGRP-induced priming of mouse mucosal mast cells

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Abstract

Functional CGRP1 receptors are present on mouse bone marrow-derived mast cells (BMMC), suggesting that these receptors play an important role in the communication between CGRP-containing afferent nerve endings and mucosal mast cells in the intestinal wall. We have investigated if BMMC can be primed, i.e. made more sensitive for a subsequent stimulation, by a pre-exposure to a physiologically relevant low concentration of CGRP. Recordings of intracellular Ca^{2+} ($[Ca^{2+}]_i$) were made from BMMC (cultured for 9-10 days) loaded with Fura-2 AM in perfused culture dishes. BMMC were pre-exposed for 20 s to CGRP 10^{-8} M or buffer (control), followed 2 min later by an application of CGRP (20 s; range from 10^{-11} to 10^{-5} M). In the control experiments, the proportion of BMMC responding to application of CGRP with a single transient rise in $[Ca^{2+}]_i$ increased concentration-dependently (range 10^{-11} to 10^{-5} M) to a maximum of about 20%. No difference was observed in the concentration-response relation or in properties of the Ca^{2+} responses (lag, amplitude and duration) between BMMC pre-exposed to CGRP 10^{-8} M or to buffer. The finding that a 20 s pre-exposure with CGRP 10^{-8} M does not influence the Ca^{2+} response of the BMMC shows that, under the experimental conditions used, CGRP does not increase the responsiveness of BMMC for stimulation.

Introduction

Inflammation and infection of the gastrointestinal tract is frequently accompanied by increased numbers of mucosal mast cells (MMC) associated with afferent extrinsic sensory nerves [1-3]. The neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) are expressed by the afferent nerves [3,4] and have been implicated in a direct nerve to MMC communication during tissue response to mucosal injury and inflammation [1,5,6]. Presumably, this communication functions to more efficiently propagate and enhance neurogenic inflammatory responses [6]. In line with these observation, the expression of functional receptors for SP (NK_1) and for CGRP has been demonstrated in bone marrow derived mucosal mast cells (BMMC) [7,8]. Activation of MMC by neuropeptides can results in an anaphylactic-type of degranulation, characterized by release of total granule content [9] but also in a very selective release of mediators [8,10,11] For instance, the response of BMMC to CGRP consisted of a single Ca^{2+} transient, which did not cause degranulation but piecemeal release [10] of mMCP-1 [8].
Another functional aspect of the activation of mast cells (MC) by neuropeptides has been proposed by Janiszewski et al in 1994 [12]. These authors used patch-clamp recording to measure outward chloride (Cl^-) currents of rat peritoneal mast cells (PMC) and determined degranulation by microscopic observation. A brief application of SP in a subthreshold concentration was found to increase the cellular responsiveness of the PMC to a subsequent stimulation with SP. The responses of the PMC to the second stimulation with SP showed an increased amplitude and a decreased threshold for degranulation [12]. This effect of the pre-exposure to SP was called "priming" by the authors (see also [13]). The decrease of the threshold for degranulation was presumably mediated by the effects of an influx of extracellular Ca^{2+} in response to the first application of SP [12]. This modulation of MMC responsiveness by SP provided a second potential rationale for the close anatomical association of MMC and sensory afferent nerves containing SP [12,13]. This phenomenon of priming has, so far as we are aware, not been confirmed by other studies. Thus, very limited information is available on the priming potential of neuropeptides and it was also not known whether CGRP has a priming action on MMC. Therefore, in this preliminary study, we have investigated if a pre-exposure of BMMC to a low concentration of CGRP (10^{-8}M) affects the response to a subsequent application of CGRP.

Material and Methods

Animals

Adult male BALB/c mice (Harlan, Horst, The Netherlands) were kept under constant housing conditions (22°C, 60% relative humidity, 12-hour light/dark cycle) and had free access to water and food. The mice were allowed to adjust to these conditions for one

week prior to the start of the experiments. The experimental design was approved by the animal experiments committee of the Utrecht University, Utrecht, The Netherlands.

Cell culture

Bone marrow-derived mast cells were cultured as previously described [14,15]. Briefly, mice at the age of 10-12 wk were euthanized by cervical dislocation, and their femurs were removed under sterile conditions. Bone marrow was washed from the femurs using a 23-gauge needle and a 5-ml syringe filled with DMEM (Invitrogen, Breda, The Netherlands) containing 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml fungizone, 2 mM L-glutamine, and 1 mM sodium pyruvate (Invitrogen) (DMEM/FCS). Cells were suspended by passing them three times through a 19-gauge needle. Cells were then centrifuged three times at 230 *g* for 7 min at room temperature (RT). After resuspension in 10 ml DMEM/FCS, viable cells were counted in a Bürker-Türk haemocytometer using trypan blue staining. Cells were cultured in a humid 5% CO_2 incubator at 37°C using 75cm^2 flat-bottomed flasks (BD Biosciences, Erembodegem, Belgium) at 5 x 10^5 cells/ml in DMEM/FCS containing 50 ng/ml SCF, 5 ng/ml recombinant mouse IL (rmIL)-9, 1 ng/ml rmIL-3 (R&D Systems, Abingdon, UK), and 1 ng/ml recombinant human transforming growth factor (TGF)-β_1 (Sigma-Aldrich, St. Louis, MO, USA). This combination of cytokines is referred to as TI3S and is known to result in high BMMC viability (93%) and mMCP-1 expression [15]. At 2- day intervals non-adherent cells were transferred into a new flask and half of the medium was replaced with fresh medium. The experiment was performed on cells cultured for 9-10 days.

Ca^{2+} imaging

BMMC were centrifuged at 150 g for 5 min at RT and resuspended twice in DMEM F-12 (Invitrogen) before being seeded (~10^5 cells in 200 µl DMEM F-12) onto the poly-D-lysine-coated glass bottom (diameter 14 mm) of a 35-mm-diameter culture dish (MatTek, Ashland, USA). Cells were loaded with the Ca^{2+}-sensitive fluorescent dye fura-2 acetoxymethyl ester (Fura-2 AM; Invitrogen; 5 µM) for 20 min. Cells were rinsed twice with DMEM F-12 and incubated for an additional 40 min in DMEM F-12. All incubations were performed at 37°C in a 5% CO2 humidified atmosphere. Dishes were transferred to an inverted microscope (Observer A1, Zeiss, Göttingen, Germany) equipped with a 40x oil objective (NA 1.3) and coupled to a TILL Photonics Polychrome IV (TILL Photonics GmBH, Gräfelfing, Germany) light source and an Image SensiCam digital camera (TILL Photonics GmBH). Imaging software (TILLvisION, v 4.01) was used for data collection and processing. Fluorescence was alternately excited with 340 and 380 nm light, and pairs of images were recorded at a frequency of 1/s using a 510 nm emission filter with 1 ms exposure time for each wavelength. Regions of interest (whole cell) were defined on subconfluent areas of the dishes and the 340/380 ratio of fluorescence intensities corrected for background signal was calculated using custom-made MS-Excel macros. Prior to each

experiment the baseline fluorescence of each cell was recorded during 30 s and normalized to 1. The relative fluorescence (RF) values of each region of interest, representing $[Ca^{2+}]_i$, were plotted as a function of time.

Cellular activation

During the experiment, the culture dishes containing BMMC were perfused with DMEM F-12 at a rate of 0.2 ml/min using a perfusion pencil (AutoMate Scientific, Berkeley, California, USA). To measure cellular Ca^{2+} responses to the application of CGRP or C48/80, the drugs were dissolved in DMEM F-12 and applied directly in the bath as a small drop (20 µl) from a micropipette. The location of the pipette tip was at least 500 µm from the nearest cells to avoid pressure-induced stretching of BMMC cell membranes. During the period from 20 s before to 20 s after the application, the perfusion was halted to allow for equal distribution of the compound in the dish. BMMC were pre-exposed to CGRP (10^{-8} M) or buffer (control), followed 2 min later by a second stimulation (CGRP; range from 10^{-11} to 10^{-5} M) (Fig. 1). At the end of each experiment C48/80 (100µg/ml) was applied to the dish. The Ca^{2+} response to C48/80 was used to determine the viability and maximal response of each cell. Cells unresponsive to C48/80 (~ 7%) were excluded from further analysis. The amplitude and lag of each CGRP-induced transient increase in $[Ca^{2+}]_i$ with an amplitude > 1.1 RF (threshold) were calculated. The lag (expressed in s) was defined as the time between drug application and the first noticeable change in the RF. The amplitude was defined as the highest RF value of the response and was expressed as percentage of the RF obtained by application of C48/80. The duration of the responses was defined as the duration (s) at half-height.

Statistical analysis

Data of all measured cells in a single dish were pooled and the percentage of responding cells and the mean values for the lag, amplitude and duration of the responses were calculated. To establish the CGRP concentration-response relationship (Fig. 2), values (mean ± SEM) obtained on ~6 dishes per concentration were used. The concentration-response relationships in Fig. 2 were fitted with a Boltzmann curve using Origin 7.5 and compared by an F-test (Origin Lab, Northampton, USA). A p value < 0.05 was considered significant.

Results

As described in detail previously [8,14-16], the BMMC cultured in the TI3S supplemented medium for 9-10 days differentiated into an MMC like phenotype expressing the characteristic chymase, mouse mast cell protease-1 (mMCP-1; data not shown). $[Ca^{2+}]_i$ was monitored in BMMC located in a culture dish perfused with buffer. GRRP (10^{-8} M) or

buffer (control) was applied for 20 s to the dish, followed after 2 min by a second application of CGRP (20 s; 10^{-11}-10^{-5} M) as shown in Fig. 1. The $[Ca^{2+}]_i$ responses to the second application were monitored during 2 min (Fig. 1).

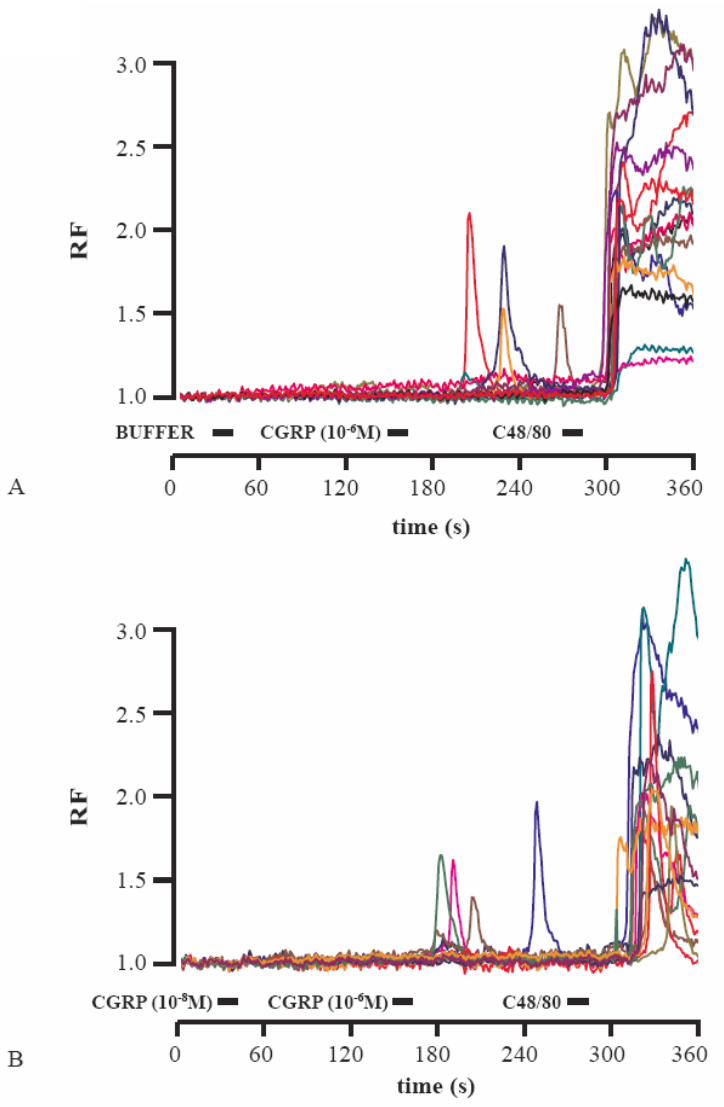

Fig. 1. $[Ca^{2+}]_i$ responses of BMMC to CGRP
BMMC were (at 30 s) either pre-exposed (20 s) to buffer in the control group (A) or to CGRP 10^{-8}M (B), as indicated by solid bars. After a 2 min recording period of $[Ca^{2+}]_i$, CGRP (in this example 10^{-6} M) was applied (at 150 s) and responses were recorded during another 2 min. The responses to CGRP consisted of a rapid increase in $[Ca^{2+}]_i$ followed by a slow exponential decrease towards the baseline. At the end of each experiment C48/80 (100ug/ml) was applied to verify the responsiveness of the BMMC. Application of C48/80 (at 270 s) resulted in an increase in $[Ca^{2+}]_i$, which settled to a temporarily sustained level and subsequently decreased slowly. The fraction of BMMC responding to CGRP was expressed as the percentage of C48/80-responding cells, in this example 4 of 16 (25%) in control BMMC (A) and 4 of 14 (29%) in pre-exposed BMMC (B), respectively. To determine if the pre-exposure did affect the second response to CGRP a comparison was made of the properties of the responses to the second stimulus between controls and pre-exposed BMMC.

From the responses to the second application (pre-exposed to GCRP or to buffer for controls), concentration-response relationships were established for the proportion of cells responding to CGRP, the lag of the response, the duration of the response and for the response amplitude. In the control group, the percentage of BMMC responding to CGRP increased from 10^{-11} M dose-dependently to a maximum of 20 ± 6 % at 10^{-5} M CGRP (Fig 2).

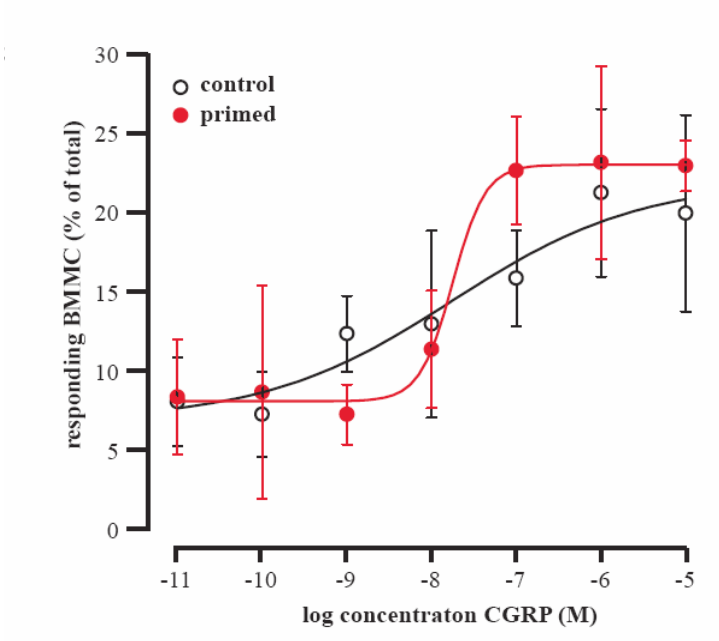

Fig. 2. Concentration-response relation for the effect of CGRP on BMMC
Concentration-response curve (-○-) showing that application of CGRP following a 20 s pre-exposure to buffer (control) evoked a concentration-dependent increase in the proportion of BMMC (re C48/80 = 100%) responding. A fit of the data (Boltzmann; R^2 0.94) revealed an EC_{50} of 10^{-8} M and a maximum response of 23%. The concentration-response curve obtained after pre-exposure of the BMMC to CGRP 10^{-8} M (20 s; -●-) was not different from the control curve (p 0.6; F-test). The data points in the graphs depict the mean values (± SEM; n ~6) obtained from in total 180 BMMC in 9 dishes, from 8 cultures.

A Boltzmann curve fit (R^2: 0.94) revealed an EC_{50} of 10^{-8} M. The concentration-response relation and the percentage of responding cells was comparable to that previously described (EC50 10^{-8}M; 19 %; [8]). BMMC which were pre-exposed to CGRP 10^{-8} M responded to CGRP with a maximum of 23 ± 2 % at 10^{-5} M and an EC_{50} of 10^{-8} M (R^2: 0.94). Comparison of the concentration-response curves (Fig. 1) by an F-test did not reveal a difference between control and pre-exposed BMMC (p= 0.6). The amplitude of the response to CGRP was expressed as the fraction of the amplitude of the maximal response

elicited by application of C48/80 (100 µg/ml) at the end of each experiment. The response amplitudes were not correlated to the CGRP concentration and ranged between 70 and 83 %. No difference was observed ($p > 0.05$) in the amplitudes between the control group (76 ± 4 %) and the group pre-exposed to CGRP 10^{-8} M (77 ± 4 %). Similarly, the lag of the responses, calculated as the time between CGRP application and the first noticeable rise in the RF, was not altered by pre-exposure to CGRP 10^{-8}M (F-test; $p = 0.35$). Thus the Ca^{2+} response to the second application of CGRP was not influenced by the pre-exposure to CGRP 10^{-8} M.

Discussion

In the present experiment we have investigated the CGRP-induced priming of BMMC by recording changes in $[Ca^{2+}]_i$. Previously we have demonstrated that the CGRP1-receptor mediated activation of BMMC results in a single transient rise of $[Ca^{2+}]_i$ [8]. In the present experiments, the concentration-response relation based on the proportion of responding BMMC was not affected by the pre-exposure to CGRP 10^{-8} M. Such a lack of priming was also apparent from the amplitude of the Ca^{2+} response to CGRP, which was not different between the control and the CGRP pre-exposed BMMC. Since also the lag and duration of the CGRP-induced Ca^{2+} transients were not influenced by the pre-exposure, our results show no change in the responsiveness of BMMC which could indicate priming by CGRP 10^{-8} M.

The activation of BMMC with CGRP resulted in a single Ca^{2+}-transient without any influx of extracellular Ca^{2+} [8]. As the CGRP-receptor belongs to the family of G_i-protein coupled receptors [14,17], the Ca^{2+}-transients are thought to result from G_i-protein dependant release of Ca^{2+} from IP_3 sensitive calcium stores, such as the endoplasmic reticulum [14,18]. Our observation that the main properties of the Ca^{2+} transients were not affected by pre-exposure to CGRP, indicates that the pre-exposure to GCRP did not induce an influx of extracellular Ca^{2+} during the second stimulation with CGRP. Since the anaphylactic-type of degranulation of mast cells requires a prolonged influx of extracellular Ca^{2+}, which is thought to initiate granule exocytosis [9,18], we conclude that pre-exposure of BMMC to CGRP 10^{-8} M does not facilitates an anaphylactic-type degranulation. Further, CGRP has been shown to induce a piecemeal [10] release of the chymase mMCP-1 from BMMC, which at EC_{50} (10^{-8}M) involves 10% only of total the cellular content [8]. The CGRP-induced release of mMCP-1 is highly correlated to the percentage of BMMC responding to CGRP [8]. Although in the present study the release of mMCP-1 was not measured, the lack of effect of the pre-exposure on the percentage of BMMC responding to CGRP indicates that it is unlikely that the mMCP-1 release was altered by the pre-exposure to CGRP.

For several reasons it is difficult to compare the present negative result on CGRP-induced priming of cultured BMMC with the data in the publication [12] in which priming of MC has

been originally described. Firstly, in the study of Janiszewski *et al* [12], the priming effects of SP were investigated on freshly isolated rat PMC, which are not mucosal MC but connective tissue MC. Thus in both studies, there were significant differences between the MC type, the priming substance and the conditions under which the cells were obtained. Secondly, in [12] the cell responses consisted of Cl^- currents measured by whole cell patch-clamp, while in the present study changes in $[Ca^{2+}]_i$ were recorded. $[Ca^{2+}]_i$ is considered the main modulator of stimulus-secretion coupling [18], while the Cl- currents reflect opening and closing of ion channels in the cell membrane. Finally, in the present study the effects of pre-exposure to CGRP 10^{-8}M were determined during a 2 min period which started 2 min after the pre-exposure to CGRP, while in [12] recordings were made during much longer times (usually 24 to 46 min). Although in [12] the recorded Cl^- currents increased rapidly in response to the priming, the priming-induced degranulation showed a lag much longer than 2 min. In the light of these experimental differences and also because of limitations of the present study, it cannot yet be excluded that CGRP can affect MC responsiveness *in-vivo*. An important limitation of the present study was that only one single concentration (10^{-8} M) CGRP was used for the pre-exposure, lower concentrations need to be tested. Additional experiments in which these and other variables will be examined should provide more information on the role of CGRP as a priming agent for BMMC and MMC.

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Chapter 6

Summarizing discussion

The principal goal of this thesis was to investigate the contribution of mucosal mast cells to the regulation of the mouse intestinal barrier function. The integrity of this barrier is highly important for homeostasis and for prevention of bacterial translocation. Although mast cells play undoubtedly an important role in AP [1, 2], in chapter 2 of this thesis the focus is on the effects of probiotics on the impairment of the integrity of the mucosal barrier during AP. In chapter 3 we investigated the contribution of the mucosal mast cell mediator mMCP-1 to impairment of the mucosal barrier during a parasitic infection. In the following 2 chapters (4 and 5) the mode of action of the neuropeptide CGRP on mouse mucosal mast cells was determined. In this chapter the major findings of our study will be summarized and discussed.

Pretreatment but not treatment with probiotics abolishes mouse intestinal barrier dysfunction in acute pancreatitis.

A serious limitation of the insight in the effects of probiotics on the mucosal barrier function in acute pancreatitis (AP) was the fact that in animal studies probiotics were applied well before the onset of AP [3, 4] whereas in the clinical setting probiotic treatment can only be started after AP is diagnosed. We have now investigated **(Chapter 2)** in an experimental mouse model of AP the effects of probiotics on intestinal barrier function, using application before AP induction (prophylactically) as well as following AP induction (therapeutically).

In the course of acute pancreatitis, an impairment of the intestinal barrier has been hypothesized to allow for intestinal bacteria and associated endotoxins to cross the intestinal wall resulting in an aggravation of the inflammatory response and in infectious complications [5, 6].

In Chapter 2, we demonstrate that AP indeed induces a functional impairment of the ileal mucosal barrier function. This impairment was similar in the early phase of AP (immediately after AP induction) and in the late phase of AP (3 days after induction). In the mouse model, the induction of AP by cerulein resulted in a decrease of the ileal electrical resistance accompanied by an enhanced permeability to the marker molecule NaFl. Since the electrical resistance of the ileal mucosa is mainly determined by the resistance of the paracellular pathway [7], we concluded that the AP-induced resistance changes result primarily from alternations in the epithelial tight junctions (TJ). This conclusion is supported by the results of a recent study, which revealed a disruption in the distribution pattern of several TJ proteins in experimental AP [8]. These results provide the first direct evidence that AP seriously affects the physical properties of the intestinal epithelium.

The prophylactic application of probiotics did not prevent AP induced damage to the epithelial integrity in the early phase of AP, but caused a complete recovery of the

epithelial integrity in the late phase of AP. This finding may be relevant for the clinical use of probiotics aimed at stabilizing the intestinal barrier function. One likely mechanism of such recovery could be that probiotics limit the inflammatory response induced by the initial damage to the mucosal barrier [9]. Further studies are necessary to determine if this is indeed the case.

The main finding of our study is that only a prophylactic application of probiotics was effective in the recovery of epithelial integrity. Once AP was induced, a treatment with probiotics did not alter the impaired epithelial integrity. The conclusion of this study is that timing of application determines if a probiotic treatment can improve the intestinal barrier function. Recently, a clinical study revealed that application of probiotics to patients suffering from severe AP increases mortality [10]. These adverse effects led to the rejection of the use of probiotics in critically ill patients [10]. The results of the present study show that clinical use of probiotics is likely to be most effective in conditions where treatment can be started prior to onset of disease.

Impairment of intestinal barrier and secretory function as well as egg excretion during intestinal schistosomiasis occur independently of mouse mast cell protease-1.

In *Schistosoma mansoni*-infected mice, egg deposition in the intestinal wall and passage to the lumen are associated with recruitment of intestinal mucosal mast cells (MMC) expressing mouse mast cell protease-1 (mMCP-1) [11]. The role of MMC during schistosomiasis is not known, nor are the mechanisms facilitating schistosome egg passage to the lumen. To determine if mMCP-1 has a role in egg passage through the intestinal wall during *S. mansoni* infection, we studied the functional relationship between mMCP-1, the integrity of the intestinal barrier and the passage of *S. mansoni* eggs **(Chapter 3)**.

The results of this first time quantification demonstrated a severe impairment of the mucosal barrier function at the peak time of schistosome egg excretion. The impairment was observed as a decrease of the electrical resistance, an increase of the intestinal permeability and a decrease of the intestinal secretory response. The lack of mMCP-1 in the KO mice did, however, not influence these impairments. Moreover, the rate of *S. mansoni* egg passage through the intestinal wall was also independent of the presence of mMCP-1. From these results it was concluded that mMCP-1 does not contribute to barrier impairment or egg clearance during infection with *S. mansoni* in the mouse. This contrast with the role of mMCP-1 during infection with *T. Spiralis* [12] is most likely due to essential differences between the parasite species. Differences between parasitic species such as their niche in the host and life cycle stage are known to result in unique expulsion mechanisms [13]. We concluded that the impairment of the mucosal barrier function during *S. mansoni* infection is not caused by mMCP-1 but possibly by other MMC mediators, or

by products secreted by the schistosome egg. The severe reduction of the epithelial secretory capacity measured during *S. mansoni* infection indicates that schistosoma egg passage is facilitated by mechanisms which affect the epithelial cells themselves rather than TJ integrity. This would be in accordance with the fact that the epithelial cells play a central role in the defence against gut nematodes [14].
The main conclusion of this Chapter is that mMCP-1 is not required for the impairment of the mucosal barrier function in *S. mansoni* infection. Thus the role of the MMC that are recruited to intestines harboring *schistosome* eggs [15] is most likely related to release of other mediators or directly to the inflammatory response to parasitic infection.

CGRP1 receptor activation induces release of mast cell protease-1 from mouse mucosal mast cells in absence of degranulation.

For the investigations of MMC activation and mediator release described in chapters 4 and 5, bone marrow derived mast cells (BMMC) were used as a model of the MMC *in-vivo*. BMMC were obtained by culturing bone marrow cells in presence of cytokines which stimulate a selective growth and maturation of mast cells expressing mMCP-1 and -2 and the integrin αEβ7 [16]. *In vivo*, both chymases and the integrin are expressed by MMC and not by connective tissue mast cells. [17-19]. BMMC are therefore considered to be true MMC homologues [16]. Nevertheless, observations made using BMMC need to be verified *in-vivo* before definitive conclusions can be drawn about MMC in the intact organism.
Using pharmacological and immunohistochemical techniques, we have demonstrated in **chapter 4** the presence and functionality of the CGRP1 receptor on BMMC. The reported EC_{50} (10^{-8}M) for activation of BMMC by CGRP is comparable to that reported for the relaxant effect of CGRP on isolated human distal coronary arteries (10^{-9}M; [20]) and for excitation of myenteric neurones in the guinea-pig ileum (5.10^{-8}M; [21]. No indications were found for a receptor-independent mode of action of CGRP on BMMC. This is a relevant and interesting finding since some other neuropeptides, such as Substance P, can at higher concentrations evoke mast cell responses by a direct activation of the G_i-protein without the intermediacy of a membrane receptor [22, 23]. The CGRP1 receptor is thus likely to be a unique component in the signal transduction pathway between sensory nerve fibers releasing CGRP, and adjacent MMC in the intestinal mucosa.
CGRP was found to stimulate a selective release of mMCP-1 from BMMC without degranulation. It can be speculated whether other mast cell mediators are released along with mMCP-1. Mast cells have been shown to selectively release several mediators such as interleukins and serotonin independent of degranulation [24]. It is therefore sugested that stimulation of MMC by CGRP results in simultaneous release of several mediators which together with mMCP-1 participate in neurogenic inflammation.

In summary, this chapter presents new mechanisms involved in the communication between afferent nerves and intestinal mucosal mast cells. This communication is important for the maintenance of homeostasis and integrity of the mucosal barrier and also provides an appropriate response to injury.

CGRP does not induce priming of mouse mucosal mast cells

There is some evidence that low doses of SP, which do not elicit MC degranulation in response to a single application, can induce degranulation when applied repeatedly [25]. This effect of SP in decreasing the threshold for MC degranulation has been named "priming" [25]. In the intestinal mucosa in which SP and CGRP are expressed by sensory nerves associated with MMC, priming of MMC by these neuropeptides could presumably enhance the neurogenic inflammatory responses by increasing MC responsiveness [26, 27]. Only limited information was available on the priming potential of neuropeptides in general, and it was not known if CGRP has a priming action on MMC.
Following the demonstration of the presence of the CGRP1 receptor on BMMC (see chapter 4), we have now investigated if CGRP can be a priming substance for BMMC. The investigation described in **chapter 5** showed that a short pre-incubation with a low concentration of CGRP does not increase BMMC responsiveness to CGRP. We thus concluded that no evidence was found for a priming action of CGRP on BMMC. However, in our study, the priming action of CGRP was only investigated for one concentration (10^{-8}M) and for limited exposure times. A more detailed and extensive investigation will be required before a definite conclusion about the priming potency of CGRP for BMMC and MMC can be drawn

To the four questions raised in the Introduction of this thesis, the following answers can be given:

1. Is the effect of probiotics on mouse intestinal barrier function during acute pancreatitis (AP) dependent on the time of application?

AP results in an impairment of the mouse intestinal barrier function. This impairment can be abolished by application of probiotics, but only if such application is started prior to the onset of AP. Proper timing of their application is therefore a condition for the beneficial effects of probiotics on the mouse intestinal barrier function in AP.

2. *Is mouse mast cell protease-1 (mMCP-1) involved in intestinal mucosal barrier impairment in the parasitic infection schistosomiasis?*

In the mouse, schistosomiasis results in severe impairment of the integrity of the intestinal mucosal barrier and also of the secretory capacity of the epithelium. No difference was found in these impairments between wildtype mice and knockout mice deficient in mMCP-1. From this finding, we concluded that in schistosomiasis mMCP-1 is not a key factor in the impairment of the intestinal mucosal barrier and the secretory capacity.

3. What is the functional effect of CGRP on mouse mucosal mast cells and is this effect receptor-mediated?

Mouse mucosal mast cells were shown to express the CGRP1 receptor. Application of CGRP to the mucosal mast cells caused a receptor-mediated release of mMCP-1 without degranulation. Interestingly, blocking of the CGRP1 receptor by a selective antagonist completely abolished the CGRP-induced activation. This demonstrates that the effect of CGRP on the mucosal mast cells is mediated exclusively by the CGRP1 receptor.

4. Can mouse mucosal mast cells be primed by CGRP?

A pre-exposure of mouse mucosal mast cells to 10^{-8} M CGRP did not affect the intracellular calcium response to a subsequent stimulation by CGRP. Under the experimental conditions used, no evidence was found that mucosal mast cells can be primed by CGRP. Additional experiments are required.

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Nederlandse samenvatting

Dit proefschrift beschrijft een onderzoek naar de bijdrage van mucosale mestcellen (MMC) aan de regulatie van de intestinale barrièrefunctie van de muis. De integriteit van deze barrière is belangrijk voor de homeostase en voor de preventie van bacteriële translocatie. In hoofdstuk 2 van dit proefschrift ligt de nadruk op de effecten van probiotica op de aantasting van de integriteit van de mucosale barrière tijdens acute pancreatitis (AP). In hoofdstuk 3 wordt de bijdrage beschreven van de MMC mediator muis mestcel protease-1 (mMCP-1) aan de aantasting van de barrièrefunctie van de darmwand tijdens een parasitaire infectie. In de volgende 2 hoofdstukken (4 en 5) wordt het werkingsmechanisme vastgesteld van het neuropeptide Calcitonin Gene-Related Peptide (CGRP) op de MMC van de muis. Hieronder zullen de belangrijkste bevindingen van het onderzoek worden samengevat en besproken.

De verzwakking van de intestinale barrière tijdens AP kan worden opgeheven door een profylactische toediening maar niet door een therapeutische toediening van probiotica

De verzwakking van de intestinale barrière tijdens AP wordt verondersteld de oorzaak te zijn voor de passage van bacteriën en gerelateerde endotoxinen door de darmwand. Deze translocatie kan resulteren in een aggravatie van de ontstekingsreactie en in infectieuze complicaties [5, 6]. Een ernstige beperking van het inzicht in de effecten van probiotica op de barrièrefunctie van de darmwand bij acute pancreatitis (AP) was het feit dat in dierstudies probiotica ruim vóór het begin van de AP werden toegediend [3, 4] terwijl in de klinische context een behandeling met probiotica pas kan worden gestart nadat AP is gediagnosticeerd. Daarom werden, in een experimenteel muizenmodel van AP, de effecten van probiotica op de intestinale barrièrefunctie onderzocht **(Hoofdstuk 2)** bij toediening vóór de AP inductie (profylactisch) alsook na AP inductie (therapeutisch).
In hoofdstuk 2 tonen we aan dat AP de barrièrefunctie van darmwand van het ileum van de muis aantast. Deze vermindering van de barrièrefunctie was niet verschillend voor de vroege fase van de AP (onmiddellijk na inductie) en de late fase van de AP (3 dagen na inductie). De inductie van AP veroorzaakte een afname van de elektrische weerstand van het ileum en een verhoogde permeabiliteit. Aangezien de elektrische weerstand van de ileale mucosa voornamelijk bepaald wordt door de weerstand van de paracellulaire route [7], hebben we geconcludeerd dat de AP-geïnduceerde veranderingen van de weerstand het

gevolg zijn van modificaties van epitheliale tight junctions (TJ). Deze resultaten vormen het eerste directe bewijs dat AP de fysische eigenschappen van het darmepitheel ernstig beïnvloedt. Deze conclusie wordt ondersteund door de resultaten van een recente studie die een verstoring demonstreert in het distributie patroon van verschillende TJ eiwitten tijdens experimentele AP [8].

De profylactische toediening van probiotica kan de AP-geïnduceerde schade aan de epitheliale integriteit niet voorkomen, maar veroorzaakt wel een volledig herstel van de epitheliale integriteit in de late fase van AP. Deze bevinding kan van belang zijn voor de klinische toepassing van probiotica gericht op stabilisatie van de intestinale barrière functie. Een mogelijk werkingsmechanisme zou kunnen zijn dat de ontstekingsreactie die veroorzaakt wordt door de initiële schade aan de mucosale barrière wordt beperkt door de probiotica. Dit zou het herstel in de late fase van AP kunnen bevorderen [9]. Verdere studies zijn nodig om te bepalen of dit inderdaad het geval is.

De belangrijkste bevinding van ons onderzoek is dat alleen een profylactische toepassing van probiotica effectief is in het herstel van de epitheliale integriteit. Als de AP eenmaal geïnduceerd is, heeft een behandeling met probiotica geen invloed op de verminderde epitheliale integriteit. De conclusie van deze studie is dat de timing van de toediening bepaalt of een behandeling met probiotica de intestinale barrière functie kan verbeteren.

Een recente klinische studie heeft uitgewezen dat de toepassing van probiotica bij patiënten die lijden aan ernstige AP de mortaliteit verhoogt [10]. Deze negatieve effecten hebben geleid tot de afwijzing van het gebruik van probiotica bij ernstig zieke patiënten [10]. De resultaten van onze studie suggereren dat klinisch gebruik van probiotica waarschijnlijk het meest effectief is in situaties waarbij de behandeling kan worden gestart voorafgaand aan het begin van de aandoening.

De afname van de integriteit en secretiecapaciteit van de mucosale barrière, evenals de ei-excretie tijdens intestinale schistosomiasis zijn onafhankelijk van muis mestcel protease-1.

In *Schistosoma mansoni* geïnfecteerde muizen is de afzetting van eieren in de darmwand en hun passage naar het lumen geassocieerd met rekrutering van intestinale mucosale mestcellen (MMC) die muis mestcel protease-1 (mMCP-1) tot expressie brengen [11]. De rol van de MMC in schistosomiasis is niet bekend, evenmin als de mechanismen die de passage van de schistosoma eieren door de darmwand faciliteren. Om te bepalen of mMCP-1 een rol speelt bij de passage van eieren door de darmwand tijdens *S. mansoni* infectie, bestudeerden we de functionele relatie tussen de aanwezigheid van mMCP-1 (mbv mMCP-1 knock-out muizen), de integriteit van de darmwand en de passage van de eieren **(Hoofdstuk 3)**. De resultaten van deze kwantificering demonstreren een ernstige aantasting van de barrièrefunctie van de darmwand tijdens de periode dat de schistosoma

ei-excretie maximaal is. De aantasting van de barrièrefunctie werd waargenomen als een daling van de elektrische weerstand, een stijging van de intestinale permeabiliteit en een afname van de intestinale secretiecapaciteit.
Het ontbreken van mMCP-1 in knock-out muizen bleek geen invloed te hebben op deze aantastingen van de barriere functie. Bovendien was ook de snelheid van de passage van *S. mansoni* eieren door de darmwand onafhankelijk van de aanwezigheid van mMCP-1. Uit deze resultaten werd geconcludeerd dat mMCP-1 niet bijdraagt aan de aantasting van de barrièrefunctie van de darmwand of aan de passage van *S. mansoni* eieren tijdens infectie met *S. mansoni* in de muis. Deze conclusie contrasteert met de beschreven rol van mMCP-1 tijdens een andere parasitaire infectie, namelijk met *Trichinella spiralis* [12] . Dit contrast is waarschijnlijk te wijten aan essentiële verschillen tussen beide parasieten. Verschillen tussen parasitaire soorten, zoals hun niche in de gastheer en de fase van de levenscyclus, resulteren in specifieke mechanismen van expulsie [13]. We concludeerden dat de aantasting van de barrièrefunctie van de darmwand tijdens infectie met *S. mansoni waarschijnlijk* wordt veroorzaakt door andere MMC-mediatoren of door stoffen die worden afgescheiden door de eieren van de parasiet. De vastgestelde ernstige vermindering van de epitheliale secretiecapaciteit tijdens de infectie met *S. mansoni* geeft aan dat de passage van de eieren vergemakkelijkt wordt door mechanismen die de epitheliale cellen beïnvloeden. Dit is in overeenstemming met het feit dat de epitheliale cellen in de darm een centrale rol spelen bij de afweer tegen nematoden [14].
De belangrijkste conclusie van dit hoofdstuk is dat mMCP-1 geen rol speelt bij de aantasting van de barrièrefunctie van de darmwand tijdens infectie met *S. mansoni.* De rol van de MMC die worden gerekruteerd naar de *S. mansoni* geïnfecteerde darm [15] is dus hoogstwaarschijnlijk gerelateerd aan de secretie van andere mediatoren uit de MMC of aan de inflammatoire respons op de parasitaire infectie.

Aktivatie van de CGRP1 receptor van de mucosale mestcel van de muis induceert “piecemeal release” van mMCP-1 zonder degranulatie

Voor het onderzoek maar de activering van MMC en secretie van hun mediatoren (hoofdstukken 4 en 5), werden uit het beenmerg van de muis gekweekte MMC (BMMC) gebruikt als een model van MMC *in-vivo*. BMMC worden volwaardige MMC homologen geacht [16]. Niettemin dienen de resultaten uit het onderzoek met BMMC *in-vivo* te worden bevestigd voordat er definitieve conclusies kunnen worden getrokken over de MMC in het intacte organisme.
In hoofdstuk 4 werden doormiddel van farmacologische en immunohistochemische technieken de aanwezigheid en functionaliteit van de CGRP1 receptor op de BMMC aangetoond. De gemeten EC_{50} (10^{-8}M) voor de activering van BMMC door CGRP is

vergelijkbaar met die voor het relaxerende effect van CGRP op geïsoleerde humane distale coronaire arteriën (10^{-9}M; [17]) en voor excitatie van myenterische neuronen in het ileum van de cavia (5.10^{-8}M; [18]).
De resultaten van experimenten met de CGRP receptor antagonist BIBN4096BS gaven geen aanwijzingen voor een receptor onafhankelijk effect van CGRP op BMMC. Dit is een relevante en interessante bevinding aangezien sommige andere neuropeptiden, zoals Substance P, bij hogere concentraties de mestcel stimuleren door een rechtstreekse aktivatie van de G_i-eiwitten zonder de tussenkomst van membraanreceptoren [19, 20]. De CGRP1 receptor is dus waarschijnlijk een unieke component in de signaaltransductie tussen de sensorische zenuwvezels die CGRP vrijmaken en de nabijgelegen MMC in de darmwand.
Een andere interessante bevinding was dat CGRP selectief het vrijkomen van mMCP-1 bleek te induceren, zonder dat de BMMC degranuleerden. Dat het vrijkomen van mMCP-1 geschiedde via “piecemeal release” werd bevestigd met behulp van electronenmicroscopie. Omdat eerder is aangetoond dat een aantal mediatoren die betrokken zijn bij neurogene inflamatie (zoals interleukines en serotonine) uit de mestcel kunnen vrijkomen onafhankelijk van degranulatie [21], kan worden gespeculeerd dat andere mestcel mediatoren vrijkomen samen met MMCP-1.
Dit hoofdstuk presenteert nieuwe mechanismen die een rol spelen in de communicatie tussen afferente zenuwvezels en intestinale MMC. Deze communicatie is belangrijk voor het behoud van homeostase en de integriteit van de mucosale barrière en draagt bij aan een passende response op beschadiging.

CGRP induceert geen “priming” in mucosale mestcellen van de muis

In de literatuur is beschreven dat lage doseringen van Substance P (SP), die bij een enkele toediening aan de mestcel geen degranulatie opwekken, wel degranulatie kunnen induceren bij herhaalde toediening [22]. Dit verminderen van de activeringsdrempel van de mestcel tot degranulatie wordt "priming" genoemd [22]. In de intestinale mucosa worden SP en CGRP tot expressie gebracht door de uitlopers van sensorische zenuwcellen die geassocieerd zijn met MMC. Deze neuropeptiden zouden door middel van priming de responsiviteit van de MMC kunnen verhogen en hierdoor bijdragen aan een neurogene inflamatie [23, 24]. De beschikbare informatie over priming door neuropeptiden was echter zeer beperkt, en er was geen informatie beschikbaar over een rol van CGRP in priming. Na de demonstratie van de aanwezigheid van de CGRP1 receptor op BMMC (zie hoofdstuk 4), hebben we onderzocht of CGRP priming van BMMC kan induceren. Uit het onderzoek beschreven in **hoofdstuk 5**, is gebleken dat een korte pre-incubatie met een lage concentratie van CGRP de responsiviteit van de BMMC niet verhoogt.

Wij hebben geconcludeerd dat er geen bewijs is gevonden voor priming van BMMC door CGRP. Echter, in onze pilotstudie werd de priming door CGRP onderzocht met slechts 1 concentratie (10^{-8} M) CGRP en met een beperkte tijd van blootstelling. Een meer gedetailleerd en uitgebreid onderzoek zal nodig zijn vooraleer een definitieve conclusie kan worden getrokken over priming van BMMC en MMC door CGRP.

Op de vier vragen gesteld in de inleiding van dit proefschrift, kunnen de volgende antwoorden worden gegeven

1. Is het effect van probiotica op intestinale barrièrefunctie van de muis tijdens acute pancreatitis (AP) afhankelijk van het tijdstip van de toediening?

In het muizenmodel resulteert AP in een verzwakking van de intestinale barrière. Deze verzwakking kan worden opgeheven door toediening van probiotica, maar alleen indien deze voorafgaat aan de inductie van AP.
Juiste timing van toediening is daarom een voorwaarde voor de gunstige effecten van probiotica op de intestinale barrière van de muis tijdens AP.

2. Is de muis mestcel protease-1 (mMCP-1) betrokken bij de aantasting van de intestinale barrièrefunctie tijdens parasitaire infectie met schistosomiasis?

Schistosomiasis in de muis resulteert in een ernstige aantasting van de integriteit van de intestinale mucosale barrière en in een sterke afname van de secretiecapaciteit van het epitheel. Beide effecten zijn in gelijke mate aanwezig in wildtype muizen en in knock-out muizen deficiënt voor mMCP-1.
Uit deze bevinding hebben we geconcludeerd dat mMCP-1 geen essentiële factor is in de aantasting van de intestinale mucosale barrière en de secretiecapaciteit tijdens schistosomiasis.

3. Wat is het functionele effect van CGRP op de mucosale mestcellen en is dit effect receptor-gemedieerd?

De aanwezigheid van de CGRP1 receptor op de MMC van de muis werd aangetoond. Stimulatie van MMC met CGRP resulteerde in een receptor gemedieerde afgifte van mMCP-1, via “piecemeal release”, in afwezigheid van degranulatie. Omdat de aanwezigheid van een selectieve CGRP-receptor antagonist de CGRP-geïnduceerde

activering volledig blokkeerde, is geconcludeerd dat het effect van CGRP op de MMC uitsluitend wordt gemedieerd door de CGRP1 receptor.

4. Kan de mucosale mestcel worden "geprimed" door CGRP?

De pre-incubatie van MMC van de muis met 10^{-8}M CGRP had geen effect op de response van de BMMC op een herhaalde stimulatie met CGRP. Onder de gebruikte experimentele condities werd geen bewijs gevonden dat MMC kunnen worden geprimed door CGRP. Aanvullende experimenten zijn echter nodig voordat een definitieve conclusie kan worden getrokken

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Curriculum Vitae

Jakub Rychter was born in Wrocław, Poland, on 11, June 1976. At the age of 14 he moved to the Netherlands. After finishing high school, he studied Biology at the Radboud University Nijmegen. During his first internship he participated in a research project on functional genomic annotation of *Lactobacillus plantarum* at the Centre for Molecular and Biomolecular Informatics. During his second internship, at the Department of Cellular Animal Physiology at Radboud University Nijmegen, he investigated the presence and distribution of the corticotropin-releasing factor recepr in the mouse spinal cord. After receiving his masters degree in 2005, he strated in 2006 as a Phd-student at the Institute for Risk Assessment Sciences (IRAS) under supervision of Dr. A.B.A. Kroese, which resulted in this thesis