Neuroimmune regulation of inflammatory responses in inflammatory bowel disease

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General Introduction

Inflammatory bowel disease

Early reports on the manifestation of inflammatory bowel disease (IBD) date from as early as 850 AD when King Alfred from England suffered from an illness which caused pain on eating, discomfort and much embarrassment, plaguing the king without remission. However, it was not until the 20th century that the term IBD became used to describe chronic inflammatory conditions of the gastro-intestinal tract. IBD is an idiopathic disease characterized by striking swings between intestinal inflammation and remission. Patients with IBD suffer from abdominal pain and cramps, diarrhea, disrupted digestion, rectal bleeding and a substantial personal burden.

IBD can be subdivided into two major representatives, Crohn's disease (CD) and ulcerative colitis (UC). Although the clinicopathological phenotype of these two disorders is similar they can be separated by different localization of the inflammation in the gastro-intestinal tract and immunological and histological pattern (1). While CD is characterized by a transmural inflammation which can be found throughout the whole gastro-intestinal tract but mainly in the terminal ileum, UC is a mucosal inflammation restricted to the colon. Moreover, CD was postulated to be a T-helper (Th)1-mediated disease (2) whereas UC is mainly a Th2-mediated disorder (3).

The exact etiology of IBD remains unknown but is thought to be a complex interaction of genetic, environmental (i.e. enteric microflora) and immunological factors (4, 5). Current investigations and observations suggest that the initial event in IBD is a result of an dysregulated inflammatory response rather than an aggressive inflammatory response by a defective intestinal immune system (1). Although it is suggested that IBD in an autoimmune disease, potential enteric antigens for the exacerbation of IBD are luminal bacteria, parasitic nematodes or food allergens (4, 6).

Innervation of the gastro-intestinal tract

The innervation of the gastro-intestinal tract is mainly organized as the enteric nervous system (ENS) (7). Often addressed as the 'brain of the gut', the ENS is the most complicated nervous structure outside the central nervous system (CNS). The structure of the ENS can be subdivided in the myenteric and submucosal plexus. The myenteric plexus, also known as the Auberbach's plexus is located between the longitudinal and circular muscle layers of the gut. Its neurons innervate the circular muscle, myenteric ganglia, submucosal ganglia, or the epithelium, and play an important role in regulating gut motility and the secretion of mediators of target organs (8). The submucosal or Meissner's plexus located in the submucosa of the intestine, contains sensory nerves communicating with the myenteric plexus, and also motor nerves regulating ion and water transport (8). Moreover, the myenteric and submucosal plexus synapse with and innervate each other forming a large self-sustaining network in the intestinal wall (9).

Besides intrinsic innervation by the ENS the gut is also extrinsically innervated by both the sympathetic and the parasympathetic autonomic nervous system, which also includes non-adrenergic non-cholinergic nerves (NANC) (7). The extrinsic sensory neurons which convey information from the gut to the brain are vagal afferents originating from the nodose ganglia and spinal afferents originating from the dorsal root ganglia (DRG) (9). Some of them also serve an efferent function by releasing neuropeptides from their peripheral endings locally affecting different processes in the gut. The majority of the axons of the extrinsic neurons is unmyelinated and can respond to a variety of noxious substances, chemical stimuli and/or functional changes in the gastro-intestinal tract. The extrinsic sensory neurons function to inform the central nervous system about processes and conditions to maintain energy balance and fluid homeostasis in the gastro-intestinal tract. Hyperalgesia and sensitization of the afferent nervous system occurs when intense and repeated stimulation is present resulting from tissue damage or inflammatory conditions. This will result in a lowered threshold for firing primary afferent neurons to originally innocuous substances or stimuli leading to chronic pain and discomfort (10). Both intrinsic and extrinsic nerves respond to a variety of mechanical, biological or chemical stimuli and release a high number of peptides such as substance P, calcitonin-gene related peptide (CGRP), neurokinin A (NKA), vaso-active intestinal polypeptide (VIP) and neuropeptide Y (11, 12). The ENS is the main source for of tachykinins in the gastro-intestinal tract (13).

Already in the 1950s a role for intestinal nerves was established in the pathogenesis of IBD (14, 15). More recently, alterations in myenteric plexus nerve function along with abnormalities in colonic motility has been observed in animal models for experimental colitis and IBD patients, which accounted for both inflamed as non-inflamed sites (16-19). While the number of ganglia in the myenteric plexus remained unaffected, the number of ganglia in the submucosal plexus was shown to be decreased by induction of experimental colitis (20). Furthermore, Boyer et al. demonstrated that experimental colitis leads to injury and apoptosis of the myenteric plexus (21).

Neurogenic inflammation and IBD

Neurogenic inflammation is a local inflammatory reaction in response to infection, toxins or trauma. It is mediated via neuropeptides released from unmyelinated afferent nerve endings, such as substance P and CGRP, and is characterized by vasodilatation, plasma extravasation and smooth muscle contraction (22, 23). Neuropeptides have been shown to modulate different aspects of mucosal function and they play a role in the recruitment of granulocytes and lymphocytes, and in the modulation of mast cell activation (13). Moreover, proliferation and growth of nerve fibers can be detected during inflammation at the site of the inflammatory response (24, 25). Neurogenic inflammation is also described in skin, airways and urogenital tract (22), but the capacity of tachykinins and CGRP in inducing a neurogenic response in

the gastro-intestinal tract is said to be ambivalent (26). However, most studies provide evidence for a role of neuropeptides and its receptors in initiating and maintaining inflammatory responses in the gastro-intestinal tract (27). For example, delayed-type hypersensitivity of the mouse ileum was diminished by depletion of sensory nerve endings and NK_1 receptor antagonism (28). In neuropeptide Y (NPY) Y1 receptor knockout mice DSS-induced colitis was attenuated (29). The functional role of NPY in intestinal inflammation was confirmed by the use of a specific Y1 receptor antagonist in both a murine and rat model for DSS-induced colitis (29, 30). Furthermore, in patients with IBD, colonic mucosal substance P concentrations and substance P binding sites on mucosal blood vessels and lymphoid tissue are increased (31-34). In contrast, levels of CGRP were also shown to be elevated during colitis, but thought to mainly mediate protective responses via specific receptors (35). The observation that the intensity and density of nerves containing substance P and VIP was increased 7 days after the induction of TNBS-colitis suggests that neuropeptides are involved in tissue repair processes in colitis (24).
Besides neuropeptides, increased production and activity of neuronal nitric oxide synthase (nNOS) was also demonstrated in both clinical and experimental intestinal inflammation (36, 37).
Overall, it can be stated that changes in neuropeptides and other neuronal mediators during inflammation of the gastro-intestinal tract imply neuronal involvement in intestinal disorders. Contradictionary observations suggest different functions in different phases of the inflammatory response. During the onset of the disease neuropeptides are likely to be involved in the initiation of the inflammatory process, while later on the neuronal function during colitis seems to be more protective and repairing.

Mast cells

Mast cells are derived from hematopoietic progenitor cells in bone marrow where they initiate their maturation process (38). Growth and proliferation takes place under the influence of interleukin (IL) -3, IL-4, IL-9, IL-10 (39, 40), stem cell factor (SCF) (41) and nerve growth factor (NGF) (42, 43). Mast cells are versatile cells that contain numerous secretory granules in the cytoplasm and are distributed throughout the body in several different tissues. They are abundantly present in the skin (44), gastro-intestinal tract (45, 46), respiratory organs (47, 48) and to a lesser extent in the brain (49). Mast cells are particularly located around blood vessels and nerve endings in all these different tissue types (50-52).
Mast cells consist of a heterogeneous population that differs in morphological and histochemical characteristics. The rodent mast cell subtypes can be divided in connective tissue mast cells (CTMC), mainly located in the skin and peritoneal cavity and mucosal mast cells (MMC), particularly found in intestinal and pulmonary mucosa (53). However, not all mast cells can be classified as classical CTMC or MMC and intermediate subtypes have been described (54). In humans, mast cells are differentiated on the base of protease

content. Cells are designated as MC_{TC} for their tryptase and chymase content and -in terms of localization- correspond closely to the rodent CTMC whereas the MC_T only contains tryptase and corresponds most closely to the rodent MMC (55).
Activation of mast cells mainly occurs by interaction of a multivalent antigen with its specific IgE antibody bound to the cell membrane via the high affinity receptor FcεRI. However, mast cells can also be activated by substances like cytokines, neuropeptides, polybasic compounds, complement components and certain drugs (53, 56). The activation of mast cells leads to degranulation and mediator release. Mediators derived from mast cells can be divided in three different classes: 1) preformed secretory mediators stored in the granules, 2) lipid-derived mediators and 3) cytokines and chemokines. Rapid release of prestored granule-associated mediators mediators like histamine, serine proteases, proteoglycans, IL-6 and tumor necrosis factor-alpha (TNFα) occurs within minutes after cellular activation (57, 58). Together with this primary response *de novo* synthesis of lipid-derived mediators like prostaglandin (PG) D_2, leukotriene (LT)C_4, LTD_4, LTE_4 and platelet-activating factor (PAF) is initiated (59, 60). In addition, the production of cytokines, growth factors and chemokines, which include IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-13, IFNγ, GM-CSF and TNFα, is also induced and are subsequently secreted by mast cells (61, 62). Noticeably, TNFα is one of the few cytokines which is prestored and synthesized *de novo* by mast cells (56).
Biological functions of mast cells are dependent on mediators released after activation and include fibrosis, angiogenesis, mucosal exudation, recruitment of leukocytes, activation of T lymphocytes and interaction with the central nervous system (53, 63-66).

Mast cells and IBD

Although mast cells are regarded as critical tissue-based effector cells in acute allergic responses they have also been shown to participate in other inflammatory conditions like delayed type hypersensitivity (67), parasite infections (68, 69), rheumatoid arthritis (70), multiple sclerosis (71, 72) and inflammatory bowel disease (IBD) (73-75).
In the human gastro-intestinal tract mast cells are distributed throughout all tissue layers of the intestinal wall. 2-3% of the intestinal lamina propria cells are whereas they represent 1% of the cell population in the submucosa (76, 77). The number of mast cells increases in intestinal disorders such as IBD, irritable bowel syndrome (IBS), food allergy and parasitic infections (78-82).
Not only can increased numbers of mast cells be found in IBD, also the cellular content of these cells was greatly changed. For example, granules contained a higher density of IL-16 (83), TNFα (84) and substance P (85). Moreover, there is abundant evidence for mast cell degranulation in the intestinal mucosa of IBD patients. Increased concentrations of mast cell mediators in the gastro-intestinal tract have been detected in these patients (77, 78, 86).
Mast cell mediators are thought to contribute to the pathogenesis of IBD (75).

As a pro-inflammatory mediator, histamine can increase ion transport across the epithelial barrier and therefore contribute to diarrhea found in IBD (87). Also TNFα was demonstrated to be an important mediator of diarrhea (88, 89). Furthermore, several mediators, like proteases, cytokines, prostaglandins and chemokines have shown to induce microvascular leakage and recruit inflammatory cells to the site of inflammation and therefore enhance the inflammatory response (53, 90).
Further proof for a role for mast cells in IBD can be found in effective therapies directed against mast cells or mast cell mediators. Treatment with corticosteroids has been demonstrated to reduce mast cell numbers (91) and mast cell-derived TNFα and IL-3 (92, 93). Furthermore, the mast cell stabilizer, ketotifen, was able to significantly reduce the severity of colitis in two different experimental models for IBD (94, 95). The most abundant therapeutic clinical evidence comes from treatment with infliximab, a mouse/human monoclonal anti-TNFα antibody. Patients with active IBD showed reduced disease activity and maintained remission after treatment with infliximab (96-99).

Mast cell-nerve interactions

Bi-directional interactions between neurons and immune cells in inflammation have been well described (100). This mutual communication is also present in the gastro-intestinal tract between the ENS and the intestinal immune system (101). There is anatomical evidence demonstrating that mucosal lymphoid tissue in the gastro-intestinal tract is innervated by noradrenergic and NANC neuronal pathways (102, 103). Mast cells can often be found in close association with nerve fibers, also in the gastro-intestinal mucosa (104-106). Besides an anatomical relation, mast cells and nerves also have a functional link. Neuronal mechanisms are involved in mast cell activation and mast cells act as principle transducers of information between peripheral nerves and local inflammatory events (107). For example, mast cell mediators can sensitize neurons, which then release neurotransmitters and neuropeptides which in turn can further activate the mast cell (108-110). These mast cell-nerve interactions have been demonstrated in several different tissue structures like skin, brain, airways and gut, and contribute to the local inflammation process (105).
Mast cells and NANC nerves both express receptors for their respective mediators. So are H_1, H_2 and H_3 receptors for histamine are localized on primary NANC nerves (105, 111) and at least the H_1 receptor was demonstrated to be upregulated under inflammatory conditions (112). It is well recognized that serotonin receptors (5HT receptors) are abundantly expressed by noradrenergic and NANC neurons (113). NGF is a growth factor that mainly acts on nerve cells and regulates neuronal survival and differentiation. It is produced and released by a range of cell types among which NANC neurons and mast cells (114). The high affinity receptor for NGF is trkA whereas the low affinity receptor p75 that belongs to the TNF receptor family plays a minor role in signal transduction (115). Both these receptors are expressed by neurons as well as mast cells (114). These two cell types also share the expression of the two TNFα

receptors, $TNFR_1$ and $TNFR_2$. Both these receptors are expressed by neurons and upregulated under inflammatory conditions on adult rat DRG neurons (116). Furthermore, mast cell tryptase can specifically signal by cleaving and activating protease-activated receptors (PAR), a family of G-protein coupled receptors. Neurons of the central and peripheral nervous system express all four PARs and activation of these receptors leads to neuronal excitation (117). Evidence is available that mast cells express receptors for neurotransmitters and neuropeptides. In regard with sensory neurons, the receptor for VIP and CGRP have been shown to display on the mast cell surface (118). And finally, mast cells are also demonstrated to functionally express the high affinity receptor NK_1 for substance P (119). The bi-directional receptor expression is depicted in figure 1.

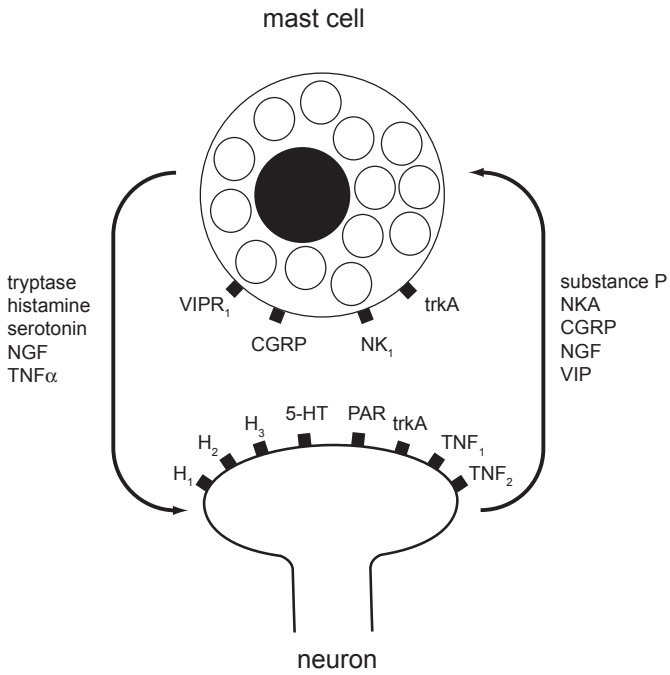

Figure 1 *Receptor expression by mast cells and nerves of their respective mediators*

Overall it can be stated that the mast cell-nerve interaction can contribute in various ways to the amplification and spreading of the inflammatory response during the pathogenesis of IBD. In this respect, it was demonstrated that the mast cell-nerve association was significantly increased during human intestinal inflammation by the anatomical observation that the distance between these two cell types was decreased (120). Moreover, in colorectal biopsies of patients with active IBD substance P was demonstrated to induce histamine release (121). Also, experimental colitis could be induced by an agonist for the tryptase

receptor PAR_2, which could be abrogated by depletion of capsaicin-sensitive nerves (122). This highly suggests that the PAR_2 induced colitis is mediated via a neuronal activation. Furthermore, stimulation of the guinea pig ENS with a mediator cocktail of human intestinal mast cells evoked a neuronal excitatory response (123). All these data indicate that mast cell-nerve communication is very relevant during inflammatory processes in the gastro-intestinal tract

Immunoglobulin-free light chains

Mast cells are classically known to induce antigen-specific IgE-mediated allergic hypersensitivity reactions (124). Delayed-type hypersensitivity (DTH) is characterized by a typical T lymphocyte-driven inflammatory cell infiltration within 12-72h after re-exposure to the antigen (125). However, mast cell degranulation was also described to be required for the elicitation of DTH reactions (126). The activation of mast cells in DTH responses was first recognized to be induced by a T cell-derived antigen-binding factor (67, 127). This factor, analogous to IgE, was demonstrated to have *in vivo* activity dependent on mast cell degranulation (128). Further research determined that this factor was not a T cell factor, but originated from B lymphocytes.

B lymphocytes are known to produce and secrete antibodies important in the humoral response of the adaptive immune system. These immunoglobulins (Ig) are potent effector molecules and exist of two heavy chains and two light chains bound by disulphide bridges. Igs can be divided into five isotypes (IgA, IgD, IgE, IgG and IgM) based on their heavy chain subclasses. The immunoglobulins all exhibit different biological functions. In the mammalian immune system two isotypes of light chains can be detected, the κ and λ light chain (129). Antigen-specificity of antibodies is determined by the variable domains of both light and heavy chains. Both light chains and heavy chains were shown to exhibit antigen binding by itself (130-132).
During the production of complete Igs B lymphocytes produce light chains in excess over heavy chains. These immunoglobulin-free light chains (IgLC) are subsequently secreted and can be detected in several different body compartments like blood, cerebrospinal fluid, synovial fluid and urine (129, 133-136). Until recently no physiological role has been ascribed to these IgLC. However, in our laboratory it was demonstrated that IgLC could mediate hypersensitivity responses by direct activation of mast cells (137). Passive immunization of mice with antigen-specific IgLC followed by topical skin application of the antigen resulted in plasma extravasation, cutaneous swelling and mast cell degranulation, which could be abolished by treatment with a specific antagonist for IgLC (137). The mechanism behind IgLC-mediated mast cell activation is depicted in figure 2.
Further evidence for IgLC-induced hypersensitivity-like responses and mast cell degranulation was provided by studies in a hapten-induced murine model for non-atopic asthma (138). Clinical relevance for IgLC was given by

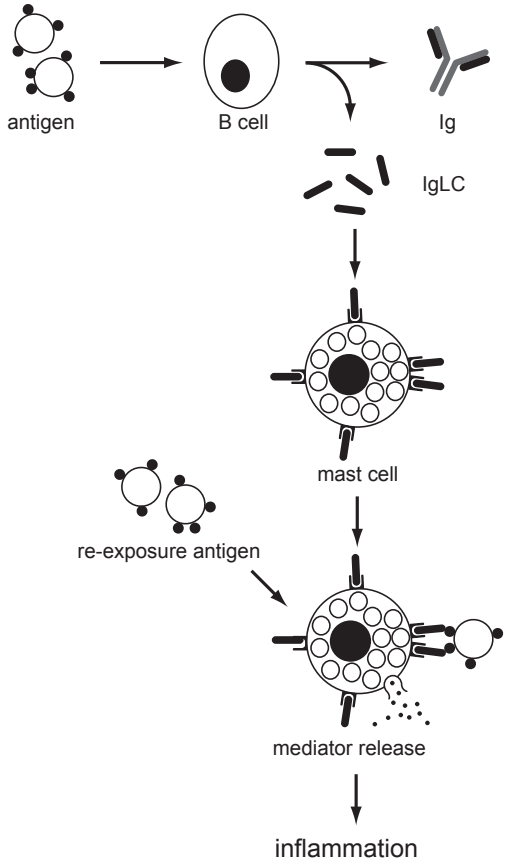

Figure 2 *Model for IgLC-mediated mast cell activation*

demonstrating significantly increased levels of IgLC in sera of non-atopic asthmatic patients compared to healthy controls (138). Moreover, increased numbers of mast cells and evidence for mast cell activation can be found in rheumatoid arthritis (70) and multiple sclerosis (72). These disorders are also accompanied with elevated levels of IgLC in synovial fluid (139) and cerebrospinal fluid (129, 136) respectively. These findings indicate that IgLC can induce hypersensitivity responses and can serve as a model to study mast cell-associated non-IgE-mediated disorders.

Although there are several lines of evidence suggesting the presence of a putative receptor for IgLC it is not identified it yet (137). Studies using knockout mice for γ-chain-associated receptors such as FcεRI, FcγRIII and PIR-A demonstrated that these receptors are not involved in IgLC-mediated mast cell activation (unpublished data). Currently we have isolated a mast cell membrane associated protein that interacts with IgLC and are in the process of identifying the protein.

Aim and scope of this thesis

It is clear from the previous paragraphs that both mast cells and NANC neurons might play an important role in the pathogenesis of inflammatory disorders of the gastro-intestinal tract. The aim of this thesis is to study the mast cell-nerve interaction, with a particular role for IgLC, in the development of IBD *in vivo* in a murine model for colonic hypersensitivity. In chapter 2 the features and characteristics for a murine model for colitis are described. This model was demonstrated to be mast cell-dependent by the use of mast cell-deficient ($WBB6F_1$ W/W^v) mice. Further validation of the newly described model is outlined in chapter 3 were the role of TNFα was established by treatment with anti-TNFα antibodies. In chapter 4 we focus on the involvement of substance P and its high affinity receptor NK_1. This role was studied using a specific NK_1 receptor antagonist.

Since mast cell degranulation could be induced by IgLC in non-IgE-mediated hypersensitivity responses, we supposed that IgLC could be involved in this colonic hypersensitivity model. The function of IgLC was explored and established in chapter 5 by treating the mice with a specific antagonist for IgLC.

As described earlier, mast cells and nerves exhibit an anatomical and functional relationship. Furthermore, both cell types have been extensively described and well acknowledged in inflammatory and hypersensitivity responses. We therefore hypothesize a function for IgLC on neurons besides its role on mast cells. In chapter 6 it is demonstrated that IgLC can specifically bind to murine dorsal root ganglion neurons and evoke an increase in intracellular calcium concentrations upon challenge with the corresponding antigen. This indicates that IgLC also plays a role in neuronal antigen-specific activation and reveals a new pathophysiological role. Chapter 7 involves preliminary data and proposes a clinical relevance for IgLC in IBD and irritable bowel syndrome (IBS). Elevated levels of serum IgLC concentrations in IBD and IBS patients compared to healthy control, and localization of IgLC in human intestinal lesions suggest a function for IgLC in these disorders. The main results are summarized and discussed in chapter 8.

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Critical role for mast cells in the pathogenesis of 2,4-dinitrobenzene- induced murine colonic hypersensitivity reaction

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Abstract

The immunological mechanisms underlying the role of mast cells in the pathogenesis of inflammatory bowel disease (IBD) are poorly defined. In this study non-IgE mediated colonic hypersensitivity responses in BALB/c mice induced by skin sensitization with dinitrofluorobenzene (DNFB) followed by an intrarectal challenge with dinitrobenzene sulfonic acid (DNS) featured as a model to study the role of mast cells in the development of IBD. Vehicle- or DNFB-sensitized mice were monitored for clinical symptoms and inflammation 72h after DNS challenge. DNFB-sensitized mice developed diarrheic stool, increased colonic vascular permeability, hypertrophy of colonic lymphoid follicles (colonic patches) and show cellular infiltration at the microscopic level. Increased numbers of mast cells were found in the colon of DNFB-sensitized mice located in and around colonic patches associated with elevated levels of mouse mast cell protease-1 (mMCP-1) in plasma indicating mast cell activation. Colonic patches of DNFB mice, stimulated *in vitro* with stem cell-factor (SCF) indicated that an increase in TNFα levels in the colon is mainly mast cell originated. Finally, neutrophil infiltration was observed in the colon of DNFB-sensitized mice. Induction of this model in mast cell deficient $WBB6F_1$ W/W^v mice shows a profound reduction of characteristics of the colonic hypersensitivity reaction. Reconstitution with bone marrow derived mast cells in $WBB6F_1$ W/W^v mice fully restored the inflammatory response. This study demonstrates the importance of mast cells in the development of clinical symptoms and inflammation in the presented murine model for IBD.

Introduction

Inflammatory bowel disease (IBD) is attended by severe abdominal pain, diarrhea, weight loss, rectal bleeding, increased presence of inflammatory cells and inflammatory products in the gastro-intestinal tract (1-3). The major representatives of chronic IBD are Crohn's disease (CD) and ulcerative colitis (UC). The pathogenesis of IBD is thought to be a complex of interactions between environmental, genetic and immunological factors. The exact etiology and pathophysiology still remains unknown, but an inadequate or prolonged activation of the intestinal immune system plays a crucial role (3). Several pathophysiological features of IBD resemble hypersensitivity-like responses in the gastrointestinal tract (4, 5).

Delayed type hypersensitivity (DTH) is classically characterized by T cell-driven antigen-specific inflammatory cell infiltration within 12-72h after a second encounter with the antigen (6). Originally, B cell-driven and IgE-mediated mast cell degranulation was only associated with immediate-type hypersensitivity reactions (7). However, mast cell degranulation is described to be required for the elicitation of DTH reactions (8). In addition, mast cell degranulation was shown to be involved in a wide range of non-IgE mediated inflammatory processes like multiple sclerosis, tuberculosis, contact dermatitis, non-atopic asthma and also IBD (9). In the gastrointestinal tract, mast cells are distributed throughout all layers of the intestinal wall and numbers increase in intestinal disorders like IBD (10). Not only is there an increased number of mast cells observed in patients with CD and UC (11, 12) but also activation in the inflamed area was found (12). Furthermore, intestinal mast cells in IBD bear a different content compared to normal subjects (10). For example they contain a higher density of the multifunctional, pro-inflammatory cytokine TNFα (13). Nowadays one of the most-promising therapies for IBD is directed against TNFα using monoclonal antibodies (14). These studies suggest a pivotal role for mast cells and its mediators in the pathogenesis of IBD.

The trinitrobenzene sulfonic acid (TNBS)-colitis model is generally accepted as a hapten-induced T cell-mediated immunological model for IBD (15). However, the role of mast cells in this model is contradictory (16, 17). Here we present a consistent novel chemically induced immunological model for colonic IBD associated with mast cell activation. Our non-IgE mediated hypersensitivity model is useful in studying immune-related processes in the course of IBD and resembles several pathophysiological features of IBD.

A hypersensitivity reaction in the colon was elicited with the low molecular weight compound (DNFB) upon skin sensitization followed by a local intrarectal challenge with the corresponding dinitrobenzene sulfonic acid (DNS). The effect of DNFB sensitization and local intrarectal challenge on mast cell activation and infiltration was examined in the hypersensitivity reaction. Furthermore, the influence of mast cells on subsequent reactions like clinical symptoms, tissue damage and cellular infiltration was monitored. Proof of principle of the crucial role for mast cells in this model is demonstrated by the use of genetic mast cell-deficient mice (WBB6F1 *W*/*W*^{v}) and congenic normal mice in this murine model for IBD.

Materials and methods

Animals Male BALB/c mice were obtained from Charles River (Maastricht, The Netherlands). These mice were 6-8 weeks of age and weighed 20-25 grams by time of use. Male mast cell-deficient mice (WBB6F$_1$ *W/W^v*) and their respective normal littermates (WBB6F$_1$ +/+) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). These mast cell-deficient mice were used at 32 weeks of age and the control littermates +/+ were age-matched.
All the animals were housed in groups not exceeding 8 mice per cage. Tap water and chow food were allowed ad libitum at a 12h day-night cycle. All experiments were conducted in accordance with The Animal Care Committee of the Utrecht University (Utrecht, The Netherlands).

Mast Cell Reconstitution Selective reconstitution of mast cells in mast cell-deficient *W/W^v* mice was carried out by the methods earlier described by Kraneveld and coworkers with slight modifications (18). In brief, bone marrow-derived mast cells (BMMC) were obtained from +/+ mice. Bone marrow was aseptically flushed from femurs of +/+ mice and cultured for 4 weeks in RPMI 1640 medium containing 10% FCS, 4 mM 1-glutamine, 0.5 μM β-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.1 mM nonessential aminoacids. Recombinant mouse IL-3 (10 ng/ml) and stem cell factor (SCF) (10 ng/ml) was added to the medium to drive bone marrow cell development to mucosal type mast cells. Medium was refreshed once a week and fresh IL-3 and SCF were added to the medium. Purity of the BMMC population was determined by flow cytometry (c-kit specific). The culture contained a uniform cell population (>90%). Furthermore staining cells with toluidin blue indicated that nearly 99% of the viable cells were mast cells after 4 weeks culture (data not shown). Mast cell-deficient *W/W^v* mice were injected via the tail vein with 5 x 10^6 cultured BMMC cells and the recipients were studied 26 weeks later. Age-matched mast cell-deficient *W/W^v* mice and congenic normal mice were used when examining the BMMC-reconstituted *W/W^v* mice.

Induction of Colonic Hypersensitivity Mice were sensitized on day 0 by application of either DNFB (0.6% in acetone:olive oil 4:1) or vehicle (acetone:olive oil 4:1) epicutaneously on the shaved abdomen (50 μl) and paws (50 μl divided over 4 paws). On day 1 the mice received a boost of DNFB or vehicle on the abdomen only (50 μl). All the animals were challenged intrarectally with DNS (0.6%) dissolved in 10% ethanol on day 5. The sensitization, boost and challenge took place under light inhalation anesthesia (halothane 3%). The mice were macroscopically scored on day 5, 6, 7 and 8 on stool consistency. Thereafter, the mice were sacrificed with an overdose of sodium pentobarbital, to determine *in vivo* mast cell activation, mast cell infiltration into colonic tissue, colonic vascular permeability, cytokine production in the colon, colonic damage and cellular infiltration and presence and number of colonic patches.

Clinical Scoring of the Disease Clinical characteristics of the inflammatory response were obtained by assessing stool consistency. The stool consistency

was scored every day at the same time point by placing the mice separately in cages without bedding. They were left in the cages until they relieved enough feces to establish the consistency till a maximum of 15 minutes to score no stool. The feces were taken out immediately by a spatula and smeared on a piece of cardboard. The scoring was as follows: 0: well-formed solid pellets, 1: easy to smear and loose stool, 2: diarrhea and watery stool, 3: bloody stool and 4: no stool.

Macroscopical Scoring of the Disease After sacrificing the animals 72h after challenge, the colon was carefully dissected from anus till cecum and placed in saline. The colon was opened longitudinally over the mesenteric border and washed gently in saline. It was placed on a rubber mat with the mucosal side up and the number of colonic patches, which appear like bulges in the tissue, was counted with the naked eye.

Determination of Colonic Vascular Permeability Vascular permeability changes were determined as described previously with slight modifications for colon tissue (19). Evans blue (1.25% in sterile saline, 50 µl) was injected i.v. in the tail vein 2h prior to the end of an experiment. After 2h the mice were killed with an overdose of sodium pentobarbital and blood samples were taken via cardiac puncture. 4% EDTA (10% v/v) was added to whole blood to obtain plasma. The colon was excised carefully and opened longitudinally. Feces were removed by gently washing in saline. The colon was dissected free from fat and placed in formamide (500 µl). Evans blue dye was extracted from the colon at 40°C for 24h. The dry weight was determined after drying the colons for 4 weeks at 40°C. The extravasation of Evans blue dye-labeled macromolecules from the blood circulation into the colonic tissues was quantified by measuring the optical density of the plasma samples and formamide extracts on a Benchmark microplate reader (Biorad, California, USA) at a wavelength of 595 nm. Vascular permeability was determined by dividing the total amount of Evans blue extracted from the colon by the concentration of Evans blue in the plasma. The vascular permeability was expressed as µl exudated plasma/mg dry weight of the colon.

Histology and Immunohistochemistry To enable the production of semi thin sections of high quality with preserved morphological characteristics, colonic tissue was embedded in glycol methylacrylate (GMA). After careful dissection of the colon it is placed in saline. The colon was then opened longitudinally over the mesenteric line and feces were removed by gently washing in saline. The colon then was placed with the serosal side up and dissected free from fat. The making of Swiss rolls was accomplished by rolling the colon from the distal to the proximal end. The roll was immediately placed in ice-cold 4% paraformaldehyde in PBS (pH 7.4) and routinely embedded in GMA for further use (20). Serial sections of 3 µm were cut using a microtome (Leica) and routinely stained with H&E to observe damage and cellular infiltration. One observer evaluated all sections and scored according to Hartmann *et al.* (21): for cellular infiltration, rare inflammatory cells in the lamina propria were

counted as 0; increased numbers of inflammatory cells in the lamina propria as 1; confluence of inflammatory cells, extending into the submucosa as 2; and a score of 3 was given to transmural extension of the infiltrate. For tissue damage, no mucosal damage was counted as 0; discrete lympho-epithelial lesions were counted as 1; surface mucosal erosion as 2; and a score of 3 was given to extensive mucosal damage and extension through deeper structures of the bowel wall. The combined histological score ranged from 0 (no changes) to 6 (extensive cell infiltration and tissue damage). Per mouse 3 different longitudinal sections of the colon tissue observed and a score was given to the total of all sections. A chloro-acetate esterase (CAE) staining was used to detect mast cells (22). However, CAE staining also detects some neutrophils. Serial sections stained with a subsequent peroxidase (PO) staining, representative for neutrophils, revealed no overlap between CAE- and PO-positive staining indicating that CAE did not stain neutrophils under our conditions. Per mouse 3 different longitudinal sections of the colon tissue were stained and analyzed. The number of CAE positive cells was quantified by microscopical visualization and manually counting. Results are expressed as median number of cells (minimum-maximum) per colon section. Infiltration of neutrophils was scored as follows: 0. no infiltration, 1. infiltration between the mucosa and submucosa, not around colonic patches, 2. extensive infiltration, especially around colonic patches; clusterformation. Results are expressed as median neutrophils score (minimum-maximum).

To enable immunohistochemical staining procedures of mucosal mast cells colon tissue was embedded in paraffin. Swiss rolls were prepared as described above, fixed in formalin for at least 24h and routinely embedded in paraffin. Sections of 5 μm were deparaffinized and immunohistochemically stained for mouse mast cell protease-1 (mMCP-1). In brief, endogenous peroxidase was blocked with 1.5% H_2O_2 in phosphate-citrate buffer for 30 min. It was washed three times with 0.05 M 0.05% v/v TBST 20. Unspecific binding of the second antibody was blocked with 10% normal goat serum in TBST for 15 min. Thereafter mMCP-1 in colonic tissue was detected with a rat Ig anti-mMCP-1 antibody. This antibody was detected with a secondary biotin-conjugated goat anti-rat Ig specific polyclonal antibody. Steptavidin-horse radish peroxidase (strep-HRP) was used to form a complex with the biotin label. The immunoreaction was visualized with AEC chromogen staining kit (Sigma). The specimens were counterstained with heamatoxylin. The primary and secondary antibodies were diluted in TBST containing 1% normal goat serum. All incubations were carried out at room temperature in a humid chamber and continued for 1h and were followed by a three times washing step with 0.05 M 0.05%v/v TBST. To ensure the preservation of the staining the slides were enclosed in an aqueous mounting medium.

Per mouse 3 different longitudinal sections of the colon tissue were stained and analyzed. The number of mMCP-1 positive cells was quantified by microscopical visualization and manually counting. Results are expressed as median average number of cells (minimum-maximum) per colon section.

Preparation of single cell suspension of colonic patch and FACS analysis The preparation of single cell suspension was adapted from Dohi *et al*. (23). In brief, colonic patch was excised from the intestinal wall and washed once with RPMI. All colonic patches originating from one mouse were pooled. Repeated cell dissociation took place with collagenase at 0.5 mg/ml in RPMI with 100 U/ml penicillin, 100 µg/ml streptomycin, 40 µg/ml gentamicin for 20 min at 37°C using fresh collagenase solution each time. After the dissociation the cells were washed twice with RPMI. Of each single cell suspension 1×10^5 cells were incubated with 1 µg of designated antibody in PBS + 10% FCS for 60 minutes on ice. After incubation the cells were washed three times with PBS. The pellet was resuspended in 200 µl PBS + 10% FCS and analysed using CellQuest (BD Biosciences). PE-conjugated hamster anti-CD3 was used to detect T cells, APC-conjugated rat anti-CD117 was used for the detection of c-kit positive cells (mast cells), PE-conjugated rat anti-B220/CD45 was used to detect B cells and finally PE-conjugated rat anti-CD11c was used to detect dendritic cells.

Preparations of Tissue Homogenates To determine mast cell infiltration into the tissue, whole colon homogenates were made. After sacrificing of the mice the colon was excised carefully and opened longitudinally. Feces were removed by gently washing in saline. The colon was placed in ice cold PBS enriched with protease inhibitors (Complete Mini, Roche) in flat bottom tubes. The tissue was dispersed on ice for 10 sec. according the rotor-stator principle (Ystral®). The homogenates were centrifuged (14,000 rpm, 4°C, 10 min) and the supernatant was frozen until further use to assess mMCP-1 and TNFα levels.

Mast cell Activation and Infiltration in vivo To monitor mast cell activation in time, blood samples of DNFB- and vehicle-sensitized mice were taken 30 min, 24h and/or 72h after intrarectal DNS challenge. Blood samples were collected via heartpunction and 4% EDTA was added (10% v/v) to obtain plasma. After centrifugation the plasma was stored at –70ºC until use. Levels of mMCP-1 in plasma were measured as described previously using a commercially available ELISA assay (18). Results are expressed as ng mMCP-1/ml plasma.
In addition, to determine mast cell infiltration in the colon mMCP-1 levels were measured in the supernatant of colon homogenates with the commercially available ELISA kit. Samples were used undiluted. Total protein content of the supernatant of colon homogenates was quantified with a commercial available detection kit (BCA protein assay, Pierce). Samples were diluted 10-fold for total protein measurements. Results are expressed as ng mMCP-1/mg total protein.

TNFα in colon tissue To determine TNFα levels in vivo TNFα was measured in the supernatant of colon homogenates 72h after DNS challenge. Samples were used undiluted. Total protein content of the supernatant of colon homogenates was quantified with a commercial available detection kit (BCA protein assay, Pierce). Samples were diluted 10-fold for total protein measurements. Results are expressed as pg TNFα/mg total protein.
In addition to *in vivo* TNFα levels, *in vitro* production of TNFα in colonic tissue

and colonic patches was determined. After sacrificing the animals the colon was dissected carefully and opened longitudinally. The feces were removed by gently washing in saline and the colon was placed on a rubber mat with the mucosal side up. With help of a dermal punch instrument colonic patches and pieces of colon tissues of 7 mm^2 were cut and placed in RPMI 1640 medium enriched with 10% FCS, 4 mM 1-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and 25 mM HEPES. The tissues specimens were cultured for 48h at 37°C with 5% CO_2 in the presence of 100 ng/ml stem cell factor or 25 μg/ml αCD3 (clone 17A2). The number of colonic patches was restricted to 3 per well for both treatment groups. After 48h supernatants were harvested and stored at –20°C until further use. TNFα levels in supernatant of colon homogenates and tissue culture samples were determined with a commercially available TNFα ELISA kit (Biosource).

Materials DNFB, olive oil, o-phenylenediamine dihydrochloride (OPD), the AEC chromogen staining kit, normal goat serum and naphthol AS-D chloroacetate (CAE) hematoxylin and eosin were all purchased from Sigma Chemical Co., St. Louis, MO. DNS was purchased from Eastman Kodak, Rochester, NY. Tween 20 was purchased from Janssen Pharmaceutica, Beerse, Belgium. Sodium pentobarbitone was purchased from Sanofi, Maassluis, the Netherlands. RPMI 1640 medium was purchased from Life Technologies, Rockville, MD. Evans blue dye was obtained from Fluka Chemie AG, Munchen, Germany. The rat Ig anti-mMCP-1 antibody was a kind gift of Dr. H.R.Miller, Royal (Dick) School of Veterinary Studies (University of Edinburgh, United Kingdom) and the biotin-conjugated goat anti-rat Ig specific polyclonal antibody was purchased by Pharmigen, Aalst, Belgium. The BCA protein assay was purchased at Pierce, Etten-Leur, The Netherlands. Collagenase IV and complete-mini protease-inhibitors were from Roche Diagnostics, Almere, The Netherlands. The TNFα ELISA kit, IL-3 and SCF were all purchased from Biosource. The mMCP-1 ELISA was from Moredun Scientific Ltd., Midlothian, UK. Maxisorp surface 96 well plates were purchased from Nunc Immuno plate, Roskilde, Denmark. All antibodies for FACS analysis are obtained from Pharmingen, the Netherlands.

Statistical analysis Stool consistency data were analyzed from raw scoring data using a distribution free Kruskal-Wallis ANOVA followed by a Dunn's multiple comparison test. Mast cell numbers, colonic patch count, tissue damage score and neutrophils scoring were all expressed as median (range) and analyzed with the use of a non-parametric test (Mann-Whitney). The following data were analyzed by one-way ANOVA: mMCP-1 content in plasma and colon tissue, and TNFα production in colonic patches. Vascular leakage was tested with a Student's t-test. In figures, group means ± sem are given. $P<0.05$ was considered to be significant. All data manipulations and statistical analysis were conducted by the usage of Graphpad Prism (version 3.0, San Diego, USA).

Results

DNFB induces a colonic hypersensitivity reaction characterized by development of diarrhea, lymphoid structure hypertrophy and increased vascular permeability

The presence of loose stool and diarrhea is indicative for the presence of a damaged colon. DNFB-sensitized animals significantly develop loose stool and diarrhea 72h after DNS challenge compared with normal well-formed pellets in vehicle-treated animals 72h after challenge (figure 1A). 24H after challenge no significant difference between the two treatment groups could be found. This can be attributed to the irritant effect of the 10% ethanol used as a mucosal barrier breaker along with the DNS challenge. Stool consistency improves again in vehicle-sensitized mice after 72h, whereas the stool of DNFB-sensitized animals deteriorates in time after challenge.

Colonic patches are small lymphoid follicles that appear at the mucosal side of the colon. They consist predominantly of B cell zones, but also T cell areas were found (23). Colonic patches are spread non-consistently and differ in size and shape in each single animal. The number of colonic patches differed significantly between the two treatment groups 72h after challenge (table I). DNFB-sensitized animals have an increased number of colonic patches present in the colon compared to vehicle-sensitized animals. In addition, enlarged sizes of colonic patches in DNFB-sensitized mice indicated hypertrophy of these lymphoid structures compared to vehicle-sensitized mice. The distribution of leukocyte population of colonic patch is presented in table II. Even though the distribution of inflammatory cells does not differ between vehicle- and DNFB-sensitized mice 72h after DNS challenge, indicated by percentages in table II, the total cell yield in DNFB-sensitized mice was significantly higher

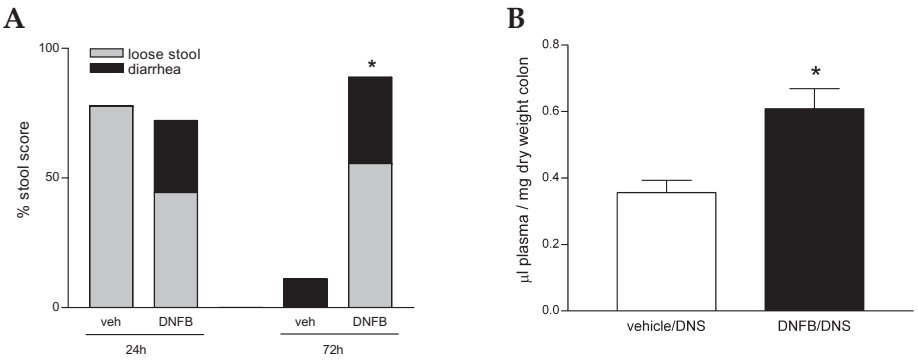

Figure 1 *Macroscopical disease characteristics. (***A***) Stool consistency 24h and 72h after DNS challenge of vehicle- and DNFB-sensitized mice. Score 0: normal well formed pellets, score 1: loose stool/easy to smear, score 2: diarrhea/watery stool. Results are expressed as cumulative percentage of total scored stool (score 0 not shown); *p<0.05, n=9 mice/group (Kruskal-Wallis followed by a Dunn's test). (***B***) Colonic vascular permeability changes measured by Evans Blue leakage from blood into colonic tissue. Open bars represent vehicle-senstized mice and closed bars DNFB-senstized 72h after DNS challenge. Results are expressed as mean µl plasma exudated/mg dry weight colon ± sem; *, p<0.05 compared to vehicle-treated mice, n=6 mice/group.*

than in vehicle-sensitized mice. This demonstrates that the total amount of inflammatory cells (i.e. T cells, B cells and dendritic cells) in colonic patches is significantly increased in DNFB-sensitized mice 72h after challenge. C-kit expression of the leukocyte population in the colonic patch was below detection limit (<1%).
Plasma extravasation quantified by the accumulation of the Evans Blue dye in the colonic tissue is a measurement for changes in vascular permeability. 72H after challenge there was a significant increase in vascular exudation from the blood into colonic tissue of DNFB-sensitized animals compared to vehicle-treated animals (figure 1B).

Table I *Number of colonic patches in BALB/c, mast cell-deficient W/W^v mice, mast cell reconstituted W/W^v mice and control littermates after vehicle or DNFB sensitization 72h after DNS challenge.*

	DNFB sensitization	colonic patches per colon	n	p
BALB/c	-	1.0 (0-3)	6	
	+	3.0 (3-6)	6	p<0.01
+/+ [a]	-	2.0 (1-4)	17	
	+	4.0 (1-5)	15	p<0.001
W/W^v	-	1.0 (0-2)	14	
	+	0.0 (0-2)	20	ns
BMMC→W/W^v	-	0.0 (0-1)	21	
	+	2.0 (0-3)	21	p<0.001

Colonic patches were counted at the mucosal side of colon of vehicle (-) or DNFB (+) sensitized mice. Results are expressed as median number of colonic patches per colon (minimum-maximum); n is number of mice; significant differences between the two treatments within one strain are indicated, ns is not significant (Wilcoxon rank sum test). [a] +/+: control littermates, W/W^v: mast cell-deficient mice and BMMC→W/W^v: BMMC-reconstituted W/W^v mice

Table II *Cell yield of colonic patch and distribution of leukocyte population in colonic patch obtained with FACS analysis.*
*[a] Values are shown as mean total number of cells ± sem, n=4 mice. *p<0.05.*
[b] Values are shown as mean percentage of total cells ± sem, n=4 mice

	vehicle	DNFB
cell yield (10^6 cells/mouse)	1.3 ± 0.1 [a]	2.1 ± 0.2*
T cells (CD3)	8 ± 2 [b]	7 ± 1
Mast cells (CD117)	<1	<1
B cells (B220)	23 ± 4	19 ± 4
Dendritic cells (CD11c)	2 ± 0.2	2 ± 0.2

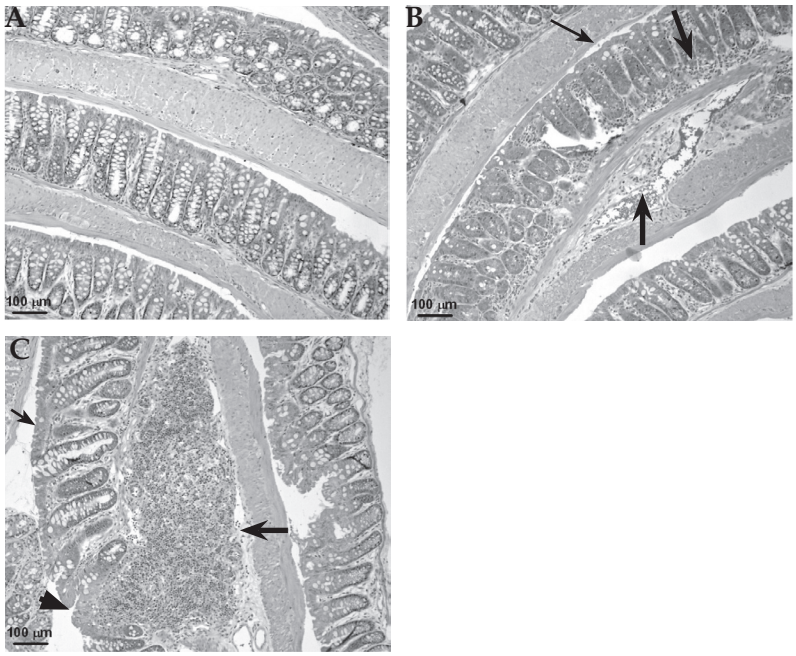

Figure 2 *Cellular infiltration and tissue damage of vehicle-and DNFB-sensitized animals 72h after DNS challenge. H&E staining of 3 µm sections of GMA-embedded colon tissue shows a normal mucosal lining in vehicle-sensitized animals (**A**) and cellular infiltration in submucosa, specifically in and around colonic patches in DNFB-sensitized animals 72h after challenge (**B,C**). Moreover figure B and C show mucosal swelling and enlarged swollen epithelial cells indicating increased mucus production (small arrows). Big arrows indicate abnormal leukocyte clusters and disruption of the mucosa from the submucosa whereas arrowheads indicate the intact epithelial lining respectively.*

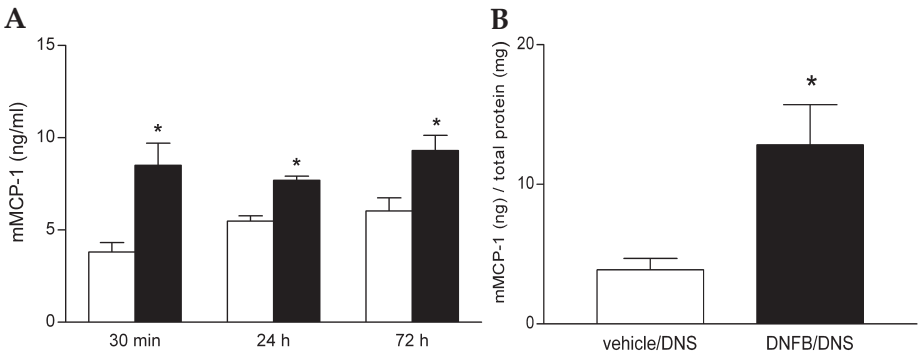

Figure 3 *Mast cell activation and infiltration in the colon of vehicle- and DNFB-sensitized mice after DNS challenge. Open bars represent vehicle-sensitized mice and closed bars DNFB-sensitized after DNS challenge. (**A**) mMCP-1 levels in plasma were assessed 30 min, 24h and 72h after DNS challenge. Results are expressed as mean ± sem; *, p<0.05 compared to vehicle-sensitized animals, n=5-12 mice/group (one-way ANOVA followed by a Bonferonni multiple comparison test). (**B**) Mast cell infiltration into the colon is indicated by changes in mMCP-1 levels in colon homogenates 72h after DNS challenge. Results are expressed as mean ± sem; *, p<0.05, n=5-6 mice/group.*

DNFB-induced colonic hypersensitivity changes colon morphology and induces cellular infiltration

To study cellular infiltration and damage of the colon serial sections of 3 μm of GMA-embedded Swiss rolls were made at three different depths of the tissue leaving at least 100 μm between two series that were stained with a standard H&E staining. DNFB-sensitized mice showed a significant higher damage score compared to vehicle-sensitized mice 72 after DNS challenge (damage score: vehicle: 0 (0-1) & DNFB: 2.5 (1-3); results are expressed as median (minimum-maximum), $p<0.05$ (Mann-Whitney), n=5-6 mice/group, 3 sections/mouse). Figure 2A demonstrates a neatly packed mucosal lining of a vehicle-sensitized mouse 72h after DNS challenge. Mucosal swelling and infiltration of inflammatory cells can be seen in DNFB-sensitized mice 72h after challenge (figure 2B, C; indicated by arrows). This infiltration was particularly seen between the mucosa and the submucosa around colonic patches at the distal end of the colon (figure 2C). Due to this infiltration the colonic tissue is damaged at some locations rupturing the mucosa from the submucosa (figure 2B). Hypertrophy of colonic patches can repulse lamina propia cells, but leaves the epithelial lining, which separates the mucosa from the lumen, intact (arrowhead in figure 2C). Furthermore, epithelial cells are enlarged and swollen in DNFB-sensitized mice 72h after challenge suggesting increased mucus production (small arrows in figure 2B and C).

The peroxidase staining method was used to detect neutrophils. Colonic tissue of DNFB-sensitized animals showed a significantly increased infiltration of these polymorphonuclear cells compared to vehicle-sensitized animals (scoring of neutrophil infiltration: vehicle/DNS: 0 (0-1), DNFB/DNS: 1 (0-2); results are expressed as median (minimum-maximum); $p<0.05$, n=5-6 mice/group, 3 sections/mouse).

DNFB-induced hypersensitivity is associated with mast cell activation and increased mast cell numbers in vivo

Mouse mast cell protease-1 (mMCP-1) is a protease specific for mouse mucosal mast cells and appears in the bloodstream after activation of mast cells (24). To assess mast cell activation mMCP-1 levels were determined in plasma 30 min, 24h and 72h after challenge. A significant rise in plasma mMCP-1 levels

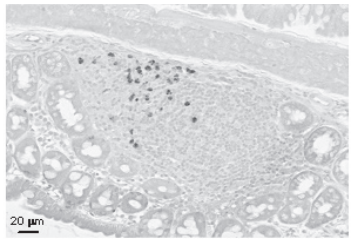

Figure 4 *Localization of mast cells in and around colonic patches 72h after DNS challenge in a DNFB-sensitized mouse. Chloroacetate esterase staining (CAE) on 3 μm sections of GMA-embedded colon tissue.*

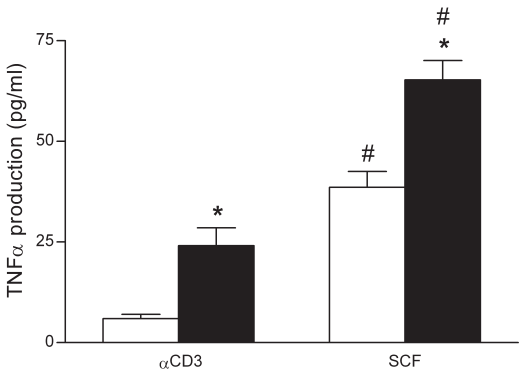

Figure 5 *TNFα production in colonic patches obtained from vehicle- or DNFB-sensitized mice 72h after DNS challenge. Colonic patches were stimulated ex vivo with SCF or αCD3 for 48h. Open bars represent colonic patches from vehicle-sensitized mice and closed bars colonic patches from DNFB-sensitized mice after DNS challenge. Results are expressed as mean ± sem per well; *, p<0.05 compared to vehicle-senstized mice; #, p<0.05 compared to αCD3-stimulated colonic patches; n=4-6 mice/group.*

was observed in DNFB-sensitized animals 30 min, 24h and 72 h after challenge compared to vehicle-sensitized mice (figure 3A). A significant increase in mMCP-1 in colon tissue of DNFB-sensitized animals, measured in supernatant of colon homogenates, was found suggesting infiltration and/or proliferation of mast cells into the colon has taken place 72h after challenge (figure 3B). An increase in mast number can be found throughout the whole colon, but predominantly at the distal end of the colon. In addition, clusters of mast cells are located around colonic patches as pictured by CAE staining in figure 4. Also the number of CAE-positive mast cells was increased after DNFB-sensitization and DNS challenge compared to vehicle-sensitized and DNS challenged mice (mast cell number per colon: vehicle/DNS 16 (6-22), DNFB/DNS 28 (22-36); results are expressed as median (range); $p<0.05$, n=6).

TNFα is one of the major cytokines released upon mast cell activation. Therefore we have determined TNFα levels in colon of vehicle and DNFB-sensitized mice 72h after challenge. Colonic tissue TNFα levels were significantly increased in DNFB-sensitized mice *in vivo* compared to vehicle-sensitized mice 72h after challenge (vehicle: 22.849 ± 2.203 & DNFB: 39.867 ± 4.399 pg/mg; results are expressed as mean TNFα (pg)/total protein (mg) ± sem; n=10-11 mice; $p<0.01$). To investigate whether colonic mast cells are the major source for TNFα colonic tissue specimens and colonic patches were collected and cultured in the presence of the specific mast cell activator SCF for 48h. SCF is known as the c-kit ligand. On mature myeloid cells c-kit is only expressed by mast cells (25). Previously Wershil et al. demonstrated that stimulation of the c-kit receptor by SCF leads to activation and degranulation of the mast cell (26). Figure 5 demonstrates that SCF-stimulated colonic patches obtained from DNFB-sensitized mice 72h after DNS challenge produce significantly more TNFα *in vitro* than colonic patches from vehicle-sensitized animals. However, mast cells are not the only source for TNFα in hypersensitivity-like responses. TNFα is also produced and secreted by T lymphocytes. Therefore a general T-cell stimulus, αCD3, was used to activate T cells in colonic patch and colon tissue *in vitro*. As shown in figure 5 TNFα levels were significantly lower compared to SCF-stimulated colonic

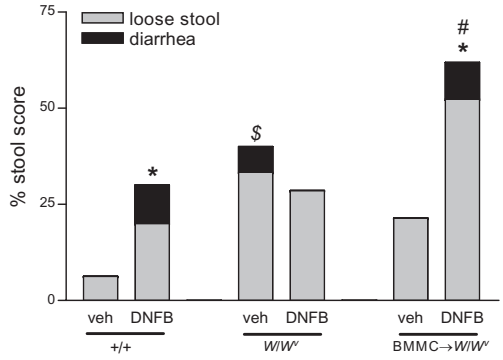

Figure 6 *Stool consistency in vehicle- and DNFB-sensitized mast cell-deficient mice, control littermates and mast cell-reconstituted mast cell-deficient mice 72h after DNS challenge. Score 0: normal well formed pellets, score 1: loose stool/easy to smear, score 2: diarrhea/watery stool. +/+: control littermates, W/Wv: mast cell-deficient mice, BMMC→W/Wv: BMMC-reconstituted W/Wv mice. Results are expressed as cumulative percentage of total scored stool (score 0 not shown); *p<0.05 compared to vehicle-sensitized mice within the same strain, n=12 mice/group; #p<0.1 compared to DNFB-sensitized +/+ and W/Wv mice, n=12 mice/group; $p<0.05 compared to vehicle-sensitized +/+ mice.*

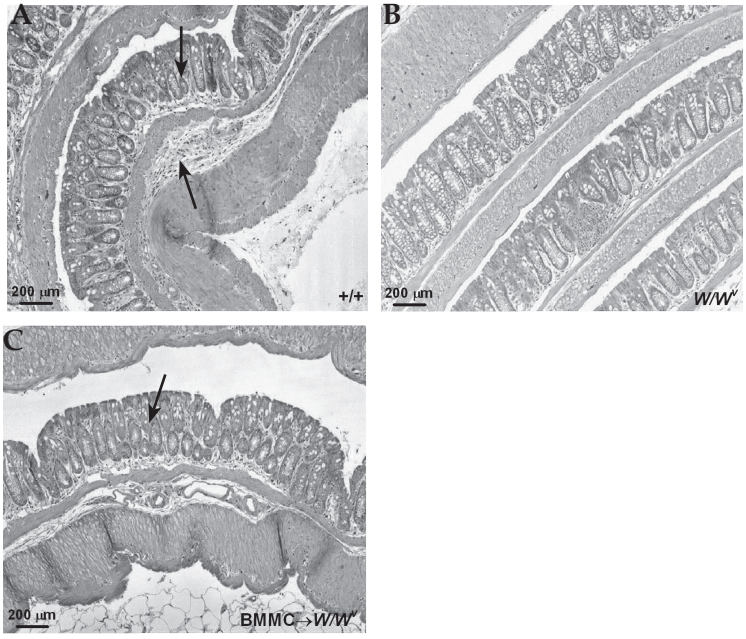

Figure 7 *Cellular infiltration and tissue damage of DNFB-sensitized mast cell-deficient mice, control littermates and mast cell-reconstituted mast cell-deficient mice 72h after DNS challenge. H&E staining of 3 μm sections of GMA-embedded colon tissue shows a normal mucosal lining in mast cell-deficient mice (**B**) and cellular infiltration and damage of colon tissue in both control littermates and BMMC-reconstituted W/Wv mice (**A,C**). Arrows indicate abnormal leukocyte clusters and disruption of the mucosa from the submucosa. +/+: control littermates, W/Wv: mast cell-deficient mice, BMMC→W/Wv: BMMC-reconstituted W/Wv mice.*

patch for both treatment groups. In comparison, supernatants of cultures from colon tissue deprived of colonic patches stimulated with either SCF or αCD3 did not show significant differences in TNFα production between the two treatment groups and both stimuli (data not shown).

Mast cells play a key role in the development of DNFB-induced colonic hypersensitivity

To assess the functional role of mast cells, several features of this hapten-induced model were studied in mast-cell deficient mice and their normal littermates. Reconstitution of bone marrow-derived mast cells in *W/W^v* served as control to confirm that the lack of functional mast cells was responsible for the failure to induce colonic hypersensitivity. The +/+ littermates and *W/W^v* mice were age-matched with the BMMC→*W/W^v* mice.

DNFB-sensitized mast cell deficient animals do not develop increased levels of loose stool and diarrhea whereas +/+ and BMMC→*W/W^v* do (figure 6). Vehicle-sensitized *W/W^v* mice have deteriorated stool consistency compared to DNFB-sensitized *W/W^v* animals. The number of colonic patches was significantly increased in DNFB-sensitized +/+ and BMMC→*W/W^v* mice compared to *W/W^v* (table I).

Damage and cellular infiltration studied at histological level was scored on GMA-embedded colon tissue (table III). Leukocyte infiltration in DNFB-sensitized +/+ was observed comparable to previously seen in BALB/c mice (figure 7A). *W/W^v* mice failed to establish such an inflammatory feature upon DNS challenge after DNFB-sensitization (figure 7B). This response however, could be restored after BMMC reconstitution (figure 7C).

In addition, in +/+ mice a significant rise in plasma mMCP-1 levels was observed in DNFB-sensitized mice 72h after challenge (figure 8A). No significant mMCP-1 levels in plasma could be detected in *W/W^v* mice confirming the fact that these animals do not have functional mast cells. The ability to detect mMCP-1 in vehicle-sensitized BMMC→*W/W^v* indicates that reconstitution of *W/W^v* with BMMC was successful. However, a difference between the two treatment groups was absent in BMMC→*W/W^v* mice. As is shown in figure 8B minor levels of mMCP-1 in supernatant of colon homogenates could be detected in either vehicle- or DNFB-sensitized *W/W^v* and BMMC→*W/W^v*

Table III *Tissue damage score in GMA-embedded colon tissue 72h after challenge. Colon sections were routinely stained with heamatoxylin and eosin and scored for cell infiltration and tissue damage. Results are expressed as median (minimum-maximum) damage score; n=3-4 mice, 3 sections/mouse. Significant differences between the two treatments within one strain are indicated, ns is not significant.*

	DNFB sensitization	damage score	
+/+ [a]	-	0 (0-1)	
	+	1.5 (1-3)	p<0.05
W/W^v	-	0 (0-1)	
	+	1 (0-1)	ns
BMMC→*W/W^v*	-	0 (0-1)	
	+	1 (1-3)	p<0.05

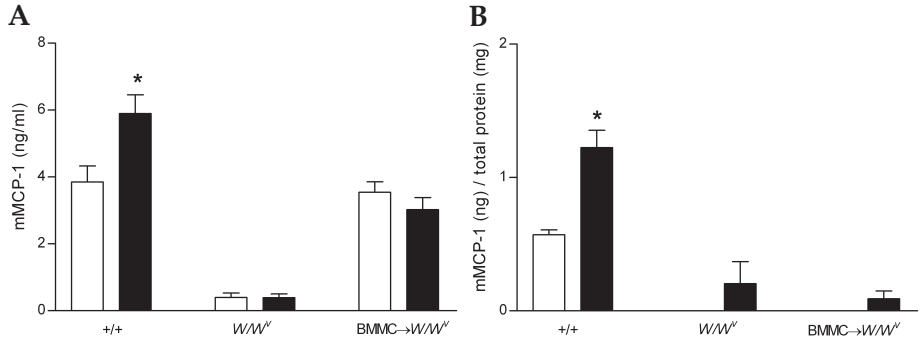

Figure 8 *Mast cell activation and infiltration in DNFB-sensitized mast cell-deficient mice, control littermates and mast cell-reconstituted mast cell-deficient mice 72h after DNS challenge. Open bars represent vehicle-senstized mice and closed bars DNFB-senstized after DNS challenge. +/+: control littermates, W/Wv: mast cell-deficient mice, BMMC→W/Wv: BMMC-reconstituted W/Wv mice. (**A**) mMCP-1 levels measured in plasma 72h after DNS challenge. Results are expressed as mean *, p<0.05 compared to vehicle-sensitized animals, n=12 mice/group. (**B**) mMCP-1 levels in colon homogenates indicate mast cell infiltration into the colon 72h after challenge. Results are expressed as mean ± sem; *, p<0.05, n=12 mice/group.*

mice. In +/+ mice however a significant difference could be detected between vehicle- and DNFB-sensitized mice. The results observed in supernatant of colon homogenates of either vehicle- or DNFB-sensitized BMMC→*W/W^v* mice corresponds with the finding of no significant differences in mMCP-1 levels in plasma in these animals.

Immunohistochemistry demonstrates a significant increase in number of CAE-positive mast cells in +/+ DNFB-sensitized animals 72h after challenge. Moreover, the number of CAE-positive mast cells is enhanced in BMMC→*W/W^v* DNFB-sensitized mice 72h after DNS challenge (table IV). However this increase is not significant compared to vehicle-sensitized BMMC→*W/W^v* mice due to a large variability. An immunohistochemical staining for mMCP-1 was performed for in depth investigation of mast cells after reconstitution. This staining specifically stains mucosal mast cells. As can be seen in table IV this immunohistochemical staining shows a similar profile as the mMCP-1 plasma levels of +/+, *W/W^v* and BMMC→*W/W^v* mice. A statistical significant difference in mMCP-1 positive mast cells could only be detected in DNFB-sensitized +/+ mice compared to vehicle-sensitized mice 72h after challenge. After mast cell reconstitution similar numbers of mMCP-1 positive cells were found in both vehicle- and DNFB-sensitized BMMC→*W/W^v* mice compared to vehicle-sensitized +/+ mice. However, no differences between the two treatment groups were present.

Even though the mMCP-1 and the CAE staining show the same profile in mast cell numbers, the mMCP-1 staining was capable of detecting more mast cells in the mouse colon. This discrepancy in mast cell numbers between the mMCP-1 and CAE staining can be explained by differences in staining methods.

Table IV *Number of CAE-positive and mMCP-1 positive mast cells in mast cell-deficient W/Wv mice, mast cell reconstituted W/Wv mice and control littermates after DNFB or vehicle sensitization 72h after DNS challenge.*

	DNFB sensitization	CAE staining			mMCP-1 staining		
		mast cells	n	p	mast cells	n	p
+/+ [a]	-	9 (5-16)	4		50 (44-71)	4	
	+	19 (17-24)	3	p<0.05	156 (120-136)	4	p<0.05
W/W^v	-	4 (3-5)	4		21 (15-27)	4	
	+	5 (3-7)	4	ns	27 (23-37)	5	ns
BMMC→W/W^v	-	6 (2-15)	4		56 (24-97)	4	
	+	6 (5-43)	4	ns	52 (21-145)	5	ns

Mast cells are counted after CAE or mMCP-1 staining. Per mouse 3 different longitudinal sections were counted and averaged. Results are expressed as median average number of CAE or mMCP-1 positive cells per colon section (minimum-maximum); n is number of mice; significant differences between the two treatments within one strain are indicated, ns is not significant.

[a] *+/+: control littermates, W/W^v: mast cell-deficient mice and BMMC→W/W^v: BMMC-reconstituted W/W^v mice*

Lower numbers of mast cells in the CAE staining could be a result of the loss of enzymatic activity as a result of the process of embedding and staining. Moreover, it could be possible that the mast cell contains less chloroacetate esterase than mMCP-1 resulting in a less intense staining.

Discussion

Several lines of evidence strongly suggest that mast cells are involved in the pathogenesis of IBD (10). The presence of increased numbers of mast cells and its mediators can be found in the mucosa of patients with IBD (13, 27). Because the gastro-intestinal tract is exposed to exogenous agents entering the body by intestinal bacteria and via food intake a constant activation of the immune system occurs. Mast cells are cells involved in recognition of the parasitic nematodes, food allergens, enterotoxigenic bacteria and invading pathogens and can therefore be found in a constitutively activated state in the gastro-intestinal tract (28). Breaking the balance between pro-inflammatory and anti-inflammatory players may cause exacerbation or remission of IBD. Profound mast cell activation may be a key event in the pathophysiology of IBD.

In the present experiments we describe a novel murine model for IBD and examine the putative role of mast cells in this model. Cutaneous sensitization with the low-molecular weight hapten DNFB, followed by an intrarectal challenge with DNS resulted in hypersensitivity responses in the colon. This DTH-like reaction is classically known as a non-IgE mediated reaction. Induction of DTH reactions with DNFB has shown to lead to successful models for contact dermatitis, non-atopic asthma and small intestinal inflammation

(18, 29, 30). An acute response occurs within 6h after challenge and is a result of direct mast cell activation and degranulation. The cell-mediated response takes place 24-72h after challenge. Increased plasma levels of mMCP-1 of DNFB-sensitized compared to vehicle-sensitized mice were present at 30 min, 24h and 72h after the challenge, indicating that mast cell activation occurs in this model.

The lack of acute effect (<6h) on stool consistency could be a result of a masking effect of ethanol, used here to break the mucosal barrier of epithelial cells. Ethanol is known to cause local irritation. Although often used in the TNBS-colitis model as vehicle, it was recently shown that 50% ethanol can induce colitis by itself and prevent oral tolerance (31). In our model 10% ethanol is used as a mucosal barrier breaker. However, histological examination and stool consistency prove that an irritant effect of ethanol is still present at 24h (previous observations by authors; data not shown). The irritant effect of ethanol seems to have faded 72h after challenge and DNFB-sensitized mice suffered from diarrhea. Therefore this time point was chosen to study colonic hypersensitivity reactions.

A prominent role in the pathogenesis of IBD can be attributed to the pro-inflammatory cytokine TNFα because increased levels can be found in inflamed tissue of IBD patients (13, 32). Significantly increased TNFα levels could be found in colon of DNFB-sensitized mice 72 after DNS challenge. SCF-induced TNFα production *in vitro* by colonic patches obtained from DNFB-sensitized mice 72h after DNS challenge, together with the observation of increased mast cell infiltration in colonic patches, strongly suggests that activated mast cells located in and around colonic patches may be an important source of TNFα. Furthermore, hypertrophy of colonic patches was previously established by Dohi et al. (23). They showed that after induction of TNBS-colitis those lymphoid follicles were thicker and contained an increased number of T- and B-lymphocytes. This is in correspondence with the hypertrophy of colonic patches after DNFB-sensitization and DNS challenge observed in our study (table II).

Besides mast cells c-kit is also expressed by interstitial cells of Cajal (ICC) in the gastro-intestinal tract (33). These cells can be addressed as the pacemaker cells of the gut and are responsible for the peristaltic movement. Until today no reports have been published indicating cytokine production by ICC. Therefore these cells are not likely to be a source for TNFα. Mast cells however, are not the only immune cells responsible for the release of TNFα and this pro-inflammatory cytokine is also produced by T lymphocytes (34). To investigate the origin of TNFα further we also *in vitro* stimulated colonic patches from DNFB-sensitized mice 72h after DNS challenge with the general T cell-activator αCD3 and measured TNFα in supernatant. And although T cells are more abundantly present than mast cells in the colonic patch, the observed significantly higher TNFα levels in colonic patch after *in vitro* SCF stimulation compared to αCD3-stimulated colonic patch suggests that TNFα produced by mast cells is quantatively more important than T cell-derived TNFα in this model. Furthermore, the observation of mast cell infiltration and

activation in and around colonic patches by immunohistochemistry suggests communication and/or interaction between mast cells and lymphocytes, both cell types involved in hypersensitivity responses. The interaction between mast cells and T cells has been shown to be bi-directional accomplishing regulatory and modulatory roles (35). Activated T cells are capable of inducing mast cell activation and degranulation (36) and of inducing cytokine and chemokine production (37), both as a result of physical cell-to-cell contact. This suggests that TNFα measured in supernatant of αCD3-stimulated colonic patches could also be derived from mast cells, which are indirectly activated by T lymphocytes.

More definite evidence for the functional role of mast cells in this murine model for colonic hypersensitivity was obtained by studies in mast cell-deficient *W/W^v* mice. The characteristic features for colonic hypersensitivity failed to establish in DNFB-sensitized mast cell-deficient *W/W^v* mice after DNS challenge. Reconstitution with *in vitro* cultured bone marrow-derived mast cells in *W/W^v* mice resulted in the appearance of a number of features like diarrhea, hypertrophy of colonic patches, mast cell infiltration and damage of colon tissue, similar to results obtained in control +/+ mice after DNFB sensitization and DNS challenge. However, even though a rise in mMCP-1 levels was present in BMMC→*W/W^v*, indicating that the reconstitution was successful, there was no significant difference between DNFB- and vehicle-sensitized mice. Furthermore, only minor mMCP-1 levels could be detected in supernatant of colon homogenates in both treatment groups of BMMC→ *W/W^v* mice as well as *W/W^v* mice. A plausible explanation could be that after reconstitution a different type of mast cell appears in the intestinal mucosa and that mMCP-1 is not a correct marker for mast cell activation in reconstituted animals. In addition, histological examination of mast cells present in the mucosa showed difference in morphology, size and granular density between +/+ and BMMC reconstituted *W/W^v* mice (personal observation). Galli (38) stressed that appropriate studies should be done to assess the number, phenotype and distribution of mast cells in BMMC recipients. Therefore, an additional mMCP-1 immunohistochemical staining was performed. This staining showed an equivalent profile of mast cell numbers as the mMCP-1 levels in plasma. This supports the observation that mMCP-1 might not be the correct marker for mast cell activation and infiltration in reconstituted mast-cell deficient mice. It is beyond the scope of this study to further investigate and identify the content of reconstituted mast cells *in vivo*.

Although significant cellular infiltration was seen in the colon of both DNFB-sensitized +/+ mice as BMMC-reconstituted W/Wv mice (figure 7A and 7C), a small lymphoid structure was also observed in the mucosa of W/Wv (figure 7B). This can be addressed as a cryptopatch, which appear in the mucosa throughout the whole gastro-intestinal tract (39, 40). These cryptopatches do not exhibit organized lymphoid tissue like Peyer's patches and colonic patches, but contain progenitors cells for intra-epithelial lymphocytes (IEL) (41). Cryptopatches are known to be the extrathymic source for the almost the whole IEL population in the murine intestine (39). It could be suggested

that these structures develop into colonic patches or serve as a source for the inflammatory cell infiltration and the hypertrophy of colonic patches.

Stool consistency of vehicle-sensitized mast cell-deficient W/W^v mice is deteriorated compared to vehicle-sensitized +/+ animals. This is probably due to the fact that W/W^v mice also have a mutation in ICC (42). It is evident that alterations in amount or function of these pacemaker cells could lead to differences in stool consistency. In BMMC→W/W^v mice the absence of ICC could contribute to the observation that the stool consistency of DNFB-sensitized animals is even more deteriorated compared to DNFB-sensitized +/+ controls. In BALB/c mice the presence of diarrhea and the number of colonic patches is of higher prevalence than in +/+ mice. These differences can be attributed to strain differences.

Conflicting studies exist about the role of mast cells in low molecular weight molecule-induced models for experimental colitis. Fukumoto et al (43) demonstrated that mast cells are not essential in the development of TNBS-induced rat model. Rectal installment of TNBS in 50% ethanol in mast cell-deficient W^s/W^s rats led to the same inflammatory conditions of the colon as seen in control W^+/W^+ littermates. Similar studies of TNBS in 50% ethanol-induced colitis in mast-cell deficient W/W^v mice showed the same results (16). Contradictory, Xu et al (17) describes an important role for mast cells in intestinal inflammation and fibrosis in TNBS in 50% ethanol-induced colitis in mice (44). Furthermore, ketotifen, an anti-histaminic drug with mast cell stabilizing properties, can attenuate the severity of TNBS-colitis (45). An explanation for these conflicting results can be found in the use of 50% ethanol, used as vehicle in the TNBS-colitis model to break the mucosal barrier. As mentioned before, 50% ethanol is capable of inducing an inflammation by itself (31). This damage-induced inflammation by ethanol is mast cell-independent and most likely masks the hapten-specific response induced by TNBS. This makes the TNBS model an unreliable model to study the role of mast cells in IBD.

The mechanism by which mast cells are activated in our model is currently under investigation. It has been suggested that upon sensitization with a hapten or other causative agents, B-lymphocytes are stimulated to produce an antigen specific lymphocyte factor. This factor has recently been demonstrated to be immunoglobulin-free light chain (IgLC) (29). Free IgLC can bind to mast cells and a subsequent second contact with the corresponding antigen (challenge) can lead to mast cell activation (29, 46, 47). This recent finding sheds a new light on the involvement of mast cells in non-atopic diseases, like IBD. Purified antigen-specific IgLC are capable of passively immunizing naive mice. Intranasal challenge resulted in acute bronchoconstriction associated with plasma leakage and mast activation (47), whereas epicutaneous challenge on the ears lead to a profound ear swelling typical for a dermal hypersensitivity reaction (29). These earlier observations in both lung and skin suggest similar mechanisms in this newly described colonic hypersensitivity model. Current investigations are started to prove this hypothesis and to establish a role for IgLC in this model.

In conclusion, although the role for mast cells is controversial in animal models for IBD this is, to our knowledge, the first study to show an important consistent role for mast cells in colonic hypersensitivity. Our findings are confirmatory for the hypothesis of the essential function for mast cells in the cellular cascade leading to an antigen-induced hypersensitive response as postulated earlier (18, 48). The presented chemically-induced immunological model for IBD can be used to develop potential therapies targeting the mast cell as an important source for the IBD-cytokine TNFα.

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TNFα is crucial for the development of mast cell-dependent colitis in mice

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Abstract

Inflammatory bowel disease (IBD) describes chronic inflammatory conditions of the gastro-intestinal tract and tumor necrosis factor alpha (TNFα) plays a pivotal role in mediating the response. TNFα is a multifunctional pro-inflammatory cytokine rapidly released by mast cells after degranulation. In the present study we hypothesized TNFα to be an important player in our recently described mast cell-dependent murine model for IBD. The effect of neutralizing anti-TNFα antibodies (clone XT 22) was studied on colonic hypersensitivity in mice induced by skin application of dinitrofluorobenzene (DNFB) followed by intrarectal challenge with dintrobenzene sulfonic acid (DNS). Features of this colonic hypersensitivity response include diarrhea, mast cell infiltration and activation, infiltration of inflammatory cells in the colon, colonic patch hypertrophy and increased mast cell-derived TNFα levels in the colon. Anti-TNFα antibodies could effectively abrogate diarrhea in DNFB-sensitized mice 72h after challenge. Moreover, the number of colonic patches and total tissue damage score were reduced by anti-TNFα antibody treatment in DNFB-sensitization 72h after challenge. However, mast cell infiltration and activation remained unaffected by neutralizing anti-TNFα antibodies. Treatment with the corticosteroid dexamethasone, a frequently used therapeutic treatment in IBD, resulted in the reduction of diarrhea, cellular infiltration and total tissue damage score in the same extent as anti-TNFα antibodies. Additionally, dexamethasone treatment could also reduce total TNFα levels in the colon, mast cell number and mast cell activation in both vehicle- and DNFB-sensitized mice 72h after challenge. These findings suggest that TNFα can play an instrumental role in causing inflammatory responses in the present murine model for IBD downstream from mast cell activation.

Introduction

Tumor necrosis factor alpha (TNFα) is a pro-inflammatory cytokine with a wide variety of biological functions in inflammation like tissue remodeling, alteration of epithelial barrier permeability, increasing vascular permeability, activation of macrophages, recruitment of inflammatory cells and up-regulation of adhesion molecules (1-3). TNFα can be produced and released by activation by monocytes, macrophages, T cells and mast cells (2, 4). More importantly, mast cells are the only cell type capable of storing pre-synthesized TNFα in the granules together with *de novo* synthesis of this cytokine (5). Indeed, during IgE-mediated allergic responses and bacterial infection mast cells secrete TNFα within minutes after challenge (6, 7) This unique ability of mast cells provides evidence that mast cells are the only readily available source of TNFα in the early onset of infection and inflammation in peripheral tissues.

As a major mediator of inflammation, TNFα is of importance in (auto)immune diseases like inflammatory bowel disease (IBD) and increased levels can locally be found at site of inflammation (8, 9). IBD is a chronic inflammatory disorder of the gastro-intestinal tract and TNFα was shown to be elevated in blood, stool and intestinal tissue of IBD patients (10-12). Not only are increased numbers of mast cells found in mucosa of IBD patients both at inflamed and non-inflamed areas (13, 14), the mast cell has also been described to be an important source for TNFα in the human intestine (3). This was further confirmed by the observation that mast cells in IBD patients contain a higher density of TNFα and increased expression of TNFα mRNA (3, 15). Moreover, therapy with infliximab, an IgG_1 murine/human chimeric monoclonal anti-TNFα antibody, in patients suffering from Crohn's disease and ulcerative colitis, the major representatives of IBD, was shown to be effective in suppressing the inflammatory response (1, 9).

In our laboratory we have developed and characterized a chemically-induced immunological murine model for colonic hypersensitivity. Hypersensitivity is evoked by skin sensitization of mice with the low molecular weight compound 2,4-dintrofluorbenzene (DNFB) followed by local intrarectal challenge with the hapten. Features of this colonic hypersensitivity model are diarrhea, hypertrophy of lymphoid structures, recruitment of inflammatory cells and infiltration and activation of mast cells in the colon (16). In this model TNFα levels in colon tissue were shown to be elevated after the induction of colonic hypersensitivity. Moreover, the increase of TNFα in colonic lymphoid structures was demonstrated to be mainly of mast cell origin (16). To investigate the importance of TNFα for the development of colonic hypersensitivity we carried out anti-TNFα antibody (Ab) treatment in this murine model. Treatment with the corticosteroid dexamethasone was conducted to assess therapeutic efficacy in DNFB-induced colonic inflammation. Consecutive treatment with both anti-mouse TNFα Ab and dexamethasone resulted in improved stool consistency, reduced infiltration of inflammatory cells and decreased hypertrophy of colonic lymphoid structures after DNFB sensitization and local challenge with the hapten. Mast cell infiltration and activation, and total TNFα levels in the colon were not affected by treatment with anti-TNFα Ab. Dexamethasone, however, could significantly reduce mast cell numbers, mast cell activation,

and total TNFα levels in both vehicle- and DNFB-sensitized mice 72h after challenge. These findings demonstrate that TNFα plays an important role in this colonic hypersensitivity model downstream from mast cell activation.

Materials and methods

Animals Male BALB/c mice were obtained from Charles River (Maastricht, The Netherlands). The mice were 6-8 weeks of age and weighed 20-25 grams by time of use. The animals were housed in groups not exceeding 8 mice per cage. Tap water and chow food were allowed ad libitum; there was a 12h day-night cycle. All experiments were conducted in accordance with The Animal Care Committee of the Utrecht University (Utrecht, The Netherlands).

Induction of Colonic Hypersensitivity Mice were sensitized on day 0 by application of either DNFB (0.6% in acetone:olive oil 4:1) or vehicle (acetone:olive oil 4:1) epicutaneously on the shaved abdomen (50 µl) and paws (50 µl divided over 4 paws). On day 1 the mice received a boost of DNFB or vehicle on the abdomen only (50 µl). All the animals were challenged intrarectally with DNS (0.6%) dissolved in 10% ethanol on day 5. The sensitization, boost and challenge took place under light inhalation anesthesia (halothane 3%). The mice were macroscopically scored on day 5, 6, 7 and 8 for stool consistency. Thereafter, the mice were sacrificed with an overdose of sodium pentobarbital, to determine *in vivo* mast cell activation, mast cell infiltration into colonic tissue, colonic vascular permeability, cytokine production in the colon, colonic damage and cellular infiltration and presence and number of colonic patches.

Treatment regimes Both vehicle- and DNFB-sensitized mice were treated with i.p. with 0.45 mg/mouse anti-TNF antibody (clone XT 22, rat IgG_1; purified in our laboratory) or rat control IgG 10 min prior to and 24h and 48h after challenge. This concentration of anti-TNFα Ab was previously shown to be successful in different murine models for experimental intestinal inflammation (17-19).
Dexamethasone treatment was accomplished in vehicle- and DNFB-sensitized mice with i.p. administration of 10 mg/kg 10 min prior to and 24h and 48h after challenge. PBS served as control for dexamethasone. This concentration of dexamethasone was been shown to be successful in a whole variety of animal models and species (17, 20).

Clinical Scoring of the Disease Clinical characteristics of the inflammatory response were obtained by assessing stool consistency. The stool consistency was scored every day at the same time point by placing the mice separately in cages without bedding. They were left in the cages until they relieved enough feces to establish the consistency till a maximum of 15 minutes to score no stool. The feces were taken out immediately by a spatula and smeared on a piece of cardboard. The scoring was as follows: 0: well-formed solid pellets, 1: easy to smear and loose stool, 2: diarrhea and watery stool, 3: bloody stool and 4: no stool.

Macroscopical Scoring of the Disease After sacrificing the animals 72h after challenge, the colon was carefully dissected from anus till cecum and placed in saline. The colon was opened longitudinally over the mesenteric border and washed gently in saline. It was placed on a rubber mat with the mucosal side up and the number of colonic patches, which appear like bulges in the tissue, was counted with the naked eye.

Histology and Immunohistochemistry After careful dissection the colon is placed in saline. The colon was then opened longitudinally over the mesenteric line and feces were removed by gently washing in saline. The colon then was placed with the serosal side up and dissected free from fat. The making of Swiss rolls was accomplished by rolling the colon from the distal to the proximal end. The roll was immediately placed in ice-cold 4% paraformaldehyde in PBS (pH 7.4) for at least 24h and routinely embedded in paraffin for further use. Serial sections of 5 μm were cut using a microtome (Leica) and routinely stained with H&E to observe damage and cellular infiltration. Per mouse 3 different longitudinal sections of the colon tissue were stained and microscopically analyzed. Pictures shown are representatives of the different treatment groups.
To detect mast cells 5 μm sections were immunohistochemically stained for mouse mast cell protease-1 (mMCP-1) as described previously (16).
Per mouse 3 different longitudinal sections of the colon tissue were stained and analyzed. The number of mMCP-1 positive cells was quantified by microscopical visualization and manually counting. Results are expressed as median average number of cells (minimum-maximum) per colon section.

Preparations of Tissue Homogenates To determine mast cell infiltration into the tissue, whole colon homogenates were made. After sacrificing of the mice the colon was excised carefully and opened longitudinally. Feces were removed by gently washing in saline. The colon was placed in ice cold PBS enriched with protease inhibitors (Complete Mini) in flat bottom tubes. The tissue was dispersed on ice for 10 sec. according the rotor-stator principle (Ystral©). The homogenates were centrifuged (14,000 rpm, 4°C, 10 min) and the supernatant was frozen until further use to assess mMCP-1 and TNFα levels by ELISA.

Mast cell Activation and Infiltration in vivo To monitor mast cell activation in time, blood samples of DNFB- and vehicle-sensitized mice were taken 30 min, 24h and/or 72h after intrarectal DNS challenge. Blood samples were collected via heartpunction and 4% EDTA was added (10% v/v) to obtain plasma. After centrifugation the plasma was stored at –70ºC until use. Levels of mMCP-1 in plasma were measured as described previously using a commercially available ELISA assay (21). Results are expressed as ng mMCP-1/ml plasma.
In addition, to determine mast cell infiltration in the colon mMCP-1 levels were measured in the supernatant of colon homogenates with the commercially available ELISA kit. Samples were used undiluted. Samples were diluted 10-fold for total protein measurements (BCA protein assay). Results are expressed as ng mMCP-1/mg total protein.

TNFα levels in vivo To determine TNFα levels *in vivo*, TNFα was measured in the supernatant of colon homogenates 72h after DNS challenge with a commercially available ELISA kit. Samples were used undiluted. Total protein content of the supernatant of colon homogenates was quantified with a commercial available detection kit (BCA protein assay). Samples were diluted 10-fold for total protein measurements. Results are expressed as pg TNFα/mg total protein.

Materials DNFB, olive oil, the AEC chromogen staining kit, normal goat serum, rat control IgG, heamatoxylin and eosin were all purchased from Sigma Chemical Co., St. Louis, MO. DNS was purchased from Eastman Kodak, Rochester, NY. Tween 20 was purchased from Janssen Pharmaceutica, Beerse, Belgium. Sodium pentobarbitone was purchased from Sanofi, Maassluis, The Netherlands. Anti-TNF monoclonal antibodies were purified from the XT22 hybrodoma. Dexamethasone was purchased from Intervet, Boxmeer, The Netherlands. The rat Ig anti-mMCP-1 antibody was a kind gift of Dr. H.R.Miller, Royal (Dick) School of Veterinary Studies (University of Edinburgh, United Kingdom) and the biotin-conjugated goat anti-rat Ig specific polyclonal antibody was purchased by Pharmigen, Aalst, Belgium. The BCA protein assay was purchased at Pierce, Etten-Leur, The Netherlands. Complete-mini protease-inhibitors were from Roche Diagnostics, Almere, The Netherlands. The mMCP-1 ELISA was from Moredun Scientific Ltd., Midlothian, UK. Maxisorp surface 96 well plates were purchased from Nunc Immuno plate, Roskilde, Denmark. The TNFα ELISA kit was purchased from Biosource, Nivelles, Belgium. Maxisorp surface 96 well plates were purchased from Nunc Immuno plate, Roskilde, Denmark.

Statistics Stool consistency data were analyzed from raw scoring data using a distribution free Kruskal-Wallis test followed by a Dunn's multiple comparison test. Tissue damage score, mast cell and colonic patch numbers were all expressed as median (range) and also analyzed with the use of a distribution free Kruskal-Wallis test followed by a Dunn's multiple comparison test. The following data were analyzed by two-way ANOVA and a Bonferroni multiple comparison test: mMCP-1 content in plasma and colon tissue, and TNFα levels in colon tissue. In figures, group means ± sem are given. $P<0.05$ was considered to be significant. All data manipulations and statistical analysis were conducted by the usage of Graphpad Prism (version 3.0, San Diego, USA).

Results

Treatment with anti-TNFα Ab and corticosteroids can abolish DNFB-induced colonic hypersensitivity

In order to investigate the role of TNFα in colonic hypersensitivity, previously shown to be associated with diarrhea, cellular infiltration and mast cell infiltration and activation (16), treatment with anti-TNFα Ab was conducted in the presented experiments. Administration of the corticosteroid dexamethasone in colonic hypersensitivity was used as reference treatment.

The formation of diarrhea is indicative for a disturbed intestinal homeostasis and can be caused by inflammation or infection and is therefore an important feature of IBD. Figure 1 demonstrates that DNFB sensitization followed by intrarectal challenge with DNS leads to significantly deteriorated stool consistency in mice treated with the control compounds 72h after challenge. However, treatment with both anti-TNFα Ab (figure 1A) and dexamethasone (figure 1B) could completely abolish the diarrhea formation in DNFB-sensitized mice 72h after challenge.

Colonic patches are small lymphoid follicles that appear on the mucosal site of the colon and are easily visible. An increase in visible colonic patches is indicative for hypertrophy of these structures (16, 22). 72h after challenge the number of colonic patches was significantly increased in DNFB-sensitized mice treated with the control Ab as well as PBS compared to vehicle-sensitized mice subjected to the same treatment protocol (table I). Treatment with anti-TNFα and dexamethasone could significantly decrease the quantity of visible colonic patches to a number not significantly different from vehicle-sensitized mice exposed to the same treatment protocol (table I) indicating that cellular recruitment to and/or proliferation of these lymphoid structures was affected by this treatment.

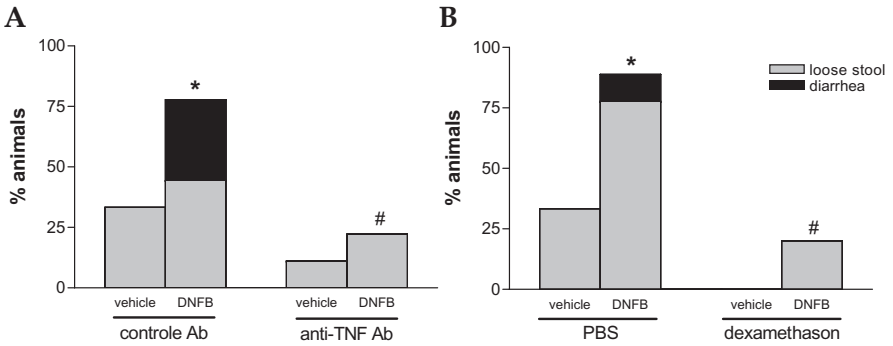

Figure 1 *Anti-TNα treatment profoundly inhibits diarrhea found in DNFB-sensitized and DNS-challenged mice. Stool consistency 72h after challenge of vehicle- and DNFB-sensitized mice treated with anti-TNFα Ab (**A**) or dexamethasone (**B**). Score 0: normal well formed pellets, score 1: loose stool/easy to smear (grey bars), score 2: diarrhea/watery stool (black bars). Results are expressed as cumulative percentage of total scored stool (score 0 not shown); *p<0.05 compared to vehicle-sensitized mice subjected to the same treatment, #p<0.05 compared to DNFB-sensitized mice treated with control compound, n=9-10 mice/group.*

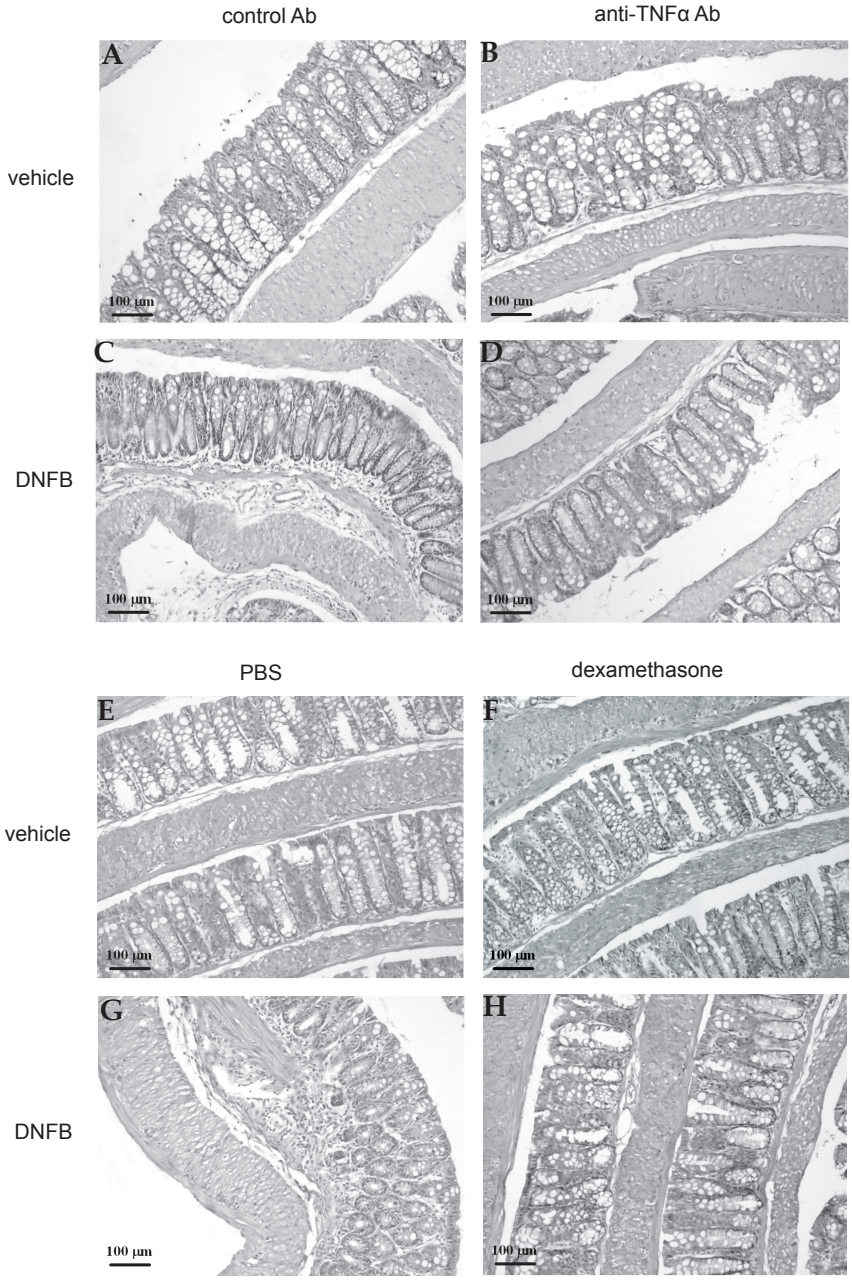

Figure 2 *Anti-TNFα and dexamethasone treatment inhibits cellular infiltration and tissue damage found in DNFB-sensitized and DNS-challenged mice. Representative samples of HE stained colon tissue of vehicle-sensitized animals treated with control Ab (**A**), anti-TNFα Ab (**B**), PBS (**E**) or dexamethasone (**F**) show normal intact colon structure. Cellular infiltration in the colon of DNFB-sensitized mice 72h after challenge treated with control Ab (**C**) or PBS (**G**) could almost completely be abolished by treatment with anti-TNFα Ab (**D**) or dexamethasone (**H**).*

This was confirmed by the observation that tissue damage and cellular infiltration, combined in total damage score, could significantly be reduced by treatment with anti-TNFα and dexamethasone in DNFB-sensitized mice 72h after challenge (table I). Figure 2 shows representative pictures of colon tissue of vehicle (figure 2A and 2B) and DNFB-sensitized (figure 2C and 2D) mice treated with either control Ab (figure 2A and 2C) or anti-TNFα Ab (figure 2B and 2D). Infiltration of inflammatory cells could be observed in control Ab-treated DNFB-sensitized mice 72h after challenge (figure 2C). This cellular infiltration could significantly be attenuated by treatment with the anti-TNFα antibody (figure 2D and table I). Dexamethasone treatment also led to significant inhibition of cellular infiltration into the colon (figure 2E-H and table I). Infiltration of inflammatory cells in PBS-treated DNFB-sensitized mice 72h after challenge (figure 2G) could also be completely abolished by treatment with dexamethasone (figure 2H). The colonic lining of vehicle-sensitized mice was not affected by treatment with either PBS or dexamethasone (figure 2E and 2F).

DNFB-induced mast cell infiltration and activation could be inhibited by dexamethasone, but not by anti-TNFα Ab treatment

Mucosal mast cells contain mMCP-1 in the granules and this protein was visualized by specific immunohistochemical staining. The number of mMCP-1-postive cells was counted and the results are expressed in table I. As this table demonstrates the number of mast cells present in the colon was not affected by anti-TNFα Ab treatment in DNFB-sensitized mice 72h after challenge. This is in agreement with the total mMCP-1 levels in the supernatant of colon homogenates. Administration of anti-TNFα Ab did not have an effect on mMCP-1 levels in the colon in DNFB-sensitized mice 72h after challenge (figure 3A). However, treatment of DNFB-sensitized mice with

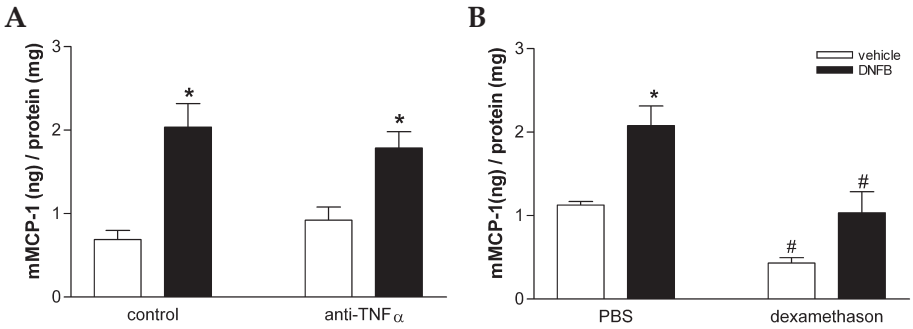

Figure 3 *Mast cell numbers in the colon of DNFB-sensitized and DNS-challenged mice are unaffected by anti-TNFα treatment. Vehicle- and DNFB-sensitized mice treated with anti-TNFα Ab (**A**) or dexamethasone (**B**) 30 min and 72h after DNS challenge. Mast cell infiltration is indicated by changes in mMCP-1 levels in supernatant of colon homogenates 72h after DNS challenge. Results are expressed as mean ± sem; *p<0.05 compared to vehicle-sensitized mice subjected to the same treatment; #p<0.05 compared to PBS-treated mice subjected to the same sensitization protocol; n=6-7 mice/group.*

Table I *Effect of neutralizing anti-TNF antibodies or corticosteroids by dexamethasone treatment on number of colonic patches, mast cell numbers in the colon and total tissue damage score in vehicle and DNFB-sensitized mice 72h after challenge.*

treatment	DNFB	colonic patch [a]	tissue damage score [b]	mast cells [c]
control Ab	-	4 (3-5)	0 (0-1)	11 (5-20)
	+	6.5 (5-8)*	2 (1-3)*	62 (32-99)*
anti-TNFα Ab	-	4 (3-5)	0 (0-1)	11 (1-27)
	+	5 (4-5)#	1 (0-2)#	58 (27-87)*
PBS	-	3.5 (3-6)	0 (0-1)	10 (6-27)
	+	6.5 (5-8)*	2 (1-4)*	142 (58-222)*
dexamethasone	-	2 (0-5)	0 (0-1)	7.5 (2-20)
	+	3 (1-5)#	0 (0-1)#	32 (8-55)#

[a] *Colonic patches were counted at the mucosal side of the colon of vehicle (-) or DNFB (+) sensitized mice. Results are expressed as median number of colonic patches per colon (minimum-maximum); n = 9-10 mice.*

[b] *Colon tissue was scored for cellular infiltration and tissue damage after HE staining. Per mouse 3 section of colon tissue were examined at three different longitudinal depths leaving at least 100 μm in between. A score was given to the total appearance of the colon. Results are expressed as median tissue damage score (minimum-maximum); n = 3 mice.*

[c] *Mast cells were counted after mMCP-1 staining. Per mouse 3 different longitudinal sections were counted and averaged. Results are expressed as median average number of mMCP-1 positive cells per colon section (minimum-maximum); n = 3 mice.*

**p<0.05 compared to vehicle-sensitized mice subjected to the same treatment protocol, #p<0.05 compared to DNFB-sensitized mice treated with control compound.*

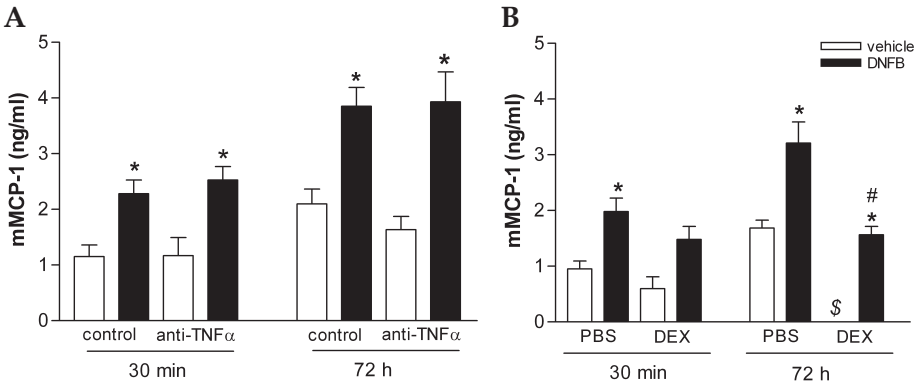

Figure 4 *Mast cell activation after DNS challenge in DNFB-sensitized mice is unaffected by anti-TNFα treatment. Vehicle- and DNFB-sensitized mice treated with control anti-TNFα Ab (**A**) or dexamethasone (**B**) 72h after DNS challenge. mMCP-1 levels in plasma were assessed 30 min and 72h after DNS challenge. Open bars represent vehicle-sensitized mice, and closed bars represent DNFB-sensitized mice. Results are expressed as mean ± sem; *p<0.05 compared to vehicle-sensitized mice subjected to the same treatment, #p<0.05 compared to DNFB-sensitized mice treated with the control compound, $p<0.05 compared to vehicle-sensitized mice treated with the control compound; n=9-10 mice/group.*

dexamethasone did result in significantly decreased levels of mMCP-1 in the colon 72h after challenge compared to PBS-treated DNFB-sensitized mice (figure 3B). Indeed, dexamethasone treatment also significantly decreased total mMCP-1 levels in the colon of vehicle-sensitized mice 72h after challenge (figure 3B). Immunohistochemical staining also demonstrated significantly reduced mMCP-1-positive cells (table I).
After activation and degranulation of the mast cell mMCP-1 is secreted into the bloodstream (23). Increased mMCP-1 levels in plasma are indicative for mast cell activation. Both 30 min and 72h after the challenge mMCP-1 levels in plasma are significantly increased in DNFB-sensitized mice compared to vehicle-sensitized mice (figure 4). Treatment with anti-TNFα Ab did not affect mMCP-1 levels in plasma of vehicle- and DNFB-senstized mice after challenge at both time-points (figure 4A).
In DNFB-sensitized mice treated with dexamethasone no significant differences in mMCP-1 plasma levels 30 min after challenge could be observed compared to PBS-treated DNFB-sensitized mice (figure 4B). 72 H after challenge, dexamethasone treatment did cause significantly decreased mMCP-1 levels in both vehicle- and DNFB-sensitized mice compared to PBS-treated mice subjected to the same sensitization protocol (figure 4B).

Anti-TNFα Ab treatment did not influence total TNFα levels in the colon
As described previously, TNFα in the colon was demonstrated to be increased and mainly derived from mast cells in our murine colitis model (16). TNFα measured in supernatant of colon homogenates is indicative for the total TNFα present in the colon. Figure 5 demonstrates that total TNFα levels were

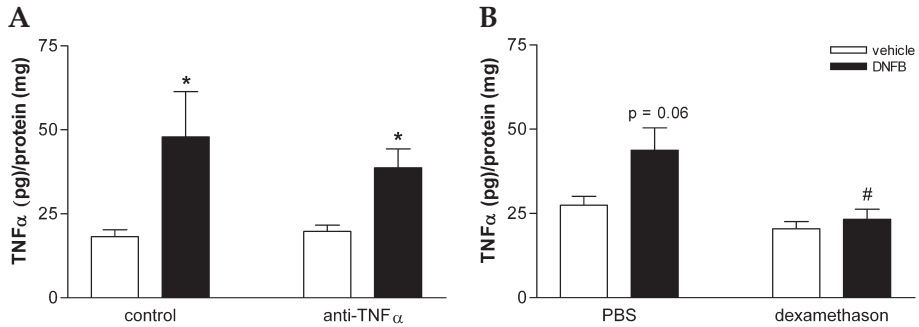

Figure 5 *Anti-TNFα treatment does not have an effect on total TNFα levels in the colon of DNFB-sensitized and DNS-challenged mice. Total TNFα levels in colon of vehicle- and DNFB-sensitized mice treated with anti-TNFα Ab (**A**) or dexamethasone (**B**) 72h after DNS challenge. TNFα levels are measured in supernatant of colon homogenates and correlated with total protein levels of the supernatant of colon homogenates. Results are expressed as mean TNFα (pg)/protein (mg) ± sem; *p<0.05 compared to vehicle-sensitized mice subjected to the same treatment; #p<0.05 compared to DNFB-sensitized mice subjected to the control treatment; n=6-7 mice/group.*

significantly enhanced in DNFB-sensitized mice subjected to treatment with the control Ab and could not be affected by treatment with anti-TNFα Ab 72h after challenge. Even though the total TNFα levels in DNFB-sensitized mice compared to vehicle-sensitized mice both treated with PBS barely reached statistical significance (p=0.06; figure 5B) dexamethasone treatment resulted in significantly decreased total TNFα levels in the colon of DNFB-sensitized mice 72h after challenge (figure 5B).

Discussion

TNFα is a pro-inflammatory cytokine known to be of importance in IBD. Patients suffering from IBD show increased levels of TNFα in the circulation, intestinal tissue at the site of inflammation and stool (10-12). Moreover, current therapeutic strategies in IBD by neutralization of TNFα show beneficial effects (9). Intestinal mast cells are suggested to be an important source for TNFα in both Crohn's disease and ulcerative colitis (3, 15). In the current study we propose a role for TNFα in a murine colitis model associated with mast cell infiltration and activation (16). To further validate this model for translational medicine from animal models to the human situation, treatment with neutralizing anti-TNFα antibodies was conducted. Our data presented here indicate that treatment with anti-TNFα could ameliorate the severity of the disease by reducing the development of diarrhea and infiltration of inflammatory cells. This occurred to the same extent as treatment with the corticosteroid dexamethasone.

TNFα has shown to be an important mediator of diarrhea in human IBD patients and rodent experimental colitis (2, 24, 25). Mast cells can rapidly release pre-stored TNFα upon activation (6). Counteracting the effect of TNFα by the use of specific antibodies contributes to improved stool consistency after induction of colitis.

Mast cell-derived TNFα is known to function as chemoattractant of leukocytes to the site of inflammation in hypersensitivity reactions (26). The mechanism of TNFα to recruit inflammatory cells to the site of inflammation can be subscribed to its ability to upregulate the expression of cellular adhesion molecules (CAM) like ICAM-1, VCAM-1, and P- and E-selectin (27) and increased expression of CAM can also be found in experimental and human IBD (28, 29). Blockade of TNFα was shown to inhibit CAM expression of endothelial cell and therefore suppress the migration of leukocytes (30, 31). This corresponds with our observation that treatment with anti-TNFα antibodies led to significantly reduced cellular infiltration into the colon and colonic damage. Furthermore, the reduced number of colonic patches observed in mice receiving anti-TNFα treatment suggests that TNFα is also involved in the enlargement of these lymphoid structures. This corresponds with the finding of McLachlan *et al*. who demonstrated that peripheral mast cells could induces the hypertrophy of draining lymph nodes via the rapid secretion of TNFα (4).

The total level of mMCP-1 in the colon is indicative for the total number of mast cells present in the colon. Treatment with anti-TNFα antibodies does

not result in reduced infiltration and proliferation of mast cells to the colon as determined by total mMCP-1 levels as well as immunohistochemical staining of mMCP-1 in the colon. Mast cell growth and proliferation is described to depend mainly on stem cell factor, but also several other factors including interleukin (IL)-3, -4, -9 and -10, but not TNFα (32, 33). Treatment with anti-TNFα antibodies will therefore not affect the increase of mast cell number provoked by the induction of colitis. Moreover, the effect of anti-TNFα treatment takes place downstream from mast cell activation since mMCP-1 levels could still be detected in plasma both 30 min and 72 h after challenge. This is supported by the observation that the increased TNFα levels in the colon during colitis after treatment with anti-TNFα are unaffected. In conclusion, it can be stated that total mast cell numbers and TNFα levels in the colon are not influenced by treatment with anti-TNFα antibodies and that the beneficial effect of this treatment is exerted downstream of mast cell activation.

As reference treatment the effect of the corticosteroid dexamethason was studied to determine the therapeutic efficacy of anti-TNFα in the DNFB-induced colitis model. Corticosteroids, like dexamethason and prednisone, are frequently used as treatment in IBD. They show beneficial immunosuppressive effects by inhibition of the production of pro-inflammatory cytokines and by reducing the upregulation of adhesion molecules (34, 35). The data obtained in the present study demonstrates that treatment with dexamethason in this colitis model also resulted in improved stool consistency, decreased cellular infiltration and reduced number of colonic patches.

Furthermore, Soda et al. demonstrated that dexamethasone can destruct mast cells without degranulation and therefore abolish the induction of inflammatory responses (36). In addition, it was also shown that dexamethasone suppresses antigen-induced FcεRI-mediated mast cell activation (37). This is in agreement with our observations that dexamethason was capable of reducing the total number of mast cells and inhibiting mast cell activation both in vehicle- and DNFB-sensitized mice. This finding together with reduced TNFα levels after dexamethasone treatment supports the proposal that TNFα is originated from mast cells in this colitis model. Furthermore, this strengthens the results obtained after treatment with anti-TNFα antibodies and sustains the suggestion that the beneficial effect of the anti-TNFα treatment occurs posterior from mast cell activation.

Because of the passage of a wide variety of pathogens (i.e. luminal microflora, food allergens, parasitic nematodes) the gastro-intestinal tract is constantly immuno-active. The immunosuppressive observations in vehicle-sensitized mice treated with dexamethason indicate that dexamethason can affect the constitutively activated state of the gastro-intestinal tract.

Taken together, we demonstrated in this study that TNFα is important for the development of mast cell-associated colitis in mice. Further validation of our recently described chemically-induced immunological model for IBD shows that this model is useful to study translational medicine from animal models to the human situation.

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4

Beneficial effect of neurokinin-1 receptor antagonism in the development of hapten-induced colitis in mice

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Abstract

The gastro-intestinal tract is highly innervated by both intrinsic and extrinsic sensory nerves and this neuronal component is thought to play a role in local inflammatory responses. This in vivo study was designed to determine the function of substance P and the neurokinin-1 (NK_1) receptor in the pathogenesis of inflammatory bowel disease (IBD) by the use of the specific antagonist RP 67580. The dinitrofluorobenzene (DNFB)-induced colonic hypersensitivity model is associated with increased levels of substance P in the colon. The NK_1 receptor antagonist RP 67580 was used to investigate the role of substance P on the development of diarrhea, mast cell infiltration and activation, colonic tissue damage, hypertrophy of colonic lymphoid structures and leukocyte infiltration. The formation of watery diarrhea could completely be abrogated by treatment with RP 67580 in DNFB-sensitized animals 72h after challenge. Antagonizing the NK_1 receptor in these animals also resulted in significantly reduced colonic patch hypertrophy, leukocyte recruitment and tissue damage. Total levels of substance P in the colon of DNFB-sensitized mice treated with the inactive enantiomer of the NK_1 receptor antagonist (RP 68651) were significantly higher compared to DNFB-sensitized mice treated with RP 67580 72h after challenge. Although RP 67580 was capable of reducing the total number of mast cells present in the colon, mast cell activation was not affected by this treatment. In conclusion, in this chemically-induced immunological model for IBD we demonstrated an important role for NK_1 receptors, and its ligand substance P, in the development of colitis downstream from mast cell activation.

Introduction

Substance P, neurokinin A and neurokinin B are the major representatives of the tachykinin mammalian neuropeptide family involved in non-adrenergic non-cholinergic (NANC) neurotransmission (1, 2). Biological effects of tachykinins are mediated trough specific receptors belonging to the G-protein-coupled receptors to promote signal transduction (1). Substance P binds with the highest affinity to neurokinin-1 (NK_1) receptor, whereas neurokinin A has the highest affinity for the NK_2 receptor and neurokinin B for the NK_3 receptor (3). Tachykinins are considered as key mediators in the communication between neurons (in particular sensory neurons) and effector cells (smooth muscle, glands and immune cells) (4).

In the gastro-intestinal tract substance P and neurokinin A are found in both intrinsic enteric neurons and extrinsic primary afferent neurons (5). Intrinsic neurons are located in the myenteric plexus and innervate all the layers of the intestinal wall whereas extrinsic neurons originate from dorsal root ganglia and mainly project to the longitudinal and circular muscle of the intestine (5, 6). They control both neuronal and non-neuronal transmission within the enteric nervous system (7). Substance P, released from NANC nerves after stimulation, interacts with NK_1 receptors on postjunctional neurons or effector cells which participate in the control of motility, vascular permeability changes, epithelial ion transport, and immune function in the gastro-intestinal tract (1). There is abundant evidence that infection and inflammation of the gastro-intestinal tract is associated with changes in neuronal activation of the gut (7). Inflammatory bowel disease (IBD) is a chronic relapsing idiopathic inflammatory disease of the gastrointestinal tract and is characterized by swings between intestinal inflammation and quiescence (8). This leads to long-term and mostly irreversible damage to the gastrointestinal structure and therefore impairment of the intestinal function (9). Increased levels of substance P has been found in human IBD and in rat models for experimental colitis during inflammation (10, 11). Furthermore, upregulated expression of NK_1 receptors was observed in inflamed and non-inflamed regions of the intestine of patients suffering from IBD (3, 12) and of rat colon (13, 14) associated with disease activity (15).

Functional expression of NK_1 receptor on lymphocytes, dendritic cells, macrophages, neutrophils, eosinophils and mast cells (1, 16, 17) and the localization of these cells in close proximity of NANC nerves (8) could indicate bi-directional communication between the immune system and the central nervous system. The interaction between mast cells and NANC neurons during inflammation has been well described and shown to be dependent on substance P (18-20). Mast cells are versatile cells capable of synthesizing and storing a large variety of pro-inflammatory mediators like histamine, serotonin, prostaglandins, tryptases, cytokines and neuropeptides (21-23). Increased numbers of mast cells can be found in the mucosa of both inflamed and non-inflamed intestinal tissue of IBD patients (24, 25) and substance P is capable of inducing histamine secretion of human mucosal mast cells *in vitro* (26).

Therefore, we propose that substance P and the NK_1 receptor play a role in the interaction between mast cells and sensory nerves in the pathogenesis of IBD. In the presented experiments this was studied by treatment with the specific NK-1 receptor antagonist RP 67580 and its inactive enantiomer RP 68651 in a murine model for colonic hypersensitivity associated with diarrhea, colonic patch hypertrophy, mast cell proliferation and activation, and infiltration of inflammatory cells (27). Colonic hypersensitivity was induced by skin application with the low molecular weight compound dinitrofluorobenzene (DNFB) followed by an intrarectal challenge with the hapten. Blockage of the NK_1 receptor resulted in improved stool consistency and reduced hypertrophy of lymphoid structures, decreased numbers of mast cells in the colon and inhibited cellular infiltration, suggesting a prominent role for tachykinins and its receptors in hapten-induced colonic hypersensitivity responses.

Materials & methods

Animals Male BALB/c mice were obtained from Charles River (Maastricht, The Netherlands). The mice were 6-8 weeks of age and weighed 20-25 grams by time of use. The animals were housed in groups not exceeding 8 mice per cage. Tap water and chow food were allowed ad libitum; there was a 12h day-night cycle. All experiments were conducted in accordance with The Animal Care Committee of the Utrecht University (Utrecht, The Netherlands).

Induction of Colonic Hypersensitivity Mice were sensitized on day 0 by application of either DNFB (0.6% in acetone:olive oil 4:1) or vehicle (acetone:olive oil 4:1) epicutaneously on the shaved abdomen (50 µl) and paws (50 µl divided over 4 paws). On day 1 the mice received a boost of DNFB or vehicle on the abdomen only (50 µl). All the animals were challenged intrarectally with DNS (0.6%) dissolved in 10% ethanol on day 5. The sensitization, boost and challenge took place under light inhalation anesthesia (isoflurane 3%). The mice were macroscopically scored on day 5, 6, 7 and 8 on stool consistency. Thereafter, the mice were sacrificed with an overdose of sodium pentobarbital, to determine *in vivo* mast cell activation, mast cell infiltration into colonic tissue, colonic vascular permeability, cytokine production in the colon, colonic damage and cellular infiltration and presence and number of colonic patches.

Treatment regime Both vehicle and DNFB sensitized mice were treated i.v. with 100 µl 10^{-9} M RP 67580 (NK_1 receptor antagonist) or RP 68651 (inactive enantiomer) 10 minutes prior to and 1, 24, 48 and 70 after DNS challenge. This concentration of RP 67580 and RP 68651 has previously shown to be effective in inhibiting NK_1 receptor-mediated responses in BALB/c mice in mouse ileum, trachea and skin (28, 29).

*Clinical Scoring of the Disease*Clinical characteristics of the inflammatory response were obtained by assessing stool consistency. The stool consistency was scored every day at the same time point by placing the mice separately in cages without bedding. They were left in the cages until they relieved enough

feces to establish the consistency till a maximum of 15 minutes to score no stool. The feces were taken out immediately by a spatula and smeared on a piece of cardboard. The scoring was as follows: 0: well-formed solid pellets, 1: easy to smear and loose stool, 2: diarrhea and watery stool, 3: bloody stool and 4: no stool.

Macroscopical Scoring of the Disease After sacrificing the animals 72h after challenge, the colon was carefully dissected from anus till cecum and placed in saline. The colon was opened longitudinally over the mesenteric border and washed gently in saline. It was placed on a rubber mat with the mucosal side up and the number of colonic patches, which appear like bulges in the tissue, was counted with the naked eye.

Histology and Immunohistochemistry After careful dissection the colon is placed in saline. The colon was then opened longitudinally over the mesenteric line and feces were removed by gently washing in saline. The colon then was placed with the serosal side up and dissected free from fat. The making of Swiss rolls was accomplished by rolling the colon from the distal to the proximal end. The roll was immediately placed in ice-cold 4% paraformaldehyde in PBS (pH 7.4) for at least 24h and routinely embedded in paraffin for further use. Serial sections of 5 μm were cut using a microtome (Leica) and routinely stained with H&E to observe damage and cellular infiltration. Per mouse 3 different longitudinal sections of the colon tissue were stained and microscopically analyzed. Pictures shown are representatives of the different treatment groups.
To detect mast cells 5 μm sections were immunohistochemically stained for mouse mast cell protease-1 (mMCP-1) as described previously (27). Per mouse 3 different longitudinal sections of the colon tissue were stained and analyzed. The number of mMCP-1 positive cells was quantified by microscopical visualization and manually counting. Results are expressed as median average number of cells (minimum-maximum) per colon section.

Preparations of Tissue Homogenates To determine mast cell infiltration into the tissue, whole colon homogenates were made. After sacrificing of the mice the colon was excised carefully and opened longitudinally. Feces were removed by gently washing in saline. The colon was placed in ice cold PBS enriched with protease inhibitors (Complete Mini, Roche) in flat bottom tubes. The tissue was dispersed on ice for 10 sec. according the rotor-stator principle (Ystral©). The homogenates were centrifuged (14,000 rpm, 4°C, 10 min) and the supernatant was frozen until further use to assess mast cell infiltration by means of measuring mMCP-1 levels. Total protein content of the supernatant of colon homogenates was quantified with a commercial available detection kit (BCA protein assay, Pierce).

Mast cell Activation and Infiltration in vivo To monitor mast cell activation in time, blood samples of DNFB- and vehicle-sensitized mice were taken 30 min, 24h and/or 72h after intrarectal DNS challenge. Blood samples were collected

via heartpunction and 4% EDTA was added (10% v/v) to obtain plasma. After centrifugation the plasma was stored at –70ºC until use. Levels of mMCP-1 in plasma were measured as described previously using a commercially available ELISA assay (30). Results are expressed as ng mMCP-1/ml plasma.
In addition, to determine mast cell infiltration in the colon mMCP-1 levels were measured in the supernatant of colon homogenates with the commercially available ELISA kit. Samples were used undiluted. Samples were diluted 10-fold for total protein measurements. Results are expressed as ng mMCP-1/mg total protein.

Quantitative measurement of substance P A commercial available substance P EIA kit was used to measure substance P levels in supernatant of colon homogenates. The EIA was performed according to the manufacturer's instructions. In brief, coated plates were incubated with primary antisera (rabbit-anti SP). Standards and samples were used undiluted and measured in duplicate. Biotinylated substance P was added to the wells to compete with substance P in samples for binding to first antibody. Biotinylated peptides were detected with streptavidin-HRP, followed by TMB substrate. Absorbance was measured at 450 nm and represents the inverse of substance P concentration present. Results are expressed as pg substance P/mg total protein.

Materials DNFB, olive oil, the AEC chromogen staining kit, normal goat serum, heamatoxylin and eosin were all purchased from Sigma Chemical Co., St. Louis, MO. DNS was purchased from Eastman Kodak, Rochester, NY. Tween 20 was purchased from Janssen Pharmaceutica, Beerse, Belgium. Sodium pentobarbitone was purchased from Sanofi, Maassluis, The Netherlands. RP67580 and RP68651 were kindly provided by Sanofi Aventis, Vitry-sur-Seine, France. The rat Ig anti-mMCP-1 antibody was a kind gift of Dr. H.R.Miller, Royal (Dick) School of Veterinary Studies (University of Edinburgh, United Kingdom) and the biotin-conjugated goat anti-rat Ig specific polyclonal antibody was purchased by Pharmigen, Aalst, Belgium. The BCA protein assay was purchased at Pierce, Etten-Leur, The Netherlands. Complete-mini protease-inhibitors were from Roche Diagnostics, Almere, The Netherlands. The mMCP-1 ELISA was from Moredun Scientific Ltd., Midlothian, UK. Maxisorp surface 96 well plates were purchased from Nunc Immuno plate, Roskilde, Denmark. The substance P EIA kit was purchased from Peninsula Laboratories Inc., Meyerside, UK.

Statistics Stool consistency data were analyzed from raw scoring data using a distribution free Kruskal-Wallis ANOVA followed by a Dunn's multiple comparison test. Tissue damage score, mast cell and colonic patch numbers were all expressed as median (range) and analyzed with the use of a non-parametric test. The following data were analyzed by one-way ANOVA followed by a Bonferroni multiple comparison test: mMCP-1 content in plasma and colon tissue, and SP levels in colon tissue. In figures, group means ± sem are given. $P<0.05$ was considered to be significant. All data manipulations and

statistical analysis were conducted by the usage of Graphpad Prism (version 3.0, San Diego, USA).

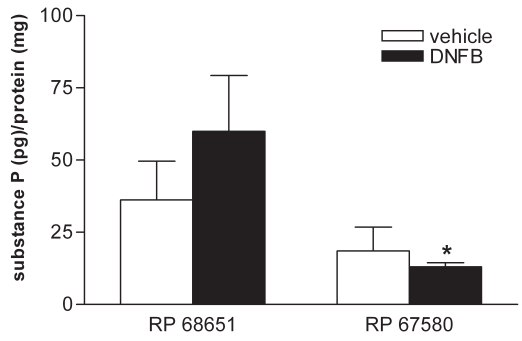
Results

The NK-1 receptor is involved in the development of colitis

Total substance P levels in colon tissue were determined in supernatants of colon homogenates by EIA. Although SP levels in DNFB-sensitized mice treated with RP 68651 were not significantly different from vehicle-sensitized RP 68651-treated mice 72 after challenge an increase could be observed (figure 1). However, treatment with RP 67580 resulted in significantly decreased substance P levels in colon tissue in DNFB-sensitized mice 72h after challenge compared to DNFB-sensitized RP 68651-treated mice (figure 1).

Intrarectal hapten application in DNFB-sensitized mice resulted in colonic hypersensitivity macroscopically characterized by the development of diarrhea. The presence of diarrhea and loose stool is indicative for a damaged and inflamed colon and therefore an important feature of IBD. In the conducted experiments the role of substance P and its receptor NK_1 was studied in this colonic hypersensitivity model by treatment with a specific antagonist for the NK_1 receptor, RP 67580. DNFB-sensitization followed by DNS challenge

Figure 1 *Substance P levels in colon of vehicle- and DNFB-sensitized mice treated with RP 68651 or RP 67580 72h after DNS challenge. Results are expressed as mean ± sem; *p<0.05 compared to DNFB-sensitized RP 68651-treated mice; n=6 mice/group.*



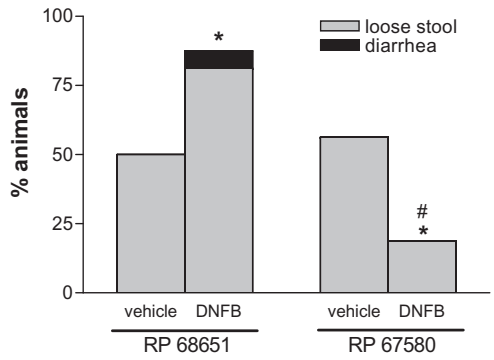


Figure 2 *Stool consistency 72h after challenge of vehicle- and DNFB-sensitized mice after treatment with RP 68651 or RP 67580. Score 0: normal well formed pellets, score 1: loose stool/easy to smear, score 2: diarrhea/watery stool. Results are expressed as cumulative percentage of total scored stool (score 0 not shown); *p<0.05 compared to vehicle-sensitized mice, #p<0.05 compared to RP68651-treated DNFB-sensitized mice, n=16 mice/group.*

Table I *Number of colonic patches, number of mMCP-1 positive cells and total damage score in vehicle- or DNFB-sensitized mice 72h after challenge treated with either the NK_1 receptor antagonist RP 67580 or its inactive enantiomer RP 68651.*

treatment	DNFB	colonic patch [a]	tissue damage score [b]	mast cells [c]
RP 68651	-	4 (2-7)	1 (0-1)	55 (27-101)
	+	8 (5-11)*	2 (1-6)*	109.5 (27-284)*
RP 67580	-	4 (1-8)	0 (0-1)	66 (41-91)
	+	4 (1-10)#	0.5 (0-2)#	58.5 (25-142)#

[a] Colonic patches were counted at the mucosal side of the colon of vehicle (-) or DNFB (+) sensitized mice. Results are expressed as median number of colonic patches per colon (minimum-maximum), n=16 mice.
[b] Colon tissue was scored for cellular infiltration and tissue damage after HE staining. Per mouse 3 section of colon tissue were examined at three different longitudinal depths leaving at least 100 μm in between. A score was given to the total appearance of the colon. Results are expressed as median tissue damage score (minimum-maximum); n = 3 mice.
[c] Mast cells were counted after mMCP-1 staining. Per mouse 3 different longitudinal sections were counted and averaged. Results are expressed as median average number of mMCP-1 positive cells per colon section (minimum-maximum), n =4 mice.
**p<0.05 compared to vehicle-sensitized mice subjected to the same treatment; #p<0.05 compared to DNFB-sensitized mice subjected to RP 68651 treatment.*

results in significantly deteriorated stool consistency compared to vehicle-sensitized mice after treatment with the inactive enantiomer RP 68651 (figure 2). Treatment with the NK_1 receptor antagonist RP 67580 prevents diarrhea in DNFB-sensitized mice 72h after challenge compared to vehicle-sensitized mice subjected to the same treatment.
Small lymphoid structures, colonic patches, are spread irregularly throughout the whole colon and differ in size and shape in each single animal. Colonic patches are present at the mucosal site of the colon and are easily visible. A significant increase in visible colonic patches was observed in DNFB-sensitized mice treated with RP 68651 72h after challenge compared to vehicle-sensitized mice (table I). This increase, which indicates hypertrophy of the lymphoid structures, could not be observed after treatment with RP 67580 in DNFB-sensitized mice 72h after challenge compared to vehicle-sensitized mice subjected to the same treatment (table I).
Furthermore, infiltration of inflammatory cells could significantly be decreased by treatment with the NK_1 receptor antagonist RP 67580 in DNFB-sensitized mice 72h after challenge compared to vehicle-sensitized mice. In DNFB-sensitized animals treated with inactive enantiomer cells are recruited to the site of inflammation after DNS challenge (figure 3C and table I). Besides infiltration in the submucosa and muscle layer also mucosal swelling can be observed (figure 3C). This was significantly decreased in DNFB-sensitized mice by treatment with RP 68651 72h after challenge (figure 3D and table I). Colon tissue of vehicle-sensitized mice was unaffected by treatment with either RP 68651 or RP 67580 (figure 3A and B).

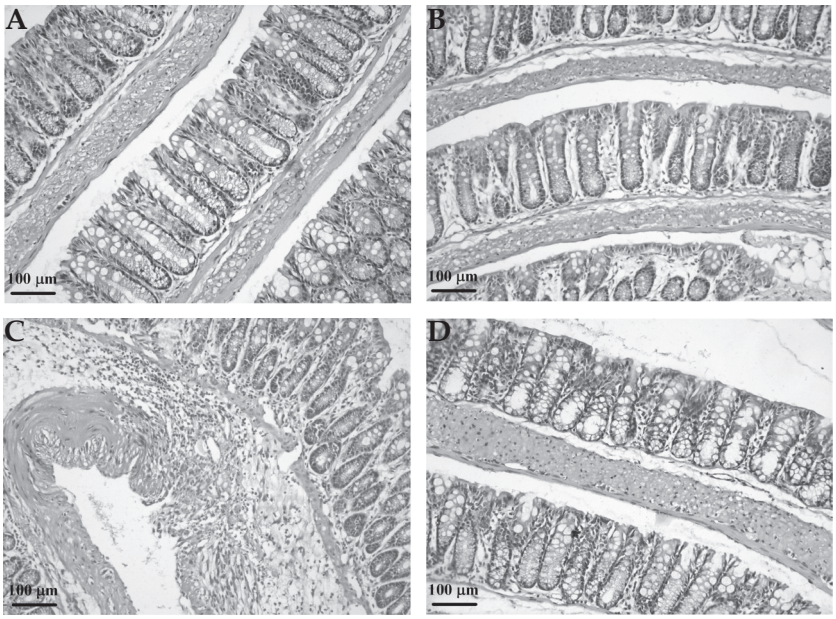


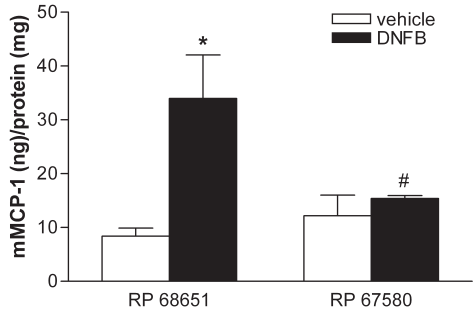
Figure 3 *HE staining of colonic tissue in vehicle- and DNFB-sensitized mice 72h after treated with either RP 67580 or RP 68651. Representative samples of colon tissue of both vehicle-sensitized animals treated with RP 68651 (**A**) or RP 67580 (**B**) shows normal intact colon structure figure. Cellular infiltration in the colon of DNFB-sensitized mice 72h after challenge treated with RP 68651 (**C**) could completely be abolished by treatment with RP 68750 (**D**).*

NK_1 receptor blockage affects DNFB-induced mast cell infiltration but not mast cell activation

Mast cells were visualized by staining mMCP-1 present in mucosal mast cells and counted. Results are presented in table I. In RP 68651-treated mice the number of mast cells was significantly increased in DNFB-sensitized mice compared to vehicle-sensitized mice. In RP 67580-treated mice no difference in mast cell number could be observed between vehicle and DNFB-sensitized animals (table I) indicating that antagonizing the NK_1 receptor inhibited mast cell infiltration into the colon. Further evidence for inhibited mast cell infiltration after RP 67580 treatment was provided by mMCP-1 levels in colon homogenates. mMCP-1 levels in supernatant of colon homogenates are indicative for total amount of mast cells present in the colon. mMCP-1 levels in supernatant of colon homogenates showed the same profile as mMCP-1 immunohistochemical staining (figure 4). DNFB-sensitized mice treated with the inactive enantiomer of the NK_1 receptor antagonist showed significantly increased levels of mMCP-1 in supernatant of colon homogenates 72h after challenge compared to vehicle-sensitized mice indicating mast cell infiltration into the colon. This increase in mMCP-1 levels in the colon in DNFB-sensitized mice could be abolished by treatment with RP 67580 (figure 4).

After mast cell activation and degranulation mMCP-1 is secreted into the serum

Figure 4 *Mast cell infiltration and/or proliferation in the colon of vehicle- and DNFB-sensitized mice treated with RP 68651 or RP 67580 72h after DNS challenge. Mast cell infiltration is indicated by changes in mMCP-1 levels in supernatant of colon homogenates 72h after DNS challenge. Results are expressed as mean ± sem; *p<0.05 compared to vehicle-sensitized RP 68651-treated mice; #p<0.05 compared to DNFB-sensitized RP 67580-treated mice; n=5-6 mice/group.*



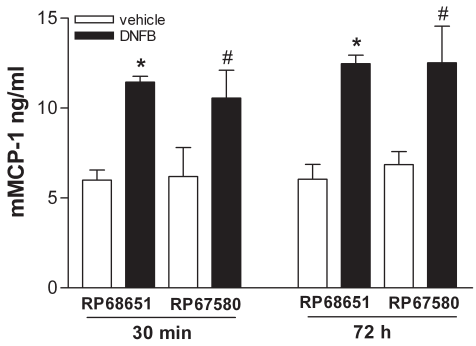


Figure 5 *Mast cell activation of vehicle- and DNFB-sensitized mice treated with RP 68651 or RP 67580 72h after DNS challenge. mMCP-1 levels in plasma were assessed 30 min and 72h after DNS challenge. Open bars represent vehicle-sensitized mice, and closed bars represent DNFB-sensitized mice. Results are expressed as mean ± sem; *p<0.05 compared to vehicle-sensitized RP 68651-treated mice; #p<0.05 compared to DNFB-sensitized RP 67580-treated mice; n=6 mice/group.*

so mMCP-1 levels in plasma are indicative for mast cell activation. Figure 5 demonstrates that mast cell activation is not affected by treatment with either RP 68651 or RP 67580 both 30 min and 72h after challenge. DNFB-sensitization results after challenge in significantly increased mMCP-1 plasma levels in both treatment regimes and time points compared to the corresponding vehicle-sensitized mice.

Discussion

The exact etiology of IBD remains unknown but is thought to be a complex interaction between genetic, environmental and immunological factors (9, 31). Additionally, both the extrinsic and intrinsic nerves of the gastro-intestinal tract are suggested to contribute to the pathogenesis of IBD. The tachykinis substance P and neurokinin A locally released from excitatory NANC nerves upon stimulation have been shown to elicit inflammatory responses via tachykinin receptors. This process is referred to as neurogenic inflammation (32). These responses include increased smooth muscle contractility, increased epithelial ion transport, enhanced plasma extravasation and infiltration and activation of effector immune cells in the gastro-intestinal tract (1). Blockade of the NK_1 receptor and capsaicin-induced depletion of sensory neurons has been shown to result in decreased vascular permeability in the mouse ileum after

DNFB sensitization and DNS challenge (29). We therefore propose a role for tachykinins (in particular substance P) in the development of hypersensitivity responses in the colon. In the present study evidence was obtained that treatment with the NK_1 receptor antagonist RP 67580 decreased important clinical features of colonic hypersensitivity. Findings in this study could provide new insights, which may be relevant to the comprehension of the role played by substance P and the NK_1 receptor in the pathogenesis of IBD.
The enteric nervous system is well recognized for its involvement in the promotion of secretory processes (33). Tachykinins released from extrinsic sensory or intrinsic enteric neurons contribute in different ways to the formation of watery diarrhea (34). In the colon, NK_1 receptor is the receptor predominantly involved in these processes (34). In different rat models associated with experimental diarrhea, substance P antagonists could prevent or markedly improve stool consistency by inhibiting substance P-induced water and electrolyte secretion (35-37). This is in agreement with the findings in our colonic hypersensitivity model showing that diarrhea induced by DNFB sensitization and DNS challenge could be abrogated by treatment with RP 67580.

Substance P has been described to be able to enhance lymphocyte proliferation (1). Furthermore, substance P can recruit leukocytes to the site of inflammation by the release of inflammatory mediators, elicitation of local vasodilation and increasing vascular permeability, thereby amplifying the inflammatory response (34). The colonic hypersensitivity model is associated with increased infiltration of inflammatory cells into the colon and hypertrophy of colonic patches (27). Cellular infiltration was observed in the colon of DNFB-sensitized mice treated with the inactive enantiomer of the antagonist 72h after challenge. This could significantly be reduced by treatment with the specific NK_1 receptor antagonist RP 67580 indicating that substance P and its NK_1 receptor are involved in the recruitment or facilitation of the recruitment of lymphocytes to the site of inflammation in this model. Increase in the number of visible colonic patches is indicative for the hypertrophy of these lymphoid structures and has previously been shown to be augmented during experimental colitis (27, 38). The capability of RP 67580 to prevent the increase of the number of colonic patches in DNFB-sensitized mice 72h after challenge further confirms that substance P and the NK-1 receptor attract leukocytes to the site of inflammation and/or induce lymphocyte proliferation.

In the investigated colitis model, the total substance P levels in the colon could significantly be reduced by the use of RP 67580 in DNFB-sensitized mice 72h after challenge. Since substance P was measured in supernatant of colon homogenates these levels indicate total amount of substance P present both in cells as in the interstitium of the colon. Moreover, NANC nerve endings are not the only source for substance P. This neuropeptide can also be synthesized and released by enterochromaffin cells, epithelial cells, fibroblasts and smooth muscle cells, but also by inflammatory cells such as macrophages, eosinophils, lymphocytes, dendritic cells and mast cells (1, 21). The observation that treatment with RP 67580 resulted in decreased infiltration and/or proliferation

of inflammatory cells and mast cells locally, supported by other authors (28, 39, 40), could explain the reduced levels of substance P in the colon implicating that inflammatory cells and mast cells are an important source for substance P in induced hypersensitivity responses.

In the intestinal mucosa of both humans and rodents mucosal mast cells can be found in close proximity of peptidergic nerves (41, 42) and NANC stimulation has been reported to result in mast cell activation (18). Furthermore, effects of substance P on skin delayed-type hypersensitivity reactions virtually entirely depended on mast cells (40). It is obvious that the interaction between mast cells and nerves is bi-directional. Mast cells and its mediators play a crucial role in IBD (21) and the presented colonic hypersensitivity responses are associated with increased mast cell numbers in the colon and increased mast cell activation (27). Using a NK_1 receptor antagonist in this murine colonic hypersensitivity model we have demonstrated that the antigen-induced mast cell proliferation and/or infiltration are dependent on the NK_1 receptor. Both decreased total mMCP-1 levels and mMCP-1 positive cells in colon tissue in DNFB-sensitized mice 72h after challenge indicate that mast cell numbers are reduced by the treatment with RP 67580. Although Moriarty *et al.* previously demonstrated that blockage of NK_1 receptors reduced the response to mast cell stimulation (15), RP 67580 was not able to decrease mMCP-1 plasma levels (generally used as marker for mast cell activation) in DNFB-sensitized mice both 30 min and 72h after challenge. It is possible that the activation of mast cells is dependent on non-neurokinin receptor-mediated mechanisms or other stimuli. High concentrations of substance P has also been reported to be able to activate mast cells in non-receptor mediated manner via G-coupled proteins (43, 44). Since mast cells and nerves are located in close association in the intestinal mucosa it is likely that local substance P concentrations here are relatively high after innervation of sensory neurons and capable of inducing mast cell activation in a non-receptor mediated manner.

The high levels of mMCP-1 72h after challenge could be ascribed to still circulating concentrations of mMCP-1 detected 30 min after challenge. Pemberton *et al.* recently demonstrated that binding of mMCP-1 to plasma serpins (serine protease inhibitors) substantially prolong the half-life of mMCP-1 in the systemic circulation (45). This serpin-protease complex is rapidly formed and is necessary to maintain the presence of mMCP-1 in the circulation. The other mouse mast cell protease (mMCP-2) shows no binding affinity for serpins and was cleared from the circulation within 15 min after peritoneal injection (45). However, mMCP-1 can still be detected in serum more than 24h after release. This observation together with our ability to detect mMCP-1 in plasma 30 min after challenge strengthens the indication that mMCP-1 in our model also forms a complex with plasma serpins. Furthermore, mMCP-1 concentrations in plasma measured 72h after challenge are of the same extent as the concentrations determined 30 min after challenge excluding a cumulative effect of mMCP-1 release in time.

In the present study we demonstrated that this reaction was dependent on NANC neuronal innervation via NK_1 receptors. Treatment with the specific

NK_1 receptor antagonist resulted in attenuated stool consistency and decreased cellular and mast cell infiltration reducing the inflammatory response induced by DNFB sensitization and rectal installment of DNS. In conclusion, the results indicate that the NK_1 receptor is crucial for the development of watery diarrhea and the recruitment and/or proliferation of leukocytes and mast cells to the site of inflammation in colonic hypersensitivity.

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Putative role for immunoglobulin-free light chain in a murine model ofinflammatory bowel disease

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Abstract

Traditionally, mast cells were regarded as key cells involved in type I hypersensitivity. However, it is now recognized that mast cells are widely involved in non-allergic (non-IgE) chronic diseases. The recent finding that immunoglobulin-free light chains (IgLC) can induce hypersensitivity-like responses, could suggest that these immunological factors may be of import in the pathophysiology of mast cell-associated diseases such as inflammatory bowel diseases (IBD). In the mouse, a colonic hypersensitivity reaction, induced by skin sensitization with dinitrofluorobenzene (DNFB) followed by an intra-rectal application of the hapten, features as a mast cell-dependent model for IBD. Using this model, we now have shown that the IgLC-antagonist, a 9-mer peptide F991, can abrogate the development of diarrhea, cellular infiltration and colonic lymphoid follicle hyperplasia. In addition, a decline in mouse mast cell protease 1 (mMCP-1) in serum was found indicating that F991 inhibits DNFB-induced mast cell activation. Furthermore, passive immunization with antigen-specific IgLC and subsequent rectal hapten challenge elicited local mast cell activation and increases in vascular permeability in the colon of mice. Our findings may provide new insights into the pathogenesis of IBD. In addition, the ability to inhibit pharmacologically IgLC- and mast cell-associated colonic hypersensitivity provides a novel therapeutic means to prevent or ameliorate the adverse gastrointestinal manifestations of IBD.

Introduction

The term inflammatory bowel disease (IBD) is used to described inflammatory disorders of the gastro-intestinal tract associated with diarrhea, rectal bleeding, weight loss and severe abdominal pain and increased presence of inflammatory cells and inflammatory products in colon (1, 2). Major representatives of chronic IBD are Crohn's disease (CD) and ulcerative colitis (UC). The exact etiology of this disorder remains unknown but is thought to be a complex of interactions between environmental, genetic and immunolgical factors (3). Potential inducing antigens for IBD are luminal bacteria, parasitic nematodes, food allergens or self-antigens (2, 4). Several inflammatory cells are involved in the pathology of IBD including the mast cell. An increased number of mast cells and mast cell activation is found in the mucosa of ileum and colon of IBD patients (5-7). Antigen-specific mast cell activation can occur via crosslinking of two IgE antibodies bound to the high affinity IgE-receptor, FcεRI. However, antigen-specific mast cell activation also occurs in the absence of IgE (8, 9). Because IBD is a disorder not associated with elevated IgE levels (10) mast cell activation is here likely to be elicited by other mechanisms.
A recent finding suggests a pathophysiological role for IgLC by demonstrating that IgLC can mediate antigen-specific mast cell-dependent hypersensitivity-like responses (11). Local application of an antigen to mice, passively immunized with antigen-specific IgLC, elicits an allergic reaction characterized by mast cell-dependent plasma extravasation and tissue swelling (11).

Based on these observations we hypothesize a function for mast cells and IgLC in the pathogenesis of IBD. In the study presented here we suggest a role for IgLC in a mast cell-dependent murine colonic hypersensitivity model for IBD (12). We administered a specific IgLC inhibitor which is based on the Tamm-Horsfall protein (THP), which has specific binding affinity for IgLC (13). F991 is a 9-mer peptide analogue of the IgLC-binding domain of THP. Administration of F991 in hypersensitivity-like reactions in skin and airways resulted in complete abolishment of mast cell-induced responses (11, 14). The therapeutic effect of this compound could therefore be attributed to the binding of IgLC, thus preventing binding of this molecule to mast cells and therefore interfering with mast cell degranulation. In the hapten-induced murine IBD model administration of F991 inhibited mast cell activation, ameliorated diarrhoea and reduced infiltration of inflammatory cells in the colon. Administration of antigen-specific IgLC led to mast cell-induced plasma extravasation after intrarectal challenge with the corresponding antigen. The results obtained in this study suggest IgLC as a potential therapeutic target in IBD.

Materials & methods

Animals Male BALB/c mice were obtained from Charles River (Maastricht, The Netherlands). The mice were 6-8 weeks of age and weighed 20-25 grams by the time of use. The animals were housed in groups not exceeding 8 mice per cage. Tap water and chow food were allowed ad libitum; there was a 12h day-night cycle. All experiments were conducted in accordance with The Animal Care Committee of Utrecht University (Utrecht, The Netherlands).

Induction of colonic hypersensitivity Mice were sensitized on day 0 by application of either DNFB (0.6% in acetone:olive oil 4:1) or vehicle (acetone:olive oil 4:1) epicutaneously on the shaved abdomen (50 µl) and all four paws (50 µl). On day 1 the mice received a boost of DNFB or vehicle on the abdomen only (50 µl). All the animals were challenged intrarectally with DNS (0.6%) dissolved in 10% ethanol on day 5. The sensitization, boost and challenge took place under light inhalation anesthesia (isoflurane 3%). The mice were macroscopically scored on day 6, 7 and 8 for stool consistency. Thereafter, the mice were sacrificed on day 8, 72h after challenge, with an overdose of pentobarbital, to determine in vivo mast cell activation, mast cell infiltration, colonic vascular permeability, cytokine production in the colon and presence and number of colonic patches.

Passive immunization with IgLC Male BALB/c mice were injected i.p. with TNP-specific IgLC (5 µg/mouse) or PBS. To determine vascular permeability changes 1.25% Evans blue dye was injected simultaneously. 30 min after passive sensitization the mice were challenged intrarectally with 0.6% picryl sulfonic acid (PSA) in 10% ethanol under light inhalation anesthesia (isoflurane 3%). The mice were sacrificed with an overdose of pentobarbital 30 min after challenge to determine *in vivo* mast cell activation, mast cell infiltration, colonic vascular permeability and number and presence of colonic patches. Optimal concentrations for IgLC sensitization have been determined in previous studies (11, 14).

F991 treatment F991 is a 9-mer peptide analogous to the specific IgLC-binding domain of THP (amino acid sequence: AHWSGHCCL). F991 was administered i.p. 500 µg in 100 µl sterile saline 24h before challenge, at time of challenge and 24h and 48h after challenge. Previous studies have demonstarted a dose-dependent inhibition of DNFB-induced cutaneous hyperresponsiveness (11).

Clinical scoring of the disease Stool consistency was scored every day at the same time point by placing the mice separately in cages without bedding. They were left in the cages until they discharged enough faeces to establish the consistency. The faeces were taken out immediately by a spatula and smeared on a piece of carton. The scoring was as follows: 0 for well-formed solid pellets, 1 for easy to smear and loose stool, and 2 for diarrhea and watery stool.

Macroscopical scoring of the disease After sacrificing the animals 72h after challenge, the colon was carefully dissected from anus till cecum and placed in

saline. The colon was opened longitudinally along the mesenteric border and washed gently in saline. It was placed on a rubber mat with the mucosal side up and the number of colonic patches, which appear like bulges in the tissue, was counted with the naked eye.

Histology and Immunohistochemistry After careful dissection the colon is placed in saline. The colon was then opened longitudinally over the mesenteric line and feces were removed by gently washing in saline. The colon then was placed with the serosal side up and dissected free from fat. Swiss rolls were formed by rolling the colon from the distal to the proximal end. The roll was immediately placed in ice-cold 4% paraformaldehyde in PBS (pH 7.4) for at least 24h and routinely embedded in paraffin for further use. Serial sections of 5 μm were cut using a microtome (Leica) and routinely stained with H&E to observe damage and cellular infiltration. For every mouse 3 different longitudinal sections of the colon tissue were stained and microscopically analyzed. Pictures shown are representatives of the different treatment groups.
To detect mast cells 5 μm sections were immunohistochemically stained for mouse mast cell protease-1 (mMCP-1) as described previously (12).
For every mouse 3 different longitudinal sections of the colon tissue were stained and analyzed. The number of mMCP-1 positive cells was quantified by microscopical visualization and manually counting. Results are expressed as median average number of cells (minimum-maximum) per colon section.

Preparations of Tissue Homogenates To determine mast cell infiltration into the tissue, whole colon homogenates were made. After sacrificing mice, the colon was excised carefully and opened longitudinally. Feces were removed by gently washing in saline. The colon was placed in ice cold PBS enriched with protease inhibitors (Complete Mini, Roche) in flat bottom tubes. The tissue was dispersed on ice for 10 sec. with a rotor-stator (Ystral®). The homogenates were centrifuged (14,000 rpm, 4°C, 10 min) and the supernatant was frozen until further use to assess mast cell infiltration by means of measuring mMCP-1 and TNFα levels.

Mast cell Activation and Infiltration in vivo To monitor mast cell activation in time, blood samples of DNFB- and vehicle-sensitized mice were taken 30 min, 24h and/or 72h after intrarectal DNS challenge. Blood samples were collected via heartpunction and 4% EDTA was added (10% v/v) to obtain plasma. After centrifugation the plasma was stored at –70ºC until use. Levels of mMCP-1 in plasma were measured as described previously using a commercially available ELISA assay (15). Results are expressed as ng mMCP-1/ml plasma.
To determine mast cell infiltration in the colon mMCP-1 levels were measured in the supernatant of colon homogenates with the commercially available ELISA kit. Samples were used undiluted. Total protein content of the supernatant of colon homogenates was quantified with a commercial available detection kit (BCA protein assay, Pierce). Samples were diluted 10-fold for total protein measurements. Results are expressed as ng mMCP-1/mg total protein.

TNFα in colon tissue To determine TNFα levels in vivo TNFα was measured in the supernatant of colon homogenates 72h after challenge with a commercially available TNFα ELISA kit (Biosource). Samples were used undiluted. Total protein content of the supernatant of colon homogenates was quantified with a commercial available detection kit (BCA protein assay, Pierce). Samples were diluted 10-fold for total protein measurements. Results are expressed as pg TNFα/mg total protein.

Isolation of antigen-specific IgLC Antigen-specific IgLC was isolated from trinitrophenol (TNP)-specific IgG (1B7-11, American Type Culture Collection, Manassas, Virginia) and purified as described previously (11). Recombinant IgLC was produced by PCR cloning of cDNA 1B7-11 in a pGEX vector (Amersham Pharmacia Biosciences, Roosendaal, The Netherlands). Fusion proteins were expressed in Escherichia coli and purified using affinity chromatography.

Determination of Colonic Vascular Permeability Vascular permeability changes were determined as described previously with small modifications (12). Evans blue (1.25% in sterile saline, 50 µl) was injected i.v. in the tail vein 1h prior to the end of an experiment and simultaneously with passive immunization with antigen-specific IgLC. After 2h the mice were killed with an overdose of sodium pentobarbital and blood samples were taken via cardiac puncture. 4% EDTA (10% v/v) was added to whole blood to obtain plasma. The colon was excised carefully and opened longitudinally. Feces were removed by gently washing in saline. The colon was dissected free from fat and placed in formamide (500 µl). Evans blue dye was extracted from the colon at 40°C for 24h. The dry weight was determined after drying the colons for 4 weeks at 40°C. The extravasation of Evans blue dye-labeled macromolecules from the blood circulation into the colonic tissues was quantified by measuring the optical density of the plasma samples and formamide extracts on a Benchmark microplate reader (Biorad, California, USA) at a wavelength of 595 nm. Vascular permeability was determined by dividing the total amount of Evans blue extracted from the colon by the concentration of Evans blue in the plasma. The vascular permeability was expressed as µl exudated plasma/mg dry weight of the colon.

Materials DNFB, olive oil, o-phenylenediamine dihydrochloride (OPD), the AEC chromogen staining kit, normal goat serum, heamatoxylin and eosin were all purchased from Sigma Chemical Co., St. Louis, MO. DNS was purchased from Eastman Kodak, Rochester, NY. Tween 20 was purchased from Janssen Pharmaceutica, Beerse, Belgium. Sodium pentobarbitone was purchased from Sanofi, Maassluis, The Netherlands. Evans blue dye was obtained from Fluka Chemie AG, Munchen, Germany. The rat Ig anti-mMCP-1 antibody was a kind gift of Dr. H.R.Miller, Royal (Dick) School of Veterinary Studies (University of Edinburgh, United Kingdom) and the biotin-conjugated goat anti-rat Ig specific polyclonal antibody was purchased by Pharmigen, Aalst, Belgium. The BCA protein assay was purchased at Pierce, Etten-Leur, The Netherlands.

Complete-mini protease-inhibitors were from Roche Diagnostics, Almere, The Netherlands. The TNFα ELISA kit was purchased from Biosource. The mMCP-1 ELISA was from Moredun Scientific Ltd., Midlothian, UK. Maxisorp surface 96 well plates were purchased from Nunc Immuno plate, Roskilde, Denmark. F991 was synthesized by Fmoc Chemistry (Bachem, Switzerland). The pGEX vector was from Amersham Pharmacia Biosciences, Roosendaal, The Netherlands.

Statistics Stool consistency data and total tissue damage score were analyzed from raw scoring data using a distribution free Kruskal-Wallis ANOVA followed by a Dunn's multiple comparison test. Mast cell and colonic patch numbers were both expressed as median (range) and analyzed with the use of a non-parametric test. The following data were analyzed by two-way ANOVA and a Bonferroni multiple comparison test: mMCP-1 content in plasma and colon tissue and TNFα concentrations in the colon. Vascular leakage was tested with a Student's t-test. In figures group means ± sem are given. $P<0.05$ was considered to be statistically significant. All data manipulations and statistical analysis were conducted by the usage of Graphpad Prism (version 3.0, San Diego, USA).

Results

Contact sensitization of BALB/c mice with DNFB followed by an intrarectal challenge with the antigen resulted in colonic hypersensitivity (12). This antigen-specific hypersensitivity reaction is associated with the development of diarrhea, lymphoid structure hypertrophy and cellular infiltration 72h after challenge (12). In the current study we investigated a possible role for IgLC in this model for IBD by treatment with a specific inhibitor for IgLC, F991.

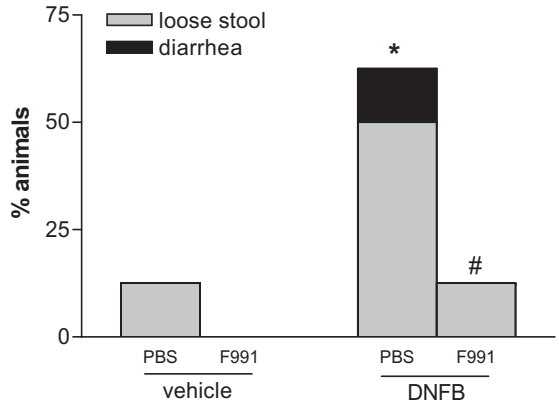

Figure 1 *The IgLC antagonist F991 inhibits antigen-induced diarrhea found 72h after challenge of vehicle- and DNFB-sensitized mice. Score 0: normal well formed pellets, score 1: loose stool/easy to smear, score 2: diarrhea/watery stool. Results are expressed as cumulative percentage of total scored stool (score 0 not shown); *p<0.05 compared to vehicle-sensitized mice, #p<0.05 compared to PBS-treated DNFB-sensitized mice, n=8 mice/group.*

Table I *Number of colonic patches, number of mMCP-1 positive cells and total damage score in vehicle- or DNFB-sensitized mice 72h after challenge treated with either PBS or F991.*

treatment	DNFB	colonic patch [a]	tissue damage score [b]	mast cells [c]
PBS	-	2.5 (1-4)	0 (0-1)	29 (16-35)
	+	6 (2-10)*	2 (1-5)*	74 (52-90)*
F991	-	3 (2-4)	0 (0-1)	14 (4-29)
	+	5.5 (3-8)^{ns}	1 (0-1)$^{\#}$	53 (30-72)*

[a] The number of colonic patches were counted at the mucosal side of the colon of vehicle (-) or DNFB (+) sensitized mice. Results are expressed as median number of colonic patches per colon (minimum-maximum), n=8 mice.

[b] Colon tissue was scored for cellular infiltration and tissue damage after HE staining. For every mouse 3 section of colon tissue were examined at three different longitudinal depths leaving at least 100 μm in between. A score was given to the total appearance of the colon. Results are expressed as median tissue damage score (minimum-maximum); n = 3 mice.

[c] Mast cells were counted after mMCP-1 staining. For every mouse 3 different longitudinal sections were counted and averaged. Results are expressed as median average number of mMCP-1 positive cells per colon section (minimum-maximum), n=3 mice.

**p<0.05 compared to vehicle-sensitized mice subjected to the same treatment; #p<0.05 compared to DNFB-sensitized mice subjected to PBS treatment; ns is not significant compared to PBS-treated DNFB-sensitized mice.*

The IgLC antagonist F991 reduces antigen-induced disease characteristics for colonic hypersensitivity

The presence of diarrhea and loose stool is indicative for a damaged and inflamed colon. As is shown in figure 1 treatment with F991 led to complete prevention of the development of diarrhea in DNFB-sensitized mice 72h after challenge. Administration of F991 had no influence on stool consistency in vehicle-sensitized mice.

Colonic patches are small lymphoid follicles that appear at the mucosal side of the colon. They consist predominantly of B-lymphocytes, but also clusters of T-lymphocytes are found (observations obtained with FACS analysis and immunohistochemistry; previously reported (12)). Colonic patches are spread irregularly and differ in size and shape between animals. The number of colonic patches increases significantly in PBS-treated DNFB-sensitized mice 72h after challenge compared to vehicle-sensitized mice (table I). Treatment of DNFB-sensitized mice with F991 had no effect on the number of colonic patches present in the colon (table I).

Moreover, in PBS-treated DNFB-sensitized mice cellular infiltration was observed 72h after DNS challenge (figure 2C). This was accompanied with loss of structure of the colonic lining and mucosal swelling. Treatment with F991 could significantly reduce this phenomenon in DNFB-sensitized mice 7h after challenge (table I and figure 2D). F991 pretreatment did not influence the colonic structure of vehicle-sensitized mice (figure 2A and 2B).

F991 reduces mast cell activation but not mast cell infiltration

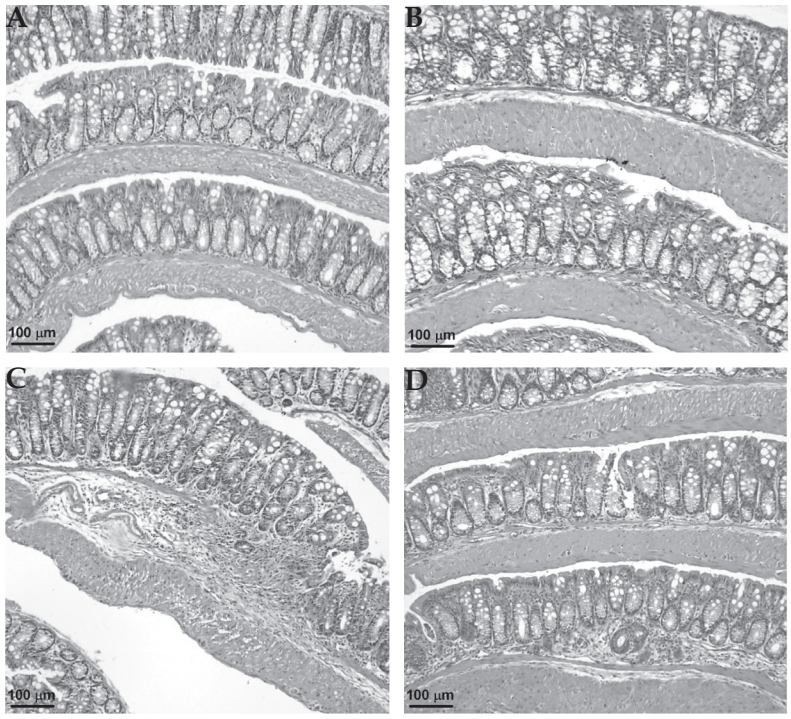

Figure 2 *The IgLC antagonist F991 inhibits antigen-induced cellular infiltration and tissue damage 72h after challenge of vehicle- and DNFB-sensitized mice. Haematoxylin and eosin staining of 5 µm sections of paraffin-embedded colon tissue shows a normal mucosal lining in vehicle-sensitized mice either treated with PBS (**A**) or with F991 (**B**). Colon tissue of DNFB-sensitized mice 72h after challenge treated with PBS shows cellular infiltration in the submucosa and disruption of the mucosal lining (**C**), which is not seen after treatment with F991 (**D**).*

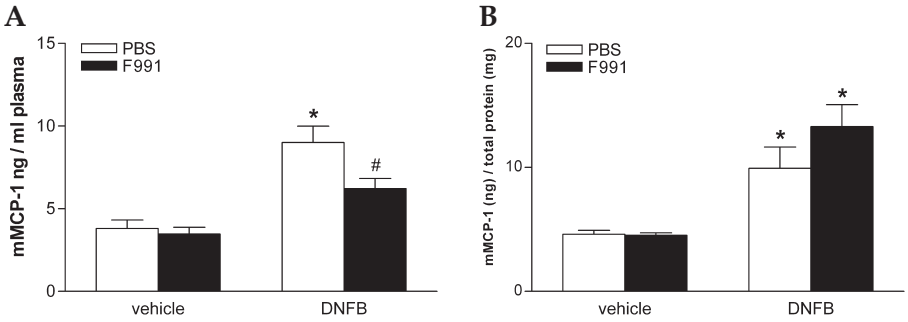

Figure 3 *Effect of IgLC antagonist F991 on mast cell activation and infiltration in the colon of vehicle- and DNFB-sensitized mice 72h after challenge. (**A**) mMCP-1 levels in plasma were assessed 30 min after DNS challenge. Results are expressed as mean ± sem; *p<0.05 compared to vehicle-sensitized animals, #p<0.05 compared to PBS-treated mice subjected to the same sensitization protocol; n=8 mice/group. (**B**) Mast cell infiltration into the colon is indicated by changes in mMCP-1 levels in supernatant of colon homogenates 72h after DNS challenge. Results are expressed as mean ± sem; *p<0.05 compared to vehicle-sensitized PBS-treated mice; n=5-6 mice/group.*

Mouse mast cell protease-1 (mMCP-1) is a protease specific for mouse mucosal mast cells and appears in the bloodstream after activation of mast cells (16). To assess mast cell activation mMCP-1 levels were determined 30 min after challenge in plasma. A significant rise in mMCP-1 was observed in DNFB-sensitized PBS-treated mice compared to vehicle-sensitized PBS-treated mice (figure 3A). This increase in mMCP-1 could significantly be attenuated by pretreatment with F991 (figure 3A). Infiltration of mast cells was determined by total mMCP-1 levels in supernatant of colon homogenates. Figure 3B shows significantly increased mMCP-1 levels in both PBS- and F991-treated DNFB-sensitized mice 72h after challenge. Immunohistochemical staining for mMCP-1 confirmed the infiltration and/or proliferation of mast cells in vivo in DNFB-sensitized mice 72h after challenge unaffected by treatment with PBS or F991 (table I).

Total TNFα levels in colon tissue increases after treatment with F991

TNFα measured in supernatant of colon homogenates is indicative for the total amount of TNFα present. DNFB sensitization leads to a significant increase in TNFα in the colon 72h after challenge compared to vehicle-sensitized mice (figure 4). Due to F991 treatment total TNFα levels in the colon of both vehicle- and DNFB-sensitized mice 72h after challenge are significantly enhanced compared to PBS treated mice subjected to the same sensitization protocol (figure 4).

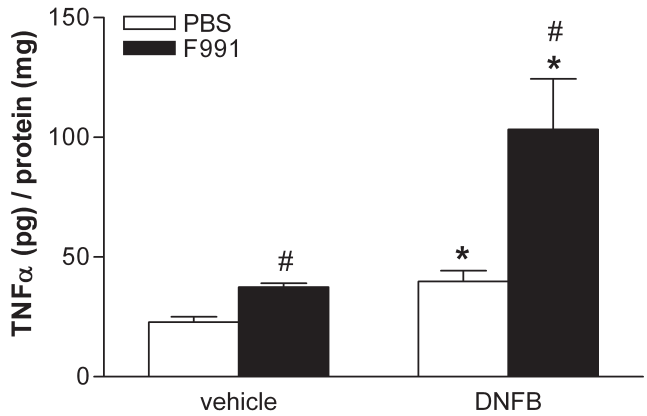

Figure 4 *Effect of IgLC antagonist F991 on total TNFα levels in colon tissue in vehicle- and DNFB-sensitized mice 72h after challenge. TNFα is determined in supernatant of colon homogenates and expressed as mean TNFα (pg)/total protein (mg) ± sem; *p<0.05 compared vehicle-sensitized mice subjected to the same treatment; #p<0.05 compared to PBS-treated mice subjected to the same sensitization protocol; n=6-12 mice.*

Passive immunization with TNP-specific IgLC leads to mast cell-induced vascular permeability after intrarectal challenge with the hapten

To confirm the role of IgLC in this colonic hypersensitivity model, mice were passively immunized i.v. with PBS or TNP-specific IgLC 30 min prior to intrarectal challenge. Mast cell activation determined by means of mMCP-1 levels in plasma 30 min after challenge was significantly increased in IgLC-immunized mice compared to PBS-immunized mice (figure 5A). This was associated with a rapid increase in vascular permeability in IgLC-immunized mice quantified by the accumulation of Evans Blue and one of the early signs of inflammation (figure 5B).

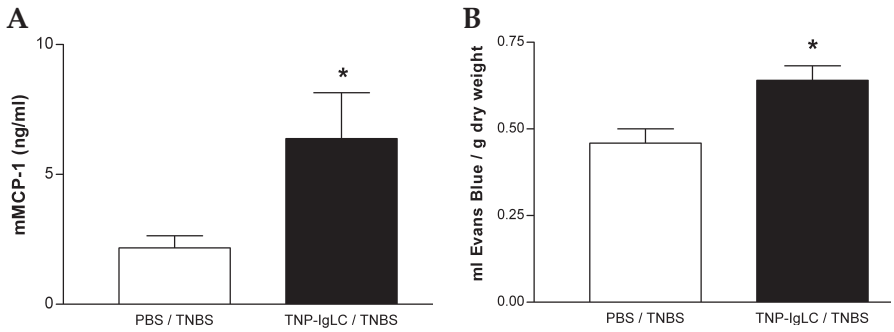

Figure 5 *Antigen-specific IgLC mediate acute mast cell-induced plasma extravasation. Open bars represent PBS-immunized mice and closed bars TNP-IgLC-immunized mice 30 min after TNBS challenge. (**A**) mMCP-1 levels in plasma were assessed 30 min after TNBS challenge. Results are expressed as mean ± sem; *p<0.05 compared to PBS-immunized mice, n=6 mice/group. (**B**) Colonic vascular permeability changes measured by Evans Blue leakage from blood into colonic tissue. Results are expressed as mean µl plasma exudated/mg dry weight colon ± sem; *p<0.05 compared to PBS-immunized mice, n=6 mice/group.*

Discussion

Several of the pathophysiological features of human IBD resemble hypersensitivity-like responses in the gastrointestinal tract (17, 18) and mast cell activation is known to play an important role in these events (5). Different pro-inflammatory mediators released after mast cell activation can induce a cascade of events initiating an inflammatory response. IgE is one of the most potent antigen-specific activators of mast cells and is the key player in immediate hypersensitivity responses. However, mast cell degranulation was also shown to be involved in non-IgE mediated inflammatory processes like multiple sclerosis, tuberculosis, contact dermatitis, non-atopic asthma and IBD (19). Because IBD is a chronic disorder not associated with elevated IgE levels (10) the suggestion for another antigen-specific non-IgE mediated pathway of mast cell activation in the pathogenesis of IBD rises.

The recent discovery that IgLC plays a pivotal role in the activation of mast cells in hypersensitivity-like responses in skin and airways (11, 14) suggests

that these molecules could also be involved in other immune disorders. We therefore hypothesized a role for IgLC in IBD, using an experimental murine model for colonic hypersensitivity associated with mast cell infiltration and activation, diarrheic stool, hypertrophy of colonic lymphoid structures and increased TNFα production (12). In the presented study, the possible role of IgLC has been investigated by the use of the specific IgLC antagonist F991 and by passive immunization with antigen-specific IgLC. We demonstrated that deterioration of stool consistency and mast cell activation after DNS challenge could be abolished by the use of F991. Furthermore, the infiltration of inflammatory cells into the tissue due to DNFB-sensitization and DNS challenge could be inhibited by administration of F991. Proof of principle was given by passive immunization with antigen-specific IgLC and intrarectal antigen challenge, which resulted in increased vascular permeability and mast cell activation in the colon. Colonic patch hypertrophy in DNFB-sensitized mice was not affected by F991 treatment indicating that IgLC is not involved in this phenomenon.

In F991-treated DNFB-sensitized mice mMCP-1 levels in plasma 30 minutes after challenge were significantly decreased compared to PBS-treated mice. This indicates that mast cell degranulation could be reduced by inhibition of IgLC-binding to mast cells. mMCP-1 levels in supernatant of colon homogenates are an indication of total amount of mast cells present in the tissue. Both PBS and F991-treated DNFB-sensitized mice showed a significant increase in mMCP-1 levels in colon tissue compared to vehicle-sensitized mice. These data are consistent with mMCP-1 staining, which also shows mast cell infiltration into the tissue. Overall it can be concluded that mast cell infiltration and/or proliferation is unaffected by F991 treatment. However, F991 can decoy IgLC via high affinity binding properties. IgLC is prevented from binding to mast cells, which leads to diminished mast cell degranulation. This therefore prevents the release of pro-inflammatory mediators.

The effect of F991 on development of diarrhea can be explained by the absence of mast cell degranulation. Activation of mast cells leads to the production and secretion of a variety of mediators like histamine, serotonin, proteases, growth factors and cytokines (20, 21). The pro-inflammatory cytokine TNFα is abundantly prestored in mast cell granules, which can be directly released and also newly synthesized after activation. TNFα has been shown to be an important inducer of diarrhea (22, 23). In relation to the presented experiments, preventing mast cell degranulation and therefore the release of TNFα could explain the positive effect of F991 on stool consistency.

Previously we have shown that TNFα present in colon tissue in this colonic hypersensitivity model is mainly derived from mast cells (12). However, measuring TNFα in supernatant of colon homogenates determines not only free TNFα but also intracellular prestored TNFα. Since TNFα is abundantly prestored in mast cells significantly higher TNFα levels in both F991-treated vehicle- and DNFB-sensitized mice 72h after challenge compared to the corresponding PBS-treated group (figure 4), could be explained by the prevention of mast cell degranulation by the IgLC antagonist. The significant increase in either

PBS- or F991-treated DNFB-sensitized mice compared to the corresponding vehicle-sensitized mice further proofs that mast cell activation is decreased, but infiltration and proliferation are not affected by F991 treatment.

We also show that mast cells were activated *in vivo* after immunization with TNP-specific IgLC and local intrarectal TNBS challenge. Increased vascular permeability can be induced by mast cell-derived histamine, serotonin and TNFα, and will permit plasma extravasation allowing macromolecules and inflammatory cell to infiltrate into the interstitium (24). This is one of the major events of the early phase of inflammation. In a non-atopic asthma model IgLC was also responsible for the mast cell-induced airway hyperresponsiveness and mucosal exudation (14). Moreover, significantly increased κIgLC serum levels have been demonstrated in both atopic and non-atopic asthma patients (14).

Luminal microflora, parasitic nematodes and food allergens are antigens involved in the initiation and exacerbation of IBD (2, 25). However, also self-antigens are described to play a role (4). In autoimmune diseases a pathophysiological role can be attributed to mast cells and IgLC (20). Therefore, evidence mounts for a key role for mast cells and IgLC in the initiating phase of IBD. In the present study we provided evidence that supports an important role for IgLC in mast cell activation in a chemically-induced immunological model for IBD. Our data indicate that by the use of an IgLC antagonist mast cell activation is abolished resulting in improvement of macroscopic disease characteristics and a decrease in cellular infiltration into the colon. These findings suggest IgLC to be an important therapeutic target in the pathogenesis of IBD.

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6

Immunoglobulin-free light chains mediate the activation of mouse dorsal root ganglion neurons by antigens

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Abstract

The immunoglobulin-free light chains (IgLC) secreted by B lymphocytes have been shown to mediate intestinal hypersensitivity by the induction of mast cell activation in an antigen-specific manner. Although both mast cells and sensory neurons contribute to the hypersensitivity response, the role of IgLC in relation to sensory neurons is unknown. We therefore aimed to investigate the effects of IgLC on murine cultures of dorsal root ganglion (DRG) neurons. Immunohistochemistry demonstrated that IgLC and IgE could specifically bind to murine cultured DRG neurons. Further, optical recordings showed that application of the corresponding antigen to IgLC- and IgE-sensitized DRG neurons induces a sustained increase in $[Ca^{2+}]_i$ in about half of these neurons. These results suggest that IgLC- and IgE-antigen complexes can act directly on mouse DRG neurons and reveal a novel potential pathway of antigen-specific neuronal activation in hypersensitivity responses.

Introduction

Immunoglobulins (Ig) are synthesized by B lymphocytes and function as potent effector molecules (antibodies) in the humoral immune response. Based on heavy chain classes, immunoglobulins can be divided into five isotypes (IgA, IgD, IgE, IgG and IgM), each of which with different biological activity. Apart from two heavy chains an antibody also possesses two light chains. Antigen specificity based on antigen binding sites in antibodies is determined by the variable domains of both light and heavy chains. Moreover, light chains have also been shown to exhibit antigen binding by themselves (1, 2). For instance, immunoglobulin-free light chain (IgLC) purified from a monoclonal antibody to vasoactive intestinal polypeptide (VIP) displayed sequence-specific binding to VIP (3). The affinity of IgLC was only 5-fold lower than the complete parent antibody (3). These findings indicate that the contribution of the variable region of IgLC to antigen-specific binding in an antibody is of high value for antigen recognition. These findings also allow to suggest a biological function for IgLC *in vivo*. Recently we have described a pathophysiological function for IgLC by demonstrating a prominent role for IgLC in cutaneous hypersensitivity responses (4). Passive immunization of mice with antigen-specific IgLC followed by topical skin application of the antigen resulted in plasma extravasation, cutaneous swelling and mast cell degranulation, which could be abolished by treatment with a specific antagonist for IgLC (4). Further evidence for IgLC-induced hypersensitivity-like responses and mast cell degranulation was provided by studies in a hapten-induced murine model for non-atopic asthma (5) and a similar model for colonic hypersensitivity (chapter 5 of this thesis). The clinical relevance of a role of IgLC in diseases, such as asthma and inflammatory bowel disease (IBD), was provided by demonstrating significantly increased levels of IgLC in sera of asthmatic patients (5) and in sera and intestinal biopsies of IBD patients (chapter 7 of this thesis) compared to healthy controls. These experiments demonstrate that IgLC-mediated hypersensitivity may serve as a model to study mast cell-associated non-IgE-mediated disorders like non-atopic asthma, rheumatoid arthritis, multiple sclerosis, IBD and cutaneous hypersensitivity.

The close proximity of mast cells to nerve endings, mostly of sensory origin, in various tissues like skin (6), dura mater (7), intestine (8, 9) and airways (6) suggests a direct bi-directional communication between mast cells and the nervous system. This neuro-immune interaction is well acknowledged and has been described extensively (10-12). In *in vitro* co-cultures with murine superior cervical ganglia (SCG) and rat basophilic leukemia (RBL) or bone marrow derived mast cells (BMMC) it was demonstrated that activation of neurites resulted in a rise in intracellular calcium in the mast cell which was mediated by the neuropeptide substance P (13, 14). Moreover, Suzuki et al. showed that this communication was bi-directional. Specific activation of the mast cell increased intracellular calcium concentrations of neurites in the co-culture system (15).

Increased numbers of mast cells and mast cell-nerve contacts are particularly found in inflamed and infected tissues (16, 17) although an anatomical

relationship between mast cells and nerves has also been demonstrated in non-inflamed areas (7). In addition to the key role attributed to mast cells in hapten-induced hypersensitivity reactions, it has been shown that sensory neurons are also important for the initiation of the response (18). Lack of the NK_1 receptor or treatment with a NK_1 receptor antagonist in different hapten-induced murine models for pulmonary hypersensitivity abolished airway hyperresponsiveness and decreased tracheal vascular permeability (19-22). In hapten-induced hypersensitivity responses in the gastrointestinal tract similar inhibitory effects of treatment with the NK_1 receptor antagonist on leukocyte accumulation and development of diarrhea were observed (23) (chapter 5 of this thesis).

Based on the role of neurons in hypersensitivity responses and the observation that IgLC plays a crucial role in the development of hapten-induced hypersensitivity responses we propose a role for IgLC in the direct activation of sensory neurons in relation to hypersensitivity reactions. We examined this by performing immunohistochemistry on cultured murine adult dorsal root ganglia (DRG) neurons. We report specific binding activity of IgLC and IgE to these neurons. Subsequently, we were able to show that significant increases in intracellular Ca^{2+} concentrations are induced by antigen-specific activation of IgLC- or IgE-sensitized DRG neurons. We therefore conclude that IgLC may contribute to the activation of sensory neurons in hypersensitivity responses.

Materials and methods

Animals Male BALB/c mice were obtained from Charles River (Maastricht, the Netherlands). Mice were 6-8 weeks of age and weighed 20-25 grams at the time of use. The animals were housed in groups not exceeding 8 mice per cage. Tap water and chow food were allowed ad libitum and a 12/12h day-night cycle was maintained. All experiments were conducted in accordance with The Animal Care Committee of Utrecht University (Utrecht, the Netherlands).

DRG neuron cultures Single neuronal cells originating from DRG from the cervical to the lumbal segments were cultured as described previously (24) with small modifications. Animals were sacrificed by i.p. injection of overdoses of sodium pentobarbital and pinned down dorsally. All organs were removed and the spinal cord was revealed. DRG were dissected one-by-one by carefully picking up the spinal cord exposing the DRG. Spinal roots were cut as close to the ganglion as possible. DRG were enzymatically digested with 10 mg/ml collagenase in L-15 medium at 37°C, 5% CO_2 for 30 min. The reaction was stopped by adding an equal volume of pre-heated FCS for 2 min. The ganglia were suspended in DRG culture medium (DMEM high glucose without glutamine and DMEM F-12 medium at a 1:1 ratio enriched with 1% N2 human serum supplement, 1% glutamine, 50 mg/ml gentamicine, 2 μM cytosine arabinoside) by mechanical dissociating with pipettes with decreasing diameters. The cell suspension was placed on serum-coated petri dishes to allow nonneuronal cells to adhere for 90 min at 37°C, 5% CO_2. After

separation neuronal cells were collected from the dishes and placed onto poly-L-lysine-coated petri dishes with glass bottoms. Cultures were maintained at 37°C, 5% CO_2 and studied at days 2-5 of the culture; medium was refreshed at day 3.

Isolation of antigen-specific IgLC, IgE and IgG TNP-specific IgG (1B7-11, American Type Culture Collection, Manassas, Virginia) was purified with G-sepharose (Amersham Bioscience, Roosendaal, the Netherlands). For antigen-specific immunoglobulin-free light chains (TNP)-specific IgG (1B7-11) was reduced, alkylated and purified as described previously (4). Recombinant IgLC were produced by PCR cloning of cDNA 1B7-11 in a pGEX vector (Amersham Pharmacia Biosciences, Roosendaal, the Netherlands). Fusion proteins were expressed in *Escherichia coli* and purified using affinity chromatography. TNP-specific IgE (H1DNPε26.82, kindly provided by J. Rivera, NIH, USA) was obtained by hapten affinity purification.

Immunohistochemistry Cultured primary DRG neurons were fixed in 4% paraformaldehyde for 24h at 4°C on the glass bottom of the poly-L-lysine-coated petri dishes. Subsequently, they were washed with and kept in PBS until further use.
Protein gene product (PGP) 9.5 is a pan-neuronal marker. This marker was used to identify primary cultured DRG cells as neuronal cells. Cells were incubated overnight with rabbit-anti-PGP 9.5 (1/5000) diluted in TBS containing 1% BSA and 0.1% Triton X-100 in a moist environment. Subsequently, after being rinsed with TBS, the cells were incubated with Cy-3-conjugated donkey-anti-rabbit antibody for 1h. Cells were enclosed in Mowiol to preserve the fluorescent staining. All incubations were performed at room temperature.
To visualize binding affinity of IgLC to DRG neurons the following staining was performed. Cells were incubated with 25 μg/ml FITC-labelled mouse IgLC diluted in TBS with 1% BSA and 0.1% Triton X-100 for 90 min. Aspecific activity of the secondary antibody was blocked with 10% normal goat serum (NGS) in TBS with 0.1% Triton X-100. Subsequently, rabbit-anti-FITC (1/1000) was incubated for 1h. The third antibody, HRP-conjugated goat-anti-rabbit, was incubated for 1h. The staining was developed with DAB enriched with 0.025% nickel ammonium sulphate to expose an intense dark-brown/black colour. To expose the morphological structure of the cells an eosin background staining was performed subsequently. All incubations were performed at room temperature in a darkened incubation chamber. The secondary and tertiary antibodies were diluted in TBS containing 1% NGS and 0.1% Triton X-100. After each incubation the cells were washed with TBS with 0.1% Triton X-100. FITC-labelled mouse IgE and IgG were used as controls.
To investigate specific binding affinity of IgLC and IgE, binding studies were performed with cold (i.e. unlabeled) ligand. In these studies the same staining protocol as described above was preceded by incubation with 250 μg/ml of unlabeled IgLC or unlabeled IgE diluted in TBS with 1% BSA and 0.1% Triton X-100 for 15 min. Without washing, the cells 25 μg/ml of FITC-labelled IgLC or IgE was added. The staining was further performed as previously described.

Sensitization with IgLC, IgE and IgG Primary cultures of DRG neurons were used at days 2-5 of the culture. Cells were washed twice with DRG medium and incubated with 25 μg/ml IgLC, IgE or IgG at 37°C, 5% CO_2 for 90 min. After this incubation cells were washed twice with DRG medium and used for optical imaging of cytosolic $[Ca^{2+}]_i$.

Solutions The buffer used in the optical Ca^{2+} recordings was artificial cerebrospinal fluid (ACSF) consisting of the following composition (in mM): 122 NaCl, 2.1 KCl, 25 $NaHCO_3$, 2.5$CaCl_2$, 1.3 $MgSO_4$, 1.2 KH_2PO_4, 11 glucose (pH 7.4; oxygenated with 95%CO_2-5%O_2). In the ACSF used to evoke brief (10 sec) membrane depolarization the concentration of KCl was increased to 70 mM, while the NaCl concentration was reduced to maintain osmolarity. To induce antigen-specific activation dinitrophenyl coupled to human serum albumin (DNP-HSA) was used (10 ng/ml in ACSF). A solution of 10 ng/ml BSA in ACSF was used as a control solution.

Optical Recording of Cytosolic $[Ca^{2+}]_i$ Intracellular calcium mobilization was assessed by confocal laser scanning microscopy (CLSM) and used as an index of neural activation. Dishes with DRG neurons were incubated with the Ca^{2+} indicator dye Fluo-4 AM (1 μM) and 0.005% of the detergent pluronic F-127 in ACSF buffer for 40 min at RT. Approximately 20–30 min after stopping dye loading by refreshing the buffer, the dishes were transferred to an inverted microscope (Axiovert 100M; Zeiss) equipped with a ×16 water immersion objective (Zeiss; Plan-neofluar; numerical aperture 0.8) in a CLSM system (Zeiss; LSM-510). Digital images (size 368.5 × 368.5 μm) were recorded (usually for a period of 40 min) at RT with a spatial resolution of 256 × 256 pixels and a temporal resolution of one image per 6 seconds. The 488-nm argon laser line (200 mW) was used to excite Fluo-4 fluorescence in the cells, which was measured using a long-pass 505-nm filter. Laser illumination intensity was kept to a minimum (max 1% of laser output) to avoid phototoxicity and photobleaching. The calcium signals of the cultured DRG neurons were recorded in the culture dishes. All fluorescence measurements were made from subconfluent areas of the dishes, enabling identification of individual cells. To study intracellular calcium changes in response to application of KCl, DNP-HSA and BSA, these compounds were dissolved in ACSF buffer and perfused through the bath in a constant flow rate (4 ml/min) by using of a 4-channel perfusion setup. Because the culture dishes with DRG neurons also contained some fibroblasts all neurons within the image were identified by visual inspection. Criteria used were shape, diameter, and thickness of the soma, presence of neurites, and neurite size. Fibroblasts were excluded from further analysis.

Image data were analyzed off-line using the Zeiss LSM510 analyzing software V2.53. A selected image in each image set was used as a template for designating each neuronal soma as a region of interest. The Ca^{2+} response to depolarization with 70mM KCl was used to localize a subconfluent area with vital neurons. At the end of each experiment depolarization with KCl was again performed and neurons lacking a Ca^{2+} response were excluded from

further analysis. Because Fluo-4 is a single-wavelength indicator, it was not possible to apply the ratiometric method for quantitative determination of $[Ca^{2+}]_i$. Therefore, data were normalized with respect to the mean fluorescence intensity (F_0) during approximately the first 3 min of the recording. The temporal fluorescence intensity of the dye during the 40-min recording was divided by F_0. The relative fluorescence (RF) obtained in this way represents integrated $[Ca^{2+}]_i$. The RF values of each region of interest were plotted as a function of time. The amplitude of the responses of neurons to application of DNP-HSA was quantified as the RF level reached at the end of the 40 min measuring period using the last 15 images of the recording.
Neurons were identified as being responsive on the basis of the following criteria: 1) a initial and transient response to application of DNP-HSA (*see Results*) followed by 2) a clear and gradual rise in RF values as a function of time and 3) a RF value at the end of the DNP-HSA application exceeding 1,2. The percentage of responsive neurons was determined by dividing their number by the total amount of vital neurons in that particular recording. The lag time of the neural response to application of specific compounds was determined by measuring the time between compound application (as recorded by computer) and the first noticeable rise in RF.

Materials L-glutamine, cytosine β-D-arabinofuranoside (ara-C), mowiol, diaminobenzidine (DAB), DNP-HSA, pluronic F-127 and BSA were all purchased from Sigma Chemical Co., St. Louis, MO, USA. Collagenase A was purchased from Roche Diagnostics, Almere, the Netherlands. The following materials were obtained from Invitrogen, Breda, the Netherlands: L15 medium, DMEM F12, DMEM high glucose without glutamine, gentamicine and N2 supplement. FCS came from Perbio, Ettenleur, the Netherlands. Glass bottom culture dishes were bought at MatTek Cultureware, Asland, MA, USA. The Cy-3-conjugated donkey anti-rabbit antibody was from Jackson ImmunoResearch Europe, Soham, UK. Rabbit anti-PGP 9.5 was purchased from Biognost, Heule, Belgium. Rabbit anti-FITC and HRP-conjugated goat anti-rabbit IgG were both from DakoCytomation, Heverlee, Belgium. Molecular Probes, Eugene, OR, USA supplied the Fluo-4 AM.

Statistics The responses to stimulation of IgLC- and IgE-sensitized DRG neurons with DNP-HSA were subjected to a two-way analysis of variance to test whether the responses on the different days of the culture could be pooled for further analysis. These statistical tests revealed no difference between the different days of culture within experimental conditions. Consequently, results obtained at different days were pooled, which resulted in mean values for the response amplitude for every experimental group. Responses in control groups and non-responsive neurons from experimental groups were compared in a one-way analysis of variance. In experimental groups (IgLC- and IgE-sensitized) responsive neurons were distinguished from non-responsive neurons (see criteria above) and comparisons were made by an unpaired *t*-test. The results in figure 4 are presented in a box-and-whisker plot. All values were expressed as means ± sd. $P<0.05$ was considered to be statistically significant.

Results

Validation of primary DRG neuron cultures

Staining with the pan-neuronal marker PGP 9.5 showed that the cultured DRG neurons consisted of a heterogeneous population with respect to neural size and shape (figure 1). After 2 days, the DRG neurons exhibited full-size neurite outgrowths contacting with other neurites and neuronal cell bodies. This observation is in agreement with previous studies in which DRG neurons were cultured in a similar way (24). Neurite growth continued up to day 5 of culture, after which degradation of the neuronal cells took place (personal observations, data not shown). Next to specialized synaptic terminals, DRG neurons in culture also developed relatively non-specialized vesicle-containing axonal enlargements, designated as varicosities (25, 26). In general, varicosities develop along unmyelinated axons and exhibit neuropeptidergic immunoreactivity (27). Newly grown dendrites originating from neuronal cell bodies in our DRG neuron culture also developed varicosities (arrows in figure 1).

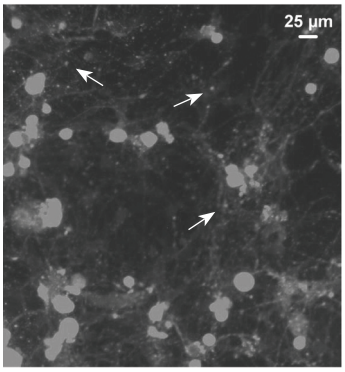


Figure 1 *Immunohistochemical staining for PGP 9.5 showing that the primary culture of DRG consists of a heterogeneous population of neurons with different soma size and shape. The neurons exhibit outgrowths of neurites that establish contacts with each other and with other neurites over long distances. The arrow in the picture indicates newly grown axonal enlargements, i.e., varicosities.*

IgLC and IgE bind specifically to DRG neurons

To determine binding affinity of IgLC, IgE and IgG to DRG neurons, binding studies were performed via immunohistochemical staining. Incubation with 25 µg/ml IgLC, IgE and IgG per DRG neuronal culture demonstrated that IgLC (figure 2A) and IgE (figure 2B) were capable of binding to all neuronal cell bodies as well as to the neurites and the varicosities. This binding was dose-dependent and 25 µg/ml per DRG neuronal culture was found to be the most effective dose, labeling most neurons without background staining (data not shown). However, DRG neurons did not show binding activity for IgG (figure 2C). The images shown in figure 2 are representatives of 4 separate experiments (different DRG neuron cultures) in which each staining condition was performed in duplicate.

Specificity of the binding activity of IgLC and IgE to the neuronal DRG cells was investigated by using of a competitive binding assay (28). Pre-incubation

Figure 2

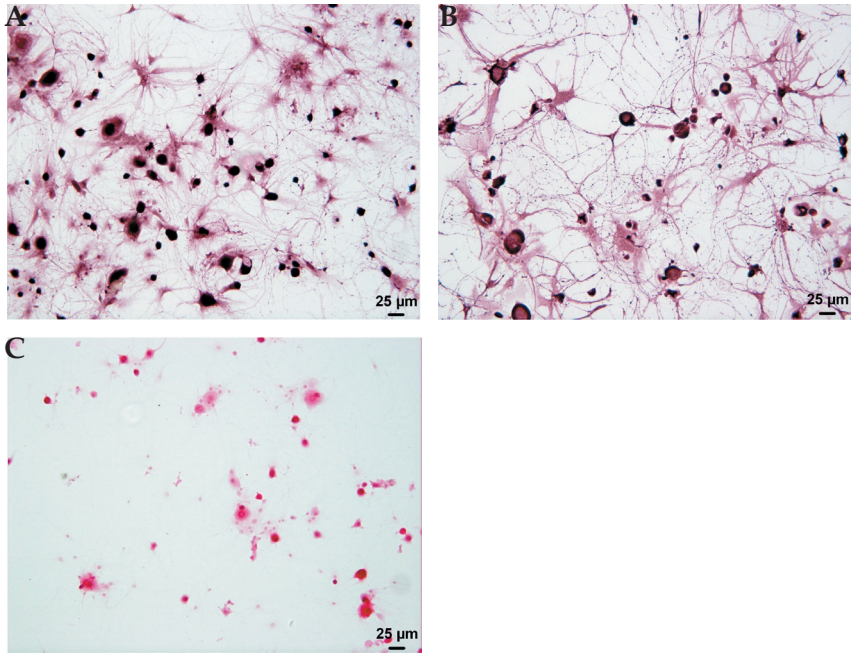
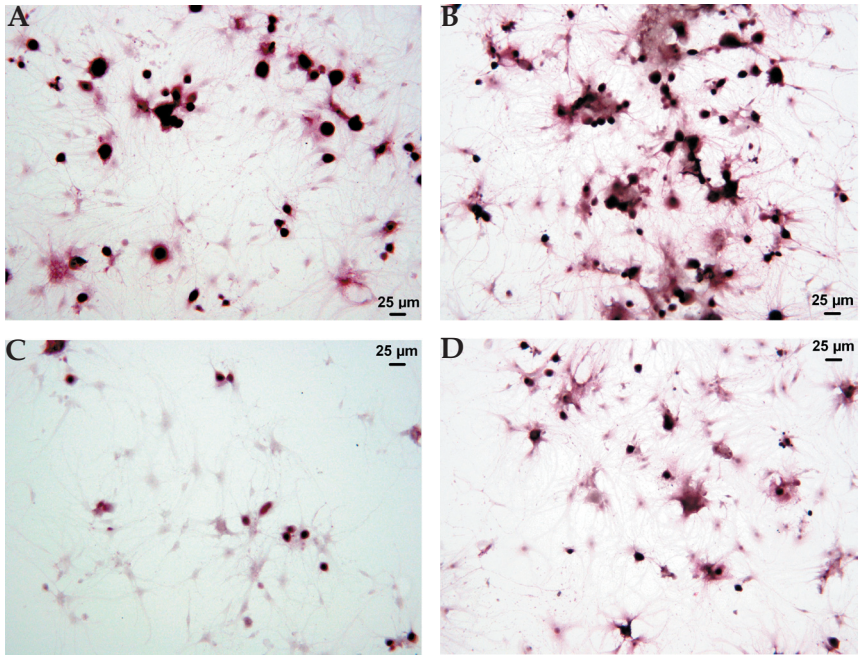


Figure 3



with 250 μg/ml of either unlabeled (i.e. cold) IgLC (figure 3C) or unlabeled IgE (figure 3D) prior to incubation with FITC-labeled IgLC or FITC-labeled IgE resulted in less intense staining (figure 3). This indicates that the labeled and unlabeled ligands compete for specific binding sites on the DRG neuronal cells and demonstrates the specificity of IgLC and IgE binding activity to the DRG neurons. The images shown in figure 3 are representative of 2 separate experiments (different DRG neuron cultures) in which each staining condition was performed in duplicate.

Antigen-specific activation of IgLC and IgE increases $[Ca^{2+}]_i$ in DRG neurons

The $[Ca^{2+}]_i$ of individual DRG neurons (day 2 to 5 of culture) loaded with Fluo-4 was recorded in culture dishes using a multi-channel perfusion system (ACSF; 4 ml/min). Recordings were made from locations (size 368.5 x 368.5 μm; one per dish) which displayed a representative number of DRG neurons (on average 20). The viability of these neurons was assured by recording their rapid and reversible Ca^{2+} response to a 10 s application of 70 mM KCl (RF 1.7 ± 0.3; n=37). Continuous perfusion of the dishes with ACSF for 40 min did not induce any change in neural $[Ca^{2+}]_i$ levels (RF 1.1 ± 0.1; n=19) (figure 4A). A few neurons and neurites showed spontaneous activity consisting of rapid rises in $[Ca^{2+}]_{I,}$ which quickly returned to baseline values.

The neurons used to measure Ca^{2+} responses to antigen-specific activation of IgLC (25 μg/ml) and IgE (25 μg/ml), were sensitized by incubation with IgLC or IgE for 90 min just prior to loading with Fluo-4 and a 40 min Ca^{2+}-recording. The duration of this sensitization was based on the optimal incubation time determined in the binding studies. After about 3 min of baseline Ca^{2+} recording the perfusion was switched to ACSF containing DNP-HSA (10 ng/ml), which induced within 10.2 ± 3.6 s (n=59) a relatively small and transient rise in neural $[Ca^{2+}]_i$ in all viable neurons, sensitized as well as non-sensitized (arrows in figure 5B and 6B). This Ca^{2+} response had a duration of about 3 min and an amplitude usually smaller than 1.2 RF. Since in control experiments application of BSA (10 ng/ml) induced a similar response in all neurons, most likely this transient Ca^{2+}-response could be ascribed to a non-specific effect of the protein coupled to DNP on the neuronal membrane. This suggestion is supported by the observation that the transient responses for all neurons in a dish had an

Figure 2 *Binding activity of FITC-labeled IgLC (**A**), IgE (**B**) and IgG (**C**) to primary cultured DRG neurons. The designated primary antibody (25 μg/ml) was incubated for 90 min after which the FITC label was detected with a secondary and a tertiary antibody, visualized with DAB/nickel staining resulting in black spots. The morphological structure was visualized with an eosin background staining revealing that all primary cultured DRG neurons show binding affinity on both cell bodies and neurites, for IgLC and IgE, but not for IgG. The images are representative of 4 separate binding experiments performed in duplicate.*

Figure 3 *Specific binding activity of FITC-labeled IgLC and IgE. Unlabeled (i.e. cold) IgLC or IgE (250 μg/ml) competed with FITC-labeled IgLC and IgE for specific binding sites on primary cultured DRG neurons. (**A**) IgLC, and (**B**) IgE staining of neuronal cells without competition for specific binding sites. After pre-incubation with cold ligand for IgLC (**C**) and IgE (**D**) 15 min before FITC-labeled IgLC or IgE was added, reduced staining activity was observed. The images are representative of two separate experiments performed in duplicate.*

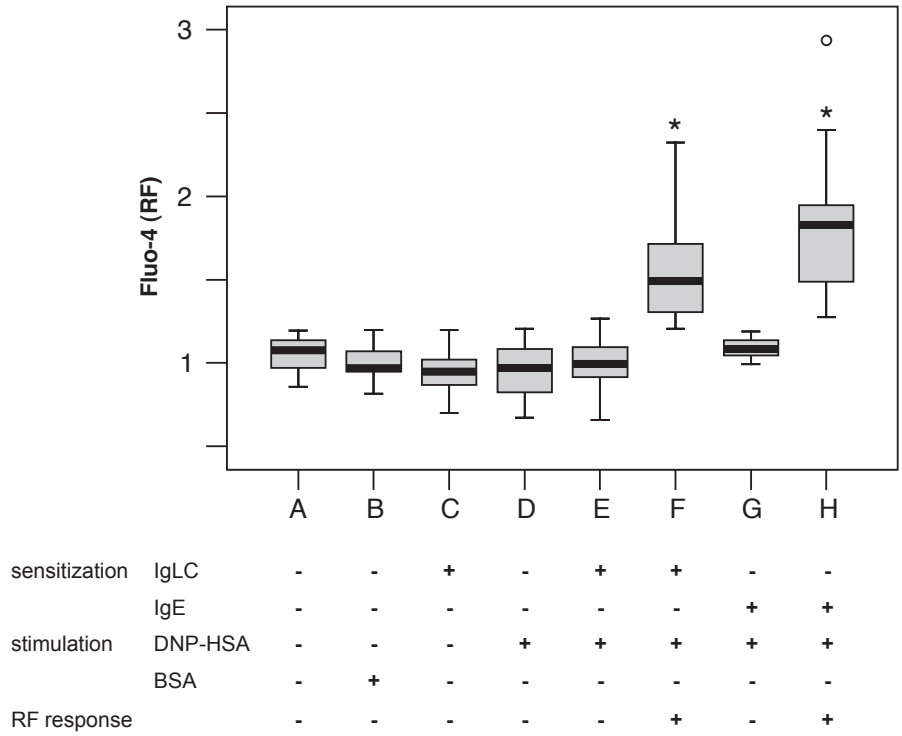


Figure 4 *RF values of non-sensitized, IgLC-sensitized and IgE-sensitized DRG neurons after 40 min of recording. The boxes represent the following applications: (**A**) non-sensitized DRG neurons, no DNP-HSA application (n=19); (**B**) non-sensitized DRG neurons, application of 10 ng/ml BSA (n=18); (**C**) IgLC-sensitized DRG neurons, no application of DNP-HSA (n=21); (**D**) non-sensitized DRG neurons, application of 10 ng/ml DNP-HSA (n=94); (**E**) IgLC-sensitized DRG neurons, non-responsive to application of 10 ng/ml DNP-HSA (n=66); (**F**) IgLC-sensitized DRG neurons, responsive to application of 10 ng/ml DNP-HSA (n=35); (**G**) IgE-sensitized DRG neurons, non-responsive to application of 10 ng/ml DNP-HSA (n=14); (**H**) IgE-sensitized DRG neurons, responsive to application of 10 ng/ml DNP-HSA (n=19). Results are presented as a box-and-whiskers plot. The n represents the number of DRG neurons. *p<0.001 compared to non-responding neurons.*

identical lag time (data not shown).
Perfusion of the dishes with DNP-HSA however, also led to a much slower and more gradual rise in $[Ca^{2+}]_i$ in about 35 % (35/101; 9 cultures) of the IgLC-sensitized DRG neurons. A representative example is shown in figure 5. This Ca^{2+} rise started after a lag time of about 11 ± 8 min (n=35) and persisted up to the end of the 40-min DNP-HSA perfusion. At the end of the recording period, the RF of the responding neurons (RF 1.6 ± 0.3; n=35) was higher than that of the non-responding neurons (RF 1.0 ± 0.1; n=66) (figure 4E/4F; P< 0.001). Control experiments showed that application of 10 ng/ml BSA (RF 1.0 ± 0.1; n=18) or DNP-HSA (RF 0.9 ± 0.1; n=94) to non-sensitized DRG neurons during

the 40-min period did not alter the $[Ca^{2+}]_i$ concentration (figures 4B and 4D respectively). In addition, sensitization of DRG neurons with IgLC alone did not result in changes in intracellular Ca^{2+} concentrations during the subsequent 40 min period with ACSF (RF 1.0 ± 0.1; n=21) (figure 4C).
Activation of IgE-sensitized DRG neurons with DNP-HSA resulted in similar responses as seen in IgLC-sensitized DRG neurons, as illustrated in figure 6. About 58% (19/33; 3 cultures) of the IgE sensitized neurons showed a gradual rise in $[Ca^{2+}]_i$ which started after a lag time of about 10 ± 7 min (n=19). At the end of the 40-min recording period, the RF of the responding IgE-sensitized neurons (RF 1.8 ± 0.4; n=19) was higher than that of the non-responding neurons (RF 1.1 ± 0.1; n=14) (figure 4G/4H; $P< 0.001$).
The data obtained in this study indicate that an antigen-induced increase in $[Ca^{2+}]_i$ of DRG neurons can be mediated via IgLC and IgE bound to the neuronal cell membrane.

Discussion

It has been discovered recently that IgLC is able to mediate hypersensitivity responses in skin and airways by provoking the activation of mast cells (4, 5). The present study demonstrates that IgLC and IgE can also specifically bind to cultured DRG neurons and induce an increase in $[Ca^{2+}]_i$ in these neurons after antigen-specific stimulation.
The binding studies demonstrated that IgLC and IgE are capable of binding to most neurons in the culture. However, not all IgLC- and IgE-sensitized neurons showed a Ca^{2+} response to the application of DNP-HSA. Most likely, this can be ascribed to the fact that cultured DRG neurons exhibit different phenotypes with different sensory function (29, 30) and may therefore not be susceptible to antigen stimulation. Phenotyping the DRG neurons that are responsive to DNP-HSA application remains to be performed.
The lag time of the response of IgLC- and IgE-sensitized DRG neurons was approximately 11 min. Since the kinetics of IgE-mediated activation of DRG neurons have not yet been described and studied, we compare the timing of the response to IgE-mediated mast cell activation. Aggregation of IgE-FcεRI on mast cells by multivalent antigen generates a complex cascade of responses and occurs within the first minute after binding of the antigen to the specific IgE (31). However, Xu *et al.* demonstrated that individual DNP-BSA groups become gradually exposed and accessible for binding to IgE when bound to serum albumin (32). Therefore, the binding of DNP-BSA to IgE was relatively slow (> 2 min) compared to the binding of monovalent DNP (32). This is in agreement with our results showing a relatively slow Ca^{2+}-response to the application of DNP-HSA. It can be concluded that this is due to the time needed for binding of DNP-HSA to the membrane-bound Ig molecules rather than to crosslinking of the receptors.
Comparison of IgLC-induced antigen-specific neuronal and mast cell activation discloses another analogy in the difference of the percentage of IgLC- and IgE-sensitized neurons responding to DNP-HSA (35% versus 58%

Figure 5

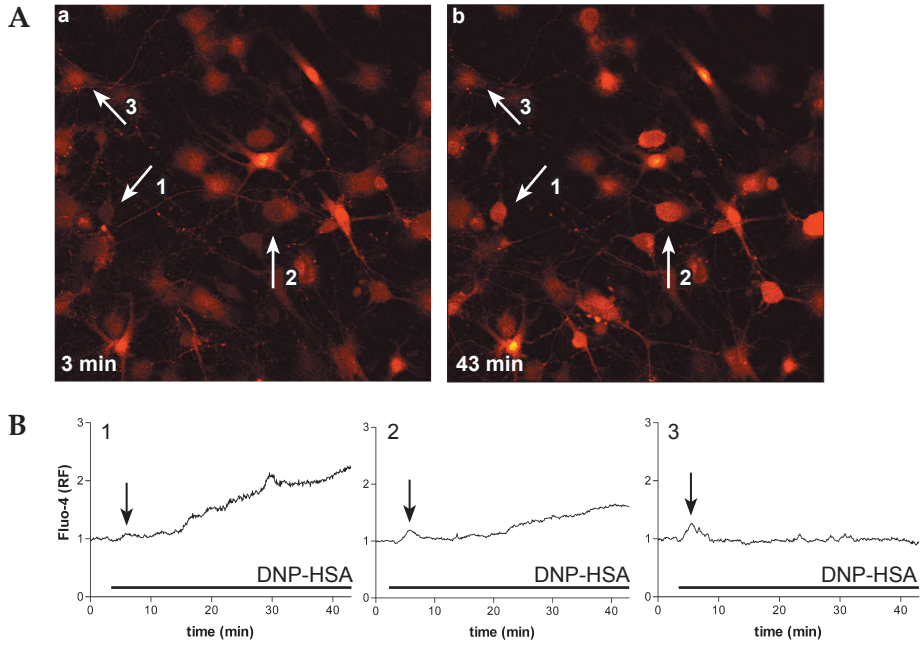
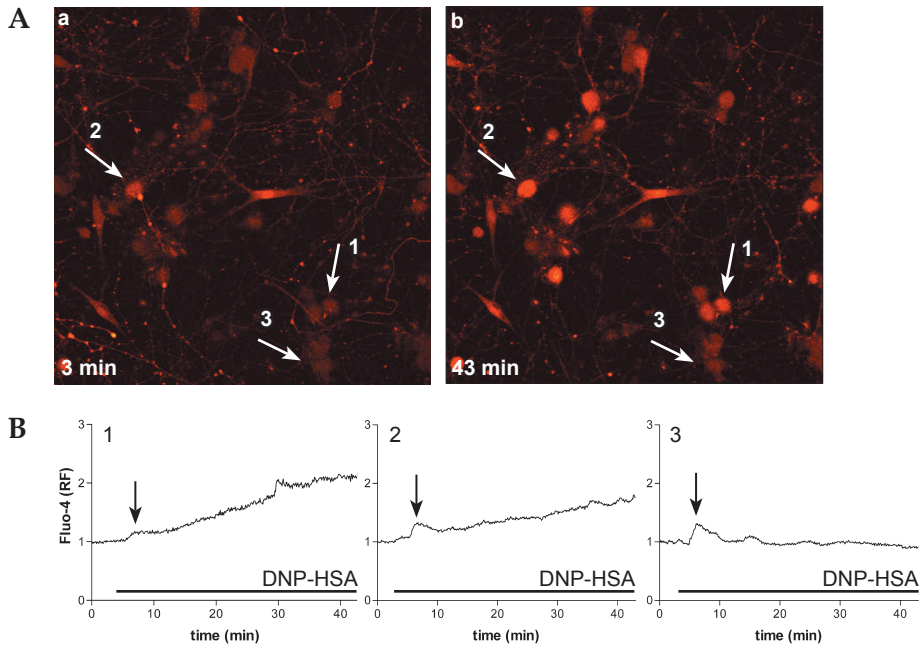


Figure 6



respectively). Measurement of mast cell activation by means of the release of β-hexasominidase demonstrated that 55% of IgE-sensitized mast cells were activated after antigen-specific stimulation compared to approximately 20% of IgLC-sensitized mast cells (4).

Our findings are in agreement with the work of Andoh and Kuraishi, who demonstrated that Ig-antigen complexes could directly enhance the influx of Ca^{2+} ions and therefore evoke activation of DRG neurons (33). Moreover, expression of the high affinity receptors for IgG and IgE, FcγRI and FcεRI respectively, on all DRG neurons was observed, regardless of size and shape (33, 34). Differences with our observations are probably due to different culture conditions.

Although there are several lines of evidence suggesting the presence of a putative receptor on mast cells for IgLC, it has not been identified yet (4). Studies using knockout mice for γ-chain-associated receptors, such as FcεRI, FcγRIII and PIR-A demonstrated that these receptors are not involved in IgLC-mediated mast cell activation (4). Currently, a mast cell membrane-associated protein that interacts with IgLC is isolated in our laboratory and investigations are directed to the identification of this protein. This IgLC-binding protein has also been established on the cultured DRG neurons (our unpublished observations). The results from this study open up new opportunities to establish the identity of the IgLC receptor.

The role of sensory neurons in hypersensitivity responses has been well established and described as non-specific neurogenic inflammation (35). Besides mechanoreceptive A-fibers, DRG neurons also exhibit unmyelinated C-fibers which are mainly of the nociceptive type (36). C fibers have an important function in transducing pain (37, 38), itch (37, 39) and flare signals (40). The observation that IgLC and IgE can bind to DRG neurons and subsequently increase intracellular calcium suggests an antigen-specific neuronal activation pathway in these responses. Complexes of Ig molecules and antigens are

Figure 5 *Typical examples of the [Ca2+]i response of IgLC-sensitized DRG neurons to application of DNP-HSA. (A) time-lapse Fluo-4 images of DRG neurons. DNP-HSA was applied to the bath at 3 min (**Aa**) and continuously perfused up to 43 min (**Ab**). In figure A three representative neurons are indicated with arrows and numbered to correspond with the activation patterns depicted in figure B. (**B**) RF as function of time of IgLC-sensitized DRG neurons. Neurons 1 and 2 are examples of neurons responding to DNP-HSA application whereas neuron 3 is representative of a non-responsive DRG neuron. The bar indicates continuous application of DNP-HSA. In this particular recording 10 out of 16 vital neurons were responsive to the application of DNP-HSA. Note the small transient response shortly after application of DNP-HSA as indicated by the arrow.*

Figure 6 *Typical examples of the [Ca2+]i response of IgE-sensitized DRG neurons to application of DNP-HSA. (A) time-lapse series of Fluo-4 images of DRG neurons. DNP-HSA was applied to the bath at 3 min (**Aa**) and continuously perfused up to 43 min (**Ab**). In figure A three representative neurons are indicated with arrows and numbered to correspond with the activation pattern in figure B. (**B**) RF as function of time of IgE-sensitized DRG neurons. Neurons 1 and 2 are examples of neurons responding to DNP-HSA application whereas neuron 3 is representative of a non-responsive DRG neuron. The bar indicates continuous application of DNP-HSA. In this particular recording 10 out of 15 vital neurons were responsive to the application of DNP-HSA. Note the small transient response shortly after application of DNP-HSA as indicated by the arrow.*

suggested to activate primary afferent sensory neurons and therefore may contribute to sensations such as pain, itch and inflammatory responses. To our knowledge, we are the first to demonstrate this new role for IgLC in the pathophysiology of inflammation.

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7

Clinical relevance of IgLC in inflammatory bowel disease and irritable bowel syndrome

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Abstract

Inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) are interrelated intestinal disorders both related with increased numbers of mast cells and mast cell activation in the gastro-intestinal tract. Immunoglobulin-free light chains (IgLC) were described to be able to mediate colonic hypersensitivity responses via mast cell activation. In this report we demonstrate increased serum concentrations of IgLC of patients suffering from IBD, which could be reduced by treatment with immunosuppressive drugs. Furthermore, we demonstrated that IgLC could be found in the intestinal mucosa and inflammatory lesions in biopsies of IBD patients associated with the presence of plasma cells and mast cells. Increased serum concentrations of IgLC were also observed in patients suffering from IBS. We propose increased levels of IgLC as potential biomarker for the development of either IBD or IBS in gastro-intestinal disorders associated with mast cell activation.

Introduction

Inflammatory bowel disease (IBD) comprises chronic, spontaneously relapsing inflammatory conditions of the gastro-intestinal tract with Crohn's disease (CD) and ulcerative colitis (UC) as major representatives (1). Patients diagnosed with this disorder are suffering from severe abdominal pain and cramps, diarrhea, rectal bleeding and a substantial personal burden. Although the exact etiology remains unclear, it is thought to be a complex interaction of genetic, environmental and immunological factors (2). IBD is interrelated with irritable bowel syndrome (IBS), a mild intestinal disorder highly associated with abdominal pain, altered bowel motility and an increase in visceral hypersensitivity (3). Most of the time the mucosal structure appears normal, however sometimes slight mucosal inflammation can be observed (4). The pathogenesis of IBS is, like IBD, poorly understood and defined, but implicate psychological factors, food hypersensitivity and gastro-intestinal infections (5). Further analogy between IBD and IBS is demonstrated by the observation of increased numbers of mast cells and mast cell activation in patients suffering from either IBD or IBS associated with disease activity (6-9).

Mast cells are versatile cells which can release a variety of pro-inflammatory mediators upon activation and degranulation (10). Traditionally, antigen-specific mast cell activation is thought to mainly occur via crosslinking of IgE antibodies bound to the high affinity IgE-receptor, FcεRI present on the cell surface of mast cells. However, antigen-specific mast cell activation can also occur in the absence of IgE (11, 12). Recently, it was discovered in our laboratory that immunoglobulin-free light chains (IgLC) were capable of mediating mast cell-dependent hypersensitivity responses in mice (13). IgLC are produced by B lymphocytes in excess over heavy chains during the production of antibodies and subsequently secreted into the circulation (14). Antigen-specific IgLC was demonstrated to bind to mast cells and succeeding local stimulation with the corresponding antigen resulted in mast cell-dependent plasma extravasation and infiltration of inflammatory cells. In the skin this is accompanied with tissue swelling (13) whereas acute bronchoconstriction and airway hyperresponsiveness is observed in the airways (15). Clinical evidence for the role of IgLC in non-IgE-mediated immune disorders was provided by demonstrating significant increased concentrations of IgLC in the circulation of non-atopic asthma patients compared to healthy controls (15). Furthermore, multiple sclerosis and rheumatoid arthritis are described to be associated with increased levels of IgLC in cerebrospinal fluid (16, 17) and synovial fluid (18) respectively. These observations combined with the observation of mast cell activation in brain (19) and synovial tissue (20) evidence mounts for an important function for IgLC in (auto)immune disorders.

A clear association between IgE and the prevalence of IBS has not been demonstrated. Some studies suggest that IgE levels are only elevated when IBS patients are suffering from food intolerance and food allergy (21). Moreover, since IBD is also not associated with increased IgE levels (22), mast cells in these disorders are likely to be elicited via other mechanisms, suggested to be IgLC.

Although UC is associated with basal plasmacytosis (i.e. infiltration of plasma B cells) (23, 24) suggesting a humoral immune response in the pathogenesis, B cells and plasma cells are not intensively studied in IBD. Hitherto, no reports have been published on humoral immunity and IBS. In chapter 5 of this thesis it was demonstrated that IgLC are involved in a mast cell-dependent murine model of IBD. In this report we therefore examined λ and κ IgLC concentrations serum levels of IBD and IBS patients. We are, to our knowledge, the first to demonstrate increased concentrations of serum IgLC in IBD patients revealing new insights in a role for B lymphocytes and plasma cells. And furthermore, the first to link a B lymphocyte product (i.e. IgLC) with IBS by the observation that serum IgLC concentrations were increased in patients suffering from this disorder. Moreover, we show that IgLC can be found in colonic lesions as well as the inflamed as normal mucosa of the colon of IBD patients. The pathophysiological role of IgLC in IBD and IBS in relation to mast cell activation needs to be investigated in further studies.

Materials and methods

Serum κ and λ IgLC assays The concentration of κ and λ IgLC in serum samples obtained from Crohn's disease (CD) patients, post-infectious IBS patients and healthy controls (see table I for characteristics) was measured by ELISA as described previously with monoclonal antibodies specific for human IgLC (25). Serum specimens were appropriately diluted to obtain at least three data points within the linear portion of the standard curve and measured in a double-blind way. Serum samples from CD patients were obtained at different time points and concentrations of λ and κ IgLC were determined at all these time points. Subsequently, the concentrations were averaged per CD patient. All CD patients received treatment with infliximab either or not co-medicated with immunosuppressive compounds like azathioprine (n=29), methotrexaat (n=23), 5-ASA and methotraxaat (n=6) or thioguanide (n=8).

Patients and tissue specimens Intestinal tissue specimens of ileum and colon were obtained during endoscopy from patients suffering from Crohn's disease (CD) (n=8) or ulcerative colitis (UC) (n=8) who underwent internal inspection. Specimens were fixed in formalin and routinely embedded in paraffin. Sequential sections of 5 μm were subjected to immunohistochemical staining

Table I *Characteristics of IBD and IBS patients of which serum concentration of κ and λ IgLC were determined.*

characteristics	IBD	IBS	healthy controls
number	91	11	11
male	36	9	6
female	55	2	5
age (mean ± sd)	35.7 ± 11.4	34.5 ± 9.8	25 ± 5.4

described below. Control tissue specimens (n=5) were obtained from patients enduring endoscopy because of suspected IBD and were defined as controls after inspection by miscroscopical observation by a pathologist based on morphology, cellular infiltration and damage of the tissue.

Immunohistochemical staining for IgLC Paraffin sections of intestinal biospies of 5 μm were routinely deparaffinized and blocked for endogenous peroxidase (DakoCytomation) for 30 min. Primary mouse antibodies directed against κ or λ IgLC (kindly provided by A. Solomon, University of Tennessee, Knoxville, TN, USA) were incubated for 60 min (concentration 1:500). After incubation with primary antibodies, the sections were incubated with anti-mouse HRP-labeled polymer (DakoCytomation, EnVision$^+$ System.) for 30 min. Color was developed with using AEC substrate chromogen (DakoCytomation). Between incubation steps the sections were intensively rinsed with 0.05 M TBS containing 0.05% Tween. To maintain the quality of the staining the sections were enclosed in Faramount mounting medium (DakoCytomation). Within each test, negative controls were included and they were all found not to contain any specific staining.
Plasma cells were detected with rabbit anti-human CD79a (Neomarkers), followed by goat anti-rabbit AP-labeled polymer (DakoCytomation, PowerVision System). Mast cells were identified with AP-labeled mouse anti-tryptase (Chemicon). Both cell types were visualized with Fast Blue.

Statistics All values were expressed as mean ± sem. λ and κ IgLC concentrations in serum of IBD and IBS patients were analyzed with a *t*-test. The analysis of infliximab treatment either or not co-medicated with azathioprine, methotrexaat, 5-ASA and methotraxaat or thioguanide was performed using a one-way ANOVA followed by a Bonferroni multiple comparison test. $p<0.05$ was considered statistically significant.

Results

IBD and IBS patients show increased levels of circulating λ and κ IgLC

Serum samples from patients suffering from CD (n=91) and healthy controls (n=11) were randomly selected from a large specimen bank for quantitive λ and κ IgLC assays. We found that the serum concentrations of λ and κ IgLC were increased in IBD patients compared to healthy controls (figure 1A). Furthermore, figure 1B demonstrated that also the serum concentrations of λ and κ IgLC were elevated in patients suffering from IBS. However, this was less pronounced as observed in sera of IBD patients.

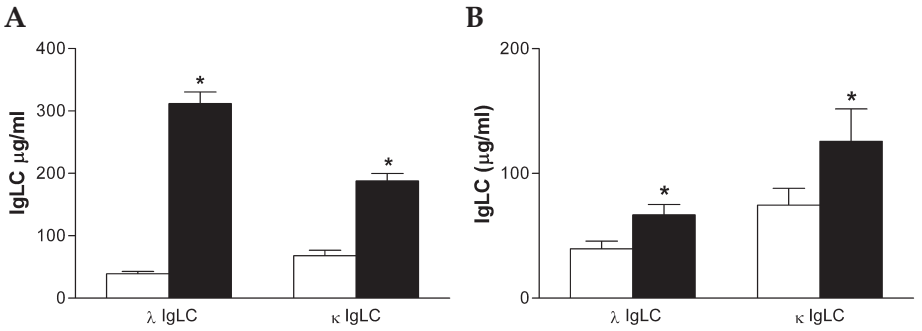

Figure 1 *Concentrations of λ and κ IgLC in serum of IBD and IBS patients. (A) Serum IgLC levels of IBD patients (λ IgLC n=91, κ IgLC n=91; black bars) were significantly increased with regard to serum IgLC levels of control subjects (n=11; white bars). (**B**) Serum IgLC levels of IBS patients (λ IgLC n=11, κ IgLC n=11; black bars) were significantly increased with regard to serum IgLC levels of control subjects (n=11; white bars); *p<0.001 and #p<0.05 compared to healthy controls.*

IgLC are located in the colon of IBD patients

To analyze the localization of IgLC, immunohistochemical staining with primary antibodies specific for λ IgLC was performed on intestinal tissue sections of both CD and UC patients. Figure 2 demonstrates representatives of positive λ IgLC staining in ileum and colon biopsies of IBD patients. Positive IgLC staining was observed in inflamed lesions as well as the normal and inflamed mucosa of the colon (figure 2A and 2B) and ileum (figure 2C and 2D) of CD patients. Colon biopsies of UC patients also demonstrated positive λ IgLC staining in the lesions and the mucosa (figure 2E en 2F). Although colonic lesions of both CD and UC showed positive staining for λ IgLC, the staining was more profoundly present in the colon of UC patients. Furthermore, positive staining for λ IgLC was co-localized with plasma B cells (figure 3A) and mast cells (figure 3B) in the mucosa.

Immunohistochemistry for κ IgLC was also performed, but no positive staining could be detected.

Immunosuppresive drugs reduce IgLC serum concentrations in Crohn's disease
All IBD patients received infliximab treatment either or not co-medicated with immunosuppressive drugs like azathioprine, methotrexaat, thioguanide or 5-ASA and methotrexaat. Figure 4 demonstrates that patients receiving only treatment with infliximab showed significantly higher concentrations of serum λ and κ IgLC compared to patients co-mediacted with azathioprine, methotrexaat or 5-ASA and methotrexaat. Although there was a descending trend in IgLC concentrations in patients treated with infliximab and thioguanide this was not significantly different from patients receiving infliximab alone (figure 4).

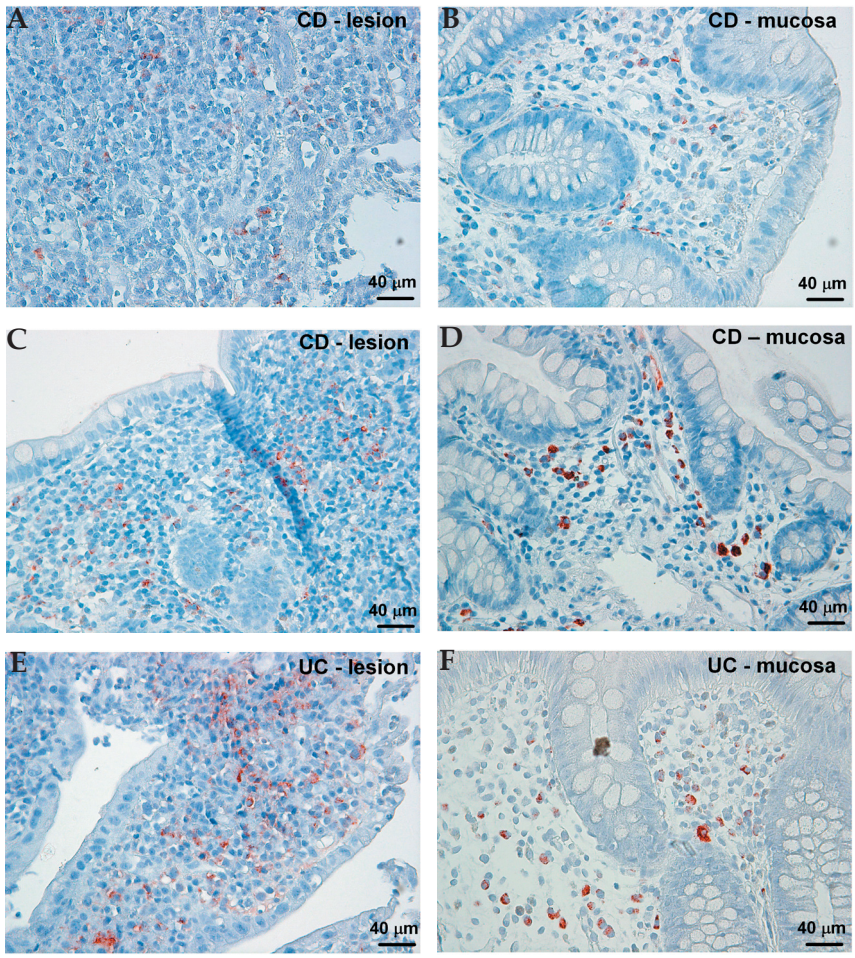

Figure 2 *Immunohistochemical staining forλ IgLC of human colon biopsies of IBD patients. (**A**) and (**B**): representative λ IgLC staining of colon biopsies of patient suffering from CD. (**C**) and (**D**): representative λ IgLC staining of ileum biopsies of patient suffering from CD. (**E**) and (**F**): representative λ IgLC staining of colon biopsies of patient suffering from UC. Figure A, C and E correspond to lesion/lymphoid structures in the human colon and ileum whereas figure B, D and F are biopsies of colon or ileum inflamed mucosa.*

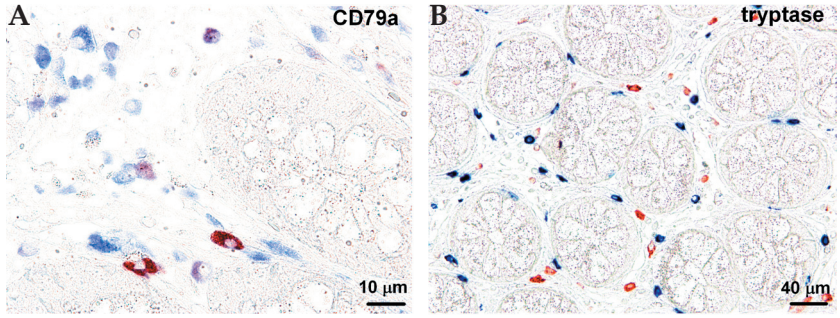

Figure 3 *Immunohistochemical staining for λ IgLC is co-localized with plasma cells and mast cells. Double-staining of λ IgLC with anti-CD79a (**A**) or anti-tryptase (**B**) demonstrates that λ IgLC is related with the presence of B cells and mast cells in the human intestinal mucosa. λ IgLC is visualized in blue whereas both plasma cells and mast cells are visualized in blue in the corresponding images.*

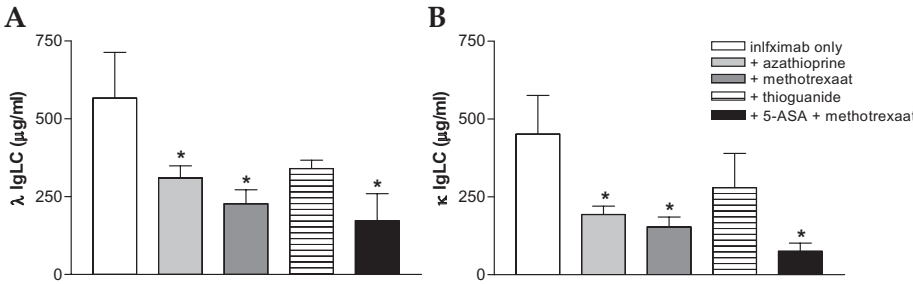

Figure 4 *IgLC serum levels of IBD patients receiving infliximab (human/mouse monoclonal anti-TNFα antibodies) medication either with or without co-medication with immunosuppressive drugs. Both λ (**A**) and κ (**B**) IgLC serum levels of IBD patients receiving infliximab with co-mediaction azathioprine (n=29), methotrexaat (n=23), or 5-ASA and methotraxaat (n=6) are significantly decreased compared to subjects receiving infliximab only. Patients taking thioguanide co-medicated with infliximab (n=8) did not show different levels from patient receiving infliximab alone. *p<0.01 (one-way ANOVA followed by a Bonferroni multiple comparison test).*

Discussion

In this report we evaluated κ and λ IgLC concentrations in serum samples of IBD and IBS patients. We are the first to report increased levels of IgLC in serum of patients suffering from these two disorders compared to healthy controls. Moreover, λ IgLC were localized in the lesions and in the mucosa of intestinal tissue of patients with ulcerative colitis (UC) and Crohn's disease (CD). The intensity of the staining observed in the inflamed lesion of the colon was more distinct than the one detected in the normal and inflamed mucosa. Furthermore, both the lesion and inflamed colon tissue specimens of UC patients showed a higher intensity in IgLC compared to patients suffering from CD. UC is a disorder associated with increased basal plamacytosis (i.e.

infiltration of plasma B cells) (23, 24). Moreover, plasma cells are the cellular source for IgLC so increased numbers of plasma cells by plasmacytosis leads inevitably to enhanced concentrations of IgLC and therefore more intense staining. In contrast, CD is a disorder not accompanied with concomitant plasmacytosis (26). However, an intense λ IgLC staining could be observed in the inflamed mucosa of the ileum of CD patients, which was not observed in the lesion of the ileum. This suggests that the inflammation observed in CD patients was more prominent in the ileum than in the colon.
Localization of free κ IgLC with immunohistochemistry was also performed; however no positive staining could be detected in all the intestinal biopsies. This could be explained as follows. First, there are no free κ IgLC present in the colon of IBD patients. This is very unlikely since high κ IgLC levels could be detected in serum samples of these patients and the same antibody was used for immunohistochemistry as for measuring serum IgLC with ELISA. Second, the conditions during the immunohistochemical staining for κ IgLC were not optimal. This is a more plausible explanation because the antibody has previously been successful in demonstrating κ IgLC in human tissue, although this was not of intestinal origin. More experiments need to be conducted to find the optimal conditions for κ IgLC staining on human intestinal biopsies.
Mast cells were identified with anti-tryptase staining in IBD. They were demonstrated to be related with the regions in which IgLC was observed. Current experiments are directed to co-localize IgLC and mast cells with immunohistochemical double-staining in more detail.

Immunosuppressive drugs like azathioprine, methotrexaat and thioguanine are widely used to dampen the inflammatory response in IBD (27). Both azathioprine and thioguanine are purine antagonists whereas methotrexaat is a folic acid antagonist. The action of these compounds is predominantly based on the abrogation of lymphocyte proliferation by inhibiting DNA synthesis. This could also lead to subduing the production of IgLC. As been demonstrated in this report, immunosuppressive drugs co-medicated with infliximab could significantly reduce the serum concentrations of IgLC in IBD patients compared to treatment with infliximab alone. Although there was a trend observed in descending IgLC serum levels after co-medication with thioguanide, this was not statistically significant.
The drug 5-ASA has an anti-inflammatory effect by the inhibition of COX-1, the enzyme responsible for the conversion of arachidonic acid into pro-inflammatory prostaglandins. In addition, 5-ASA has no effect on lymphocyte proliferation. The decrease observed after co-medication with 5-ASA and methotrexate can therefore be described to immunosuppression by methotrexate.

As described earlier, antigen-specific IgLC is able to bind to mast cells and re-exposure to the corresponding antigen leads to activation and degranulation of the mast cell resulting in the release of pro-inflammatory mediators (13). The increased concentrations of IgLC measured in serum of IBD and IBS patients suggest this IgLC-mediated mechanism for mast cell activation associated with these disorders.

Moreover, a bi-directional relation exists between IBS and IBD (28, 29). IBS patients can develop chronic inflammation characterized by an increase in mast cells, enteroendocrine cells, T lymphocytes and gut permeability accompanied with increased mucosal permeability (30, 31). And conversely, IBD patients in remission are reported to have IBS-like symptoms (32). Moreover, mast cells and mast cell activation remain present during the remission phase in UC patients (33).

Increased concentrations of serum IgLC in IBD and IBS patients could suggest a mechanism of the antigen-specific mast cell activation in these diseases. Mediators released by mast cells after moderate degranulation could induce hyperalgesia by activation of sensory nerves or diarrhea by increasing epithelial permeability resulting in IBS-related symptoms (3). However, more pronounced mast cell activation can also initiate severe inflammatory responses like accumulation and activation of inflammatory cells, and tissue damage as observed during the exacerbation of IBD (10). The measured serum concentrations of IgLC were 2-4 fold higher in IBD patients compared to IBS patients (figure 1). The level of IgLC serum concentrations could be indicative for the development of either severe (IBD) or mild (IBS) manifestation of the inflammatory response in the gastro-intestinal tract.

Based on the findings presented in this report, we hypothesize a functional role for IgLC associated with mast cell activation in the relation between IBD and IBS. We suggest increased IgLC serum concentrations as potential biomarker for the development or exacerbation of either IBD or IBS in gastro-intestinal inflammatory disorders.

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8

General Discussion

Introduction

Mast cells and sensory nerves are located in close proximity in a variety of tissues and the interaction between these two cell types is well described (1, 2). Besides an anatomical relation, mast cells and nerves also exhibit a functional link (3). Mast cell mediators have been demonstrated to sensitize and activate sensory nerves, which accordingly release neuronal mediators. These neuropeptides can in turn induce activation of mast cells. In general, it can be stated that mast cell-nerve interactions can contribute considerably to the amplification and spreading of inflammatory conditions as observed in inflammatory bowel disease (IBD).

The term IBD is used to describe idiopathic conditions of the gastro-intestinal tract characterized by swings between intense inflammation and periods of remission. Patients suffering from IBD experience abdominal pain and cramps, disrupted digestion, diarrhea, rectal bleeding and a substantial personal burden.

Although a variety of inflammatory cells and mediators are involved and intensively studied in IBD, little is known about the role of mast cells and nerves and the neuroimmune interaction in this inflammatory condition. However, in mucosal lesions of IBD patients increased numbers of mast cells can be found and evidence for mast cell activation is presented (4, 5). Proliferation of sensory nerve fibers is also observed at sites of intestinal inflammation (6). This is associated with neuronal activation since elevated concentrations of peptidergic mediators can be detected (7). Moreover, it was demonstrated that the proximity of mast cell and nerves becomes even closer during human intestinal inflammation (8).

Even though the pathophysiology of IBD is gradually being unraveled, it remains a complex interaction of genetic, environmental and immunological factors (9, 10). Hence, insight in the relation between mast cells and nerves could help to obtain a better understanding of the etiology of chronic inflammatory intestinal disorders. This thesis focuses on the mast cell-nerve interaction in the development of IBD.

Mouse model for colonic hypersensitivity

A wide variety of animal models exists to study intestinal inflammation, reviewed in (11) and (12), and the list is still growing. The different animal models can roughly be divided into four groups (11, 12): 1) spontaneous colitis as a result of naturally occurring genetic abnormalities, 2) spontaneous colitis as a result of defects in particular genes obtained by gene targeting or introduction of a transgene, 3) induction of colitis via deregulation of the immune response by transferring T-cell populations lacking regulatory lymphocytes into immune-incompetent animals, and 4) colitis induced by exposure to an haptenating or other causative agent.

The last group consists of a wide variety of different noxious substances ranging from damage-induced to more immunological-mediated colitis.

The most intensively studied models are chemically-induced models like trinitrobenzene sulfonic acid (TNBS)-induced and dextran sodium sulfate (DSS)-induced colitis.
DSS-induced colitis is characterized by disruption of the epithelial lining resulting in luminal bacterial translocation. Since DSS can generate colitis in the absence of lymphocytes (13), this animal model is more appropriate to study epithelial responses to mucosal injury and reactions associated with the acute phase of the inflammatory responses rather than to investigate the immunological processes involved in the inflammation.
The TNBS-induced colitis model is an antigen-induced model used to investigate the biochemical pathways of the inflammation. The administration of TNBS is usually accompanied with 50% ethanol to break the mucosal barrier. However, it has been demonstrated that 50% ethanol alone is also capable of inducing colitis (14) suggesting that this TNBS model is not only an immunological model induced by an antigen but also a damage-induced model dealing with a change in epithelial barrier function. Furthermore, this TNBS-induced model is predominantly associated with infiltration and proliferation of Th1 lymphocytes and the role of mast cells in this model is debatable (11). Several authors showed that mast cells do not play a significant part in TNBS colitis (15, 16), while Xu *et al.* demonstrated that the mast cell is essential in the development of this antigen-induced experimental colitis model (17).
Conflicting data also exist about the role of NK_1 receptors and substance P in TNBS-induced colitis. In the rat TNBS-induced colitis model, treatment with the NK_1 receptor antagonist RP 67580 was demonstrated to reduce granulocyte infiltration into the colon during the initial phase of the response. However, long-term treatment failed to affect the severity of tissue injury (18). McCafferty *et al.* also showed that granulocyte infiltration induced by TNBS in the rat colon could be attenuated by a substance P receptor antagonist (19). In contrast with the above observations, Reinshagen *et al.* demonstrated by the use of specific antagonists that substance P and calcitonin gene-related peptide (CGRP) mediated a protective effect of sensory neurons in TNBS colitis (20).
The contradictory observations in the TNBS-colitis model are likely to be the result of the perspective that this model is besides an immunological model induced by the antigen TNBS also a damage-induced model by 50% ethanol. This makes the TNBS-colitis model in its classical form (i.e. accompanied with 50% ethanol) not a useful model to study mast cells and sensory nerves, and the interaction between them in the initiating phase of the inflammatory response.

To study the role of mast cells and nerves in the onset of IBD, a novel murine antigen-induced colitis model was setup based on local non-IgE-mediated hypersensitivity responses in the colon. The administration of the antigen in this model is accompanied with 10% ethanol to break the mucosal barrier. This concentration of ethanol was not shown to induce intestinal tissue damage. The colonic hypersensitivity model and its validation in respect to mast cells and nerves is presented in this thesis in chapter 2-4.

The reaction evoked in the mouse colon to study mast cells and nerves is based on a delayed type hypersensitivity response. Delayed-type hypersensitivity is a non-IgE-mediated and T cell-dependent immune reaction that is manifested by an inflammatory response at the site of antigen provocation (21). Mast cells are widely recognized to also contribute to this reaction (22). This hypersensitivity response consists of an early and late phase, which are both mast cell-dependent. After first exposure to the antigen, B lymphocytes are stimulated to produce a factor identified as immunoglobulin-free light chain (IgLC) (23). This factor subsequently binds to the mast cell surface. In addition, antigen-specific T cells are produced. Re-exposure to the antigen results in stimulation of the antigen-specific IgLC that are bound to the mast cell, resulting in activation and degranulation. Prestored mediators released by mast cells (i.e. histamine, serotonin, TNFα, IL-6) can induce mucosal exudation and vascular permeability promoting the infiltration of antigen-specific T lymphocytes to the site of inflammation (early phase). Locally, these T cells can recognize the antigen presented by MHC class II on antigen-presenting cells. This T cell activation results in the production and release of cytokines, which further recruit inflammatory cells to the affected tissue (late/effector phase).
Skin sensitization with a low-molecular weight compound followed by local application with the corresponding antigen has long been known to result in hypersensitivity responses that resemble pathophysiological conditions of mast cell-dependent non-IgE-mediated disorders like cutaneous swelling, non-atopic asthma and gastro-intestinal inflammatory disorders (24-27). The requirement for mast cells to elicit these responses was demonstrated by the observation that hypersensitivity responses could not be induced in mast cell-deficient mice (24, 28, 29). Moreover, sensory neurons and neuropeptides are also demonstrated to contribute to the DTH reaction since depletion of sensory neurons of neuropeptides by capsaicin results in the abolishment of the inflammatory response (30). The use of specific NK_1 receptor antagonists and NK_1 receptor deficiency further established a functional role for neuropeptides in hypersensitivity responses in skin, airways and gut (31-34).

Chapter 2 of this thesis demonstrates that skin application of DNFB followed by an intrarectal challenge with DNS dissolved in ethanol results in the manifestation of hypersensitivity responses in the colon 72h after challenge. In contrast to the TNBS-induced colitis model where 50% ethanol is used, DNFB administration in this model is accompanied with 10% ethanol. Although deteriorated stool consistency in both control and experimental groups suggested that an irritant effect of ethanol is still present at 24h and 48h (personal observations; data not shown), this irritant effect of ethanol seems to have faded 72h after challenge. The immunological response at 72h after challenge was still profoundly present; therefore this time-point was chosen to study mast cells in colonic hypersensitivity.
The response at 72h after challenge is characterized by diarrhea formation, increased vascular permeability, colonic patch hypertrophy, infiltration of

inflammatory cells, proliferation of mast cells, activation of mast cells and increased TNFα production. Mast cells were demonstrated to be crucial for the development of the characteristics of colonic hypersensitivity since the features were absent in mast cell-deficient mice ($WBB6F_1$ W/W^v) and could be restored by reconstitution with *in vitro* cultured bone marrow-derived mast cells. Moreover, TNFα was shown to be mainly derived from mast cells. Therefore, an initiating role for TNFα in these responses is suggested in chapter 3.

The function of TNFα was established by the finding that treatment with anti-TNFα antibodies led to the abrogation of diarrhea, colonic patch hypertrophy and infiltration and accumulation of inflammatory cells (chapter 3). Because an increased number of mast cells, mast cell activation and elevated TNFα levels in the colon were still present irrespective of anti-TNFα treatment, it can be concluded that this protective effect of anti-TNFα took place downstream of mast cell activation. Because mast cell infiltration and activation is still present, but infiltration of inflammatory cells is abolished, these data further indicate that mast cell-derived TNFα plays a role in the infiltration of inflammatory cells. Treatment with the anti-inflammatory corticosteroid dexamethasone induced a comparable therapeutic effect on the development of DNFB-induced colitis in mice as anti-TNFα. (chapter 4). This demonstrates the therapeutic efficacy of the treatment with anti-TNFα antibodies.

A role for the NK_1 receptor and therefore indirectly for its ligand substance P is subsequently investigated by treating vehicle- and DNFB-induced mice with a specific NK_1 receptor antagonist (chapter 4). Blockage of the NK_1 receptor resulted in abolishment of diarrhea formation, inhibition of colonic patch hypertrophy, decreased number of mast cells in the colon and reduced infiltration of inflammatory cells.

The data obtained in this novel murine model for IBD establish an important role for both mast cells and neurons in the development of colonic hypersensitivity. Reducing the function of one of these cell types by either using mast cell-deficient mice, treatment with anti-TNFα antibodies or with a NK_1 receptor antagonist, is sufficient to abolish features of this response, like diarrhea formation, colonic patch hypertrophy and cellular infiltration. These findings suggest an important role for mast cell-nerve interactions in the colon during the development of local inflammatory responses. This chemically-induced immunological murine model for colitis is valuable to study this potential interaction in the onset of IBD.

Role for IgLC in colonic hypersensitivity

Although mast cells traditionally play a role in allergic responses, the involvement of mast cells in non-IgE-mediated hypersensitivity reactions has been well accepted (22). The activation of mast cells in non-IgE-associated disorders has long been thought to be mediated by an antigen-specific T cell

factor (35). However, further research has demonstrated that this T cell factor was equivalent to B cell-derived immunoglobulin-free light chains (IgLC) (23). IgLC were shown to be responsible for eliciting mast cell activation in non-IgE-mediated hypersensitivity reactions (23, 36).
In chapter 5 we investigated whether there a role could be ascribed to IgLC in colonic hypersensitivity responses. The compound F991 has high binding affinity for IgLC. Administration of F991 will decoy IgLC and therefore prevent this molecule from binding to mast cells. Therefore, F991 will inhibit antigen-induced IgLC-mediated mast cell activation. The effect of F991 has previously been shown to be effective in IgLC-mediated mast cell-dependent cutaneous swelling and acute bronchoconstriction (23, 36). Treatment with F991 in colonic hypersensitivity led to the abrogation of diarrhea formation, prevention of mast cell activation and inhibition of cellular infiltration (chapter 5).

As a possible local source for IgLC colonic patches can be addressed. Colonic patches consist mainly of large B lymphocyte follicles and smaller T cell clusters (chapter 2) (37, 38). Because IgLC is produced by plasma B cells, colonic patches could be a likely source of IgLC. Since colonic patch hypertrophy is unaffected by treatment with F991 this might indicate that production of IgLC is still occurring in F991-treated DNFB-sensitized mice. It could also be possible that IgLC is systemically produced by intestinal draining lymph nodes. Further research is necessary to elucidate on the source for IgLC in this murine colonic hypersensitivity model.

The number of mast cells present in the colon also remains unaffected by treatment with F991. However, mast cell activation was decreased in DNFB-sensitized F991-treated mice after challenge. These data demonstrate that F991 indeed was capable of preventing IgLC-induced mast cell activation, supposedly by binding this molecule with high affinity.

Role for IgLC in neuronal activation

In addition to the established function on mast cells, the mast cell-nerve interaction suggests a possible role for IgLC in neuronal activation. We therefore investigated the activity of IgLC on cultured dorsal root ganglion (DRG) neurons in chapter 6. Specific binding of IgLC to the somas as well as newly grown dendrites of the DRG neurons was observed. Stimulation of neuron-bound IgLC with the corresponding antigen led to an increase of intracellular calcium concentrations in the neurons. These data demonstrate that IgLC can mediate antigen-specific neuronal responses. However, it needs to be elucidated whether stimulation of IgLC results in excitation of DRG neurons and the release of proinflammatory neuropeptides. In addition, the slow and persistent response mediated by IgLC could also indicate that the effect of IgLC on nerves is involved in modulation of nerve function (2). The threshold for firing neurons of sensory nerves can be influenced under inflammatory conditions and nerves can become hyperresponsive to

inflammatory mediators (39). Moreover, the response can also sustain for a longer period (40). Mast cell products like histamine, serotonin, prostanoids and TNFα have been demonstrated to sensitize and prime sensory nerves to make them more susceptible to other noxious substances (41-43). This could also be the function of IgLC on neurons.
Nerve proliferation has also been observed during gastrointestinal inflammation (44). It could also be plausible that IgLC induces nerve growth via its action on neurons itself or by making nerves more susceptible to growth-stimulating substances such as nerve growth factor (NGF). The high affinity receptor for NGF, trkA, is expressed by sensory nerves (45).

Overall, the results obtained in chapter 6 suggest that IgLC can contribute to maintenance or amplification of the antigen-induced inflammatory response by neuronal activation, either directly or indirectly. Although more experiments need to be conducted to elucidate the mechanism of action of IgLC on neurons, these data reveal a new function for nerves in antigen-specific inflammatory responses (figure 1).

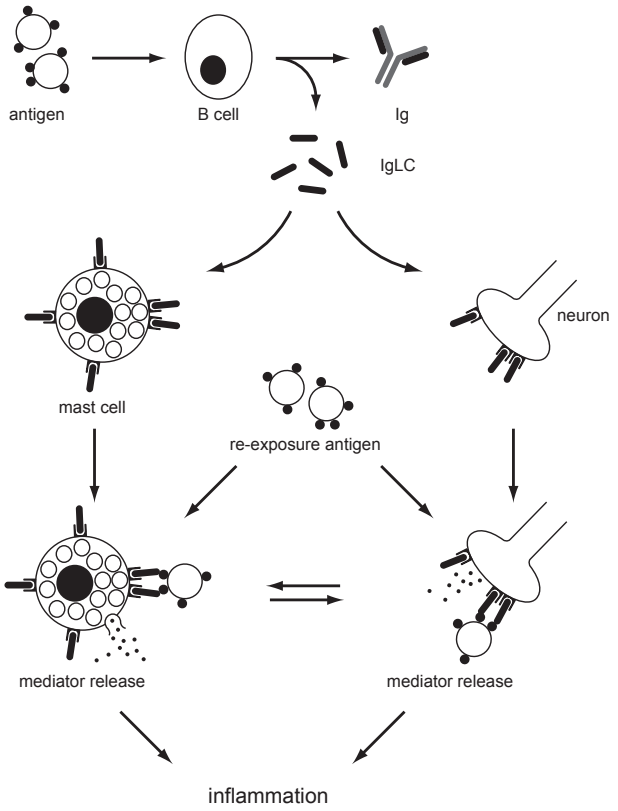

Figure 1 *Proposed role for IgLC in mast cell-nerve interaction in non-IgE-mediated hypersensitivity responses*

It is likely that IgLC affects the afferent nervous system by its action on DRG neurons. Extrinsic primary afferent neurons involve nociception, mechanoreception and thermal reception and its cell bodies are located in DRG (46). DRG neurons predominantly function as neurons that alert modifications in the environment where they project to and communicate this information to the brain to notify the central nervous system about processes and conditions to maintain energy balance, to preserve fluid homeostasis and to experience pain sensation in the body. Dorsal root ganglions are located outside the blood brain barrier within the reach of IgLC and circulating antigens. Regarding the findings presented in chapter 6 it is appealing to study the role of IgLC in affecting the afferent nervous system. Hence, more research needs to be conducted to elucidate the ability of IgLC to reach and bind to DRG *in vivo*.

In addition to a predominant role in afferent signaling, DRG neurons are also addressed as neurons with efferent-like functions (47). Moreover, it is also possible that IgLC can bind locally to neuronal cells in tissue. Both the antigen-specific effect mediated by IgLC on the efferent pathway of DRG and on other local tissue neuronal cells could result in the activation of sensory nerve endings causing the release of neuropeptides like substance P, VIP, and CGRP that exhibit proinflammatory properties such as the induction of plasma extravasation, recruitment and accumulation of leukocytes and activation of mast cells (48). In addition, substance P has been demonstrated to induce differentiation of B lymphocytes and secretion of immunoglobulins (49, 50). These findings propose a positive feedback mechanism in which substance P can stimulate B lymphocytes to further secrete IgLC and therefore augment the inflammatory response.
In conclusion, the data obtained in chapter 6 could disclose more information on the mechanism behind the involvement of sensory nerves in hypersensitivity responses and suggests a variety of possible future directions for research on IgLC.

Proposed role for IgLC in the relation between IBS and IBD

Irritable bowel syndrome (IBS) is a mild intestinal disorder highly associated with abdominal pain, altered bowel motility and an increase in visceral hypersensitivity (51). IBD and IBS are interrelated intestinal disorders. IBD patients have demonstrated IBS-like symptoms during the remission phase of the disease (52). And although IBS is mainly correlated with an unchanged mucosal structure, infiltration of inflammatory cells can be observed which can evolve into a chronic inflammatory condition (53). Since both disorders are associated with increased mast cell numbers, nerve growth and activation of both mast cells and nerves, we evaluated serum IgLC concentrations in IBD and IBS patients in chapter 7. In this chapter we report increased concentrations of IgLC in serum of both IBD and IBS patients compared to healthy controls. However, the serum IgLC concentrations in IBD patients were 2-4 fold higher compared to concentrations measured in IBS patients. Moreover, we

demonstrated that IgLC could be found in the normal and inflamed mucosa and in lesions of the intestine of patients suffering from Crohn's disease (CD) and ulcerative colitis (UC).

A functional role for IgLC in the relation between IBD and IBS is hypothesized. Ongoing IBD and IBS induce increased serum levels of IgLC (chapter 7). IgLC can subsequently bind to mast cells (23) and sensory nerves (chapter 6). Activation of mast cells and nerves mediated by IgLC takes place after a second contact with the corresponding antigen resulting in the release of mast cell mediators and neuropeptides. These mediators can bring about a variety of responses. Both mast cell and neuronal mediators can induce increased vascular permeability, infiltration of inflammatory cells and diarrhea. Moreover, the bi-directional interaction between mast cells and nerves demonstrated that mast cell mediators can stimulate sensory nerves and neuropeptides can induce

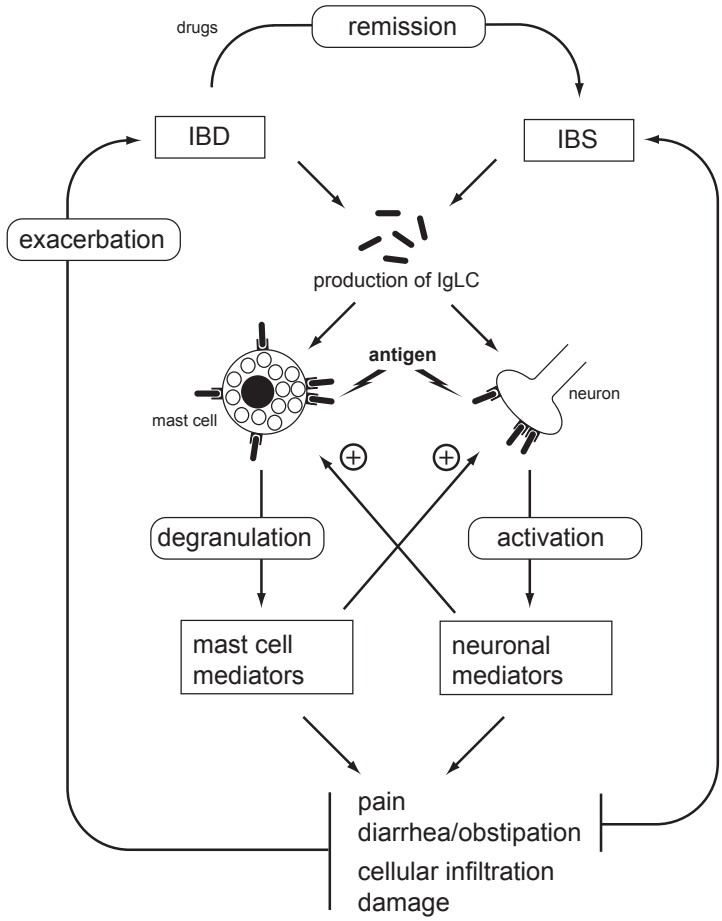

Figure 2 *Hypothesized role for IgLC in the relation between IBD and IBS*

mast cell activation. This all can lead, dependent on the extent of the response, to the exacerbation of the inflammation in IBD or remission state of IBD with symptoms defined as IBS. This proposed role for IgLC is schematically depicted in figure 2.
The concentration IgLC measured in serum of patients suffering from IBD was 2-4 fold higher than the concentrations of IBS patients (chapter 7). These data suggest that the levels of IgLC in intestinal disorders could be indicative for the manifestation of either mild (IBS) or severe (IBD) aggravation.

In conclusion

This thesis provides new insights and substantial evidence for mast cell-nerve interactions in the onset of gastro-intestinal inflammatory disorders like IBD. Potential antigens to initiate the inflammatory response are luminal bacteria, parasitic nematodes, food allergens or self-antigens. Continuous or repeated exposure to these antigens could induce IgLC-mediated hypersensitivity reactions characterized by mast cell and nerve activation, potentially leading to (the exacerbation of) chronic inflammatory conditions. The newly described and validated *in vivo* model presented in this thesis is valuable to further elucidate on the function of IgLC on hypersensitivity reactions of the gastro-intestinal tract mediated by mast cells and neurons, and the interaction between those two cell types.

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Nederlandse Samenvatting

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De ziekte van Crohn en colitis ulcerosa zijn de meest voorkomende vormen van chronische ontstekingen van het maag-darm-kanaal. In het engels wordt deze groep van aandoeningen aangeduid met inflammatory bowel disease, afgekort IBD. Aangezien er geen overeenkomstige nederlandse vertaling is voor deze verzamelnaam zal de afkorting IBD in de verdere nederlandse samenvatting gebruikt worden.
Patienten die lijden aan IBD ondervinden symptomen als hevige buikpijn en buikkrampen, diarree of obstipate, bloedingen uit de anus en een grote sociale last en beperking. In ernstige gevallen wordt een chirurgische ingreep gedaan en een deel van de ontstoken darm verwijderd en/of een stoma geplaatst.
Er is geen directe oorzaak toe te schrijven aan IBD. Het ontstaat uit een complexe interactie van genetische (erfelijk bepaalde), immunologische (een disfunctionerend immuunsysteem) en omgevingsfactoren (aan- of afwezigheid van bacteriën).
In het ontstekingsproces bij IBD zijn veel verschillende ontstekingscellen (witte bloedcellen/leukocyten) en andere celtypes betrokken. In dit proefschrift gaat de aandacht naar mestcellen en pijngevoelige (sensorische) zenuwen, en de interactie tussen deze 2 celtypes in het ontstaan van IBD.

Mestcellen

Mestcellen zijn ontstekingscellen die in het beenmerg worden aangemaakt en een rol spelen in het eerste afweermechanisme van het immuunsysteem. Mestcellen zijn derhalve uitbundig aanwezig in huid, maag-darm-kanaal en longen waar lichaamsvreemde stoffen (antigenen) het lichaam binnen kunnen dringen. In mestcellen kunnen een hoop verschillende (ontstekings)mediatoren aangemaakt en opgeslagen worden. De opslag van mediatoren vindt plaats in blaasjes in de cel die granules worden genoemd en de cel zijn specifieke karakter geven. Wanneer een mestcel geprikkeld (gestimuleerd) wordt door de aanwezigheid van antigenen wordt de cel geactiveerd en zullen de granules openbarsten (degranulatie) en komen de ontstekingsmediatoren vrij. Deze ontstekingsmediatoren kunnen een aantal processen in gang zetten die het ontstaan van een ontstekingsproces mediëren. Zij zorgen onder andere voor het verwijden van het vaatbed waardoor leukocyten makkelijker vanuit de bloedbaan het getroffen weefsel binnen kunnen dringen.
Mestcellen spelen een grote rol bij allergische en overgevoeligheidsreacties. Wanneer het lichaam in contact komt met een allergeen worden B leukocyten aangezet tot het maken van specifieke antistoffen tegen dit allergeen, zogenoemde antilichamen. Deze antilichamen zullen binden aan specifieke receptoren aanwezig op het oppervlak van de mestcel. Bij een 2^{e} contact met het antigeen zal deze direct binden aan de antigeen-specifieke antilichamen op de mestcel en de cel activeren. De mediatoren aanwezig in de mestcel zullen worden vrijgezet en kunnen allergische reacties veroorzaken, maar ook ontstekingscellen aantrekken vanuit het bloed. Leukocyten zullen het

antigeen herkennen en daardoor geactiveerd worden. De cellen geactiveerd door het antigeen zijn verantwoordelijk voor het verder tot stand komen van de ontstekingen en het handhaven er van.
In allergische reacties is er sprake van verhoogde concentraties antigeen-specifieke antilichamen in het bloed. Bij overgevoeligheidsreacties kunnen er geen verhoogde levels van het antilichaam gevonden worden en lang is het onbekend gebleven wat de mestcel gevoelig maakte voor de lichaamsvreemde stof. Onlangs is in ons laboratorium ontdekt dat een klein gedeelte van het antilichaam ook in staat is eenzelfde reactie teweeg te brengen. Hier gaat om het zogenaamde vrije lichte keten. Een antilichaam is opgebouwd uit twee zware en twee lichte ketens van eiwitten. Tijdens de productie van antilichamen door B cellen worden lichte ketens geproduceerd in overmaat en tot voor kort kon er geen fysiologische rol worden toegeschreven aan deze lichte ketens. Nu is aangetoond dat antigeen-specifieke lichte ketens betrokken zijn bij de mestcel activatie in overgevoeligheids reacties. In dit proefschrift wordt gekeken naar de rol van lichte ketens bij de mestcel-activatie in het ontstaan van IBD.

Voor het bestuderen van mestcellen in het ontstaan van IBD is in dit proefschrift een muismodel opgezet. De muizen worden als eerste in contact gebracht met een antigeen, in dit geval is dat DNFB, op de huid. Dit proces wordt de sensibilisatie genoemd. Het 2^{e} contact met het allergeen wordt lokaal aangebracht en aangezien we een ontstekingsreactie in de dikke darm (colon) willen opwekken, wordt het antigeen via de anus (intrarectaal) toegediend. Dit proces heet de challenge. Drie dagen na de challenge hebben de dieren diarree en een ontsteking in het colon ontwikkeld en worden ze opgeofferd. In het bloed van deze muizen kan bewijs voor de activatie van mestcellen worden aangetoond. En in het colon kan infiltratie van mestcellen en ontstekingscellen worden waargenomen. In hoofdstuk 2 worden de karateristieken van de reactie gepresenteerd. In dit hoofdstuk staat ook beschreven dat de reactie niet optreedt in muizen die geen mestcellen bevatten (deficiënt zijn). Toediening van gekweekte mestcellen in deze mestcel-deficiente muizen herstelde de reactie in het colon. Dit bewijst de belangrijke rol van mestcellen in deze reactie.

Eén van de belangrijkste mediatoren van mestcellen is tumor necrosis factor-α (TNFα). Ook in IBD speelt deze mediator een grote rol. Patienten die lijden aan IBD worden behandeld met antilichamen tegen TNFα (de functie van TNFα wordt weggevangen) met positief resultaat. In het muismodel voor IBD worden verhoogde levels TNFα in het colon gevonden, voornamelijk afkomstig uit mestcellen (hoofdstuk 2). Om de rol van deze mediator in het muismodel te bestuderen zijn in hoofdstuk 3 de muizen behandeld met een antilichaam tegen TNFα. Behandeling met antilichamen tegen TNFα leidde tot de verhindering van het ontstaan van diarree en van de infiltratie van ontstekingscellen in het colon. Dit geeft aan dat TNFα belangrijk is bij het ontstaan van de overgevoeligheidsreactie in het colon die leidt tot ontsteking in dit orgaan.

De rol van lichte ketens in dit proces is bestudeerd in hoofdstuk 5. Hierbij is gebruik gemaakt van een substantie die grote bindingsaffiniteit heeft voor lichte keten, F991. Toediening van F991 vangt geproduceerde lichte ketens weg waardoor deze niet meer aan de mestcel kunnen binden en activatie van dit celtype kunnen veroorzaken. Behandeling van de muizen met F991 leidde tot verhindering van het ontstaan van diarree, van de mestcelactivatie en van de infiltratie van leukocyten in het colon. Dit geeft aan dat lichte ketens verantwoordelijk zijn voor de activatie van mestcellen in de overgevoeligheidsreactie in het colon zoals beschreven in hoofdstuk 2.

Sensorische zenuwen

Sensorische zenuwen geven prikkels veroorzaakt door schadelijke stoffen door aan de hersenen. Van sensorische zenuwen is bekend dat ze ook een rol spelen bij ontstekingsprocessen. Zo kan er verhoogde activiteit en uitgroei van zenuwen gevonden worden op plaatsen van ontsteking. Ook kunnen zenuwuiteinden mediatoren (neuropeptides) vrijzetten die het ontstekingsproces beïnvloeden. Een belangrijke neuropeptide is substance P. Substance P heeft een specifieke receptor waar het met grote affiniteit aan kan binden. Dit is de neurokinine-1 (NK_1) receptor. Deze NK_1 receptor is o.a. aanwezig op endotheelcellen (deze cellen bekleden de binnenkant van bloedvaten). Stimulatie van de NK_1 receptor door substance P kan door verhoging van de vaatdoorlaatbaarheid van bloedvaten de infiltratie van leukocyten veroorzaken. De NK_1 receptor is ook aanwezig op verschillende onstekingscellen waaronder ook de mestcel. Dit betekent dat substance P activatie en dus de vrijzetting van mestcel mediatoren kan veroorzaken.
In hoofdstuk 4 is gekeken naar de rol van sensorische zenuwen en in het bijzonder naar substance P. Hierbij is gebruik gemaakt van een antagonist voor de NK_1 receptor. Deze antagonist blokkeert de NK_1 receptor waardoor substance P niet meer kan binden en een werking kan uitvoeren. Behandeling van de muizen met deze NK_1 antagonist leidde wederom tot de verhindering van het ontstaan van diarree en van de infiltratie van leukocyten en mestcellen. Deze resultaten tonen aan dat sensorische zenuwen en in het bijzonder substance P betrokken zijn bij het ontstaan van overgevoeligheidsreacties in het colon in dit muismodel.

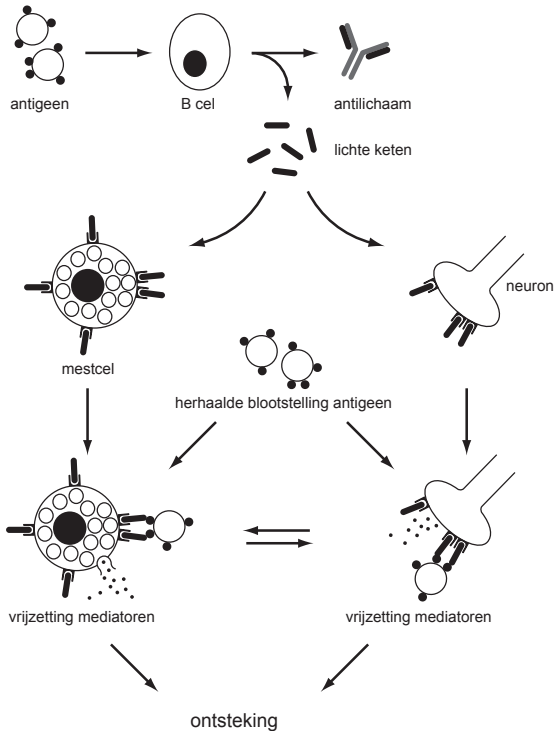
Mestcel-zenuw interactie

Mestcellen en zenuwen worden vaak dicht bij elkaar gelokaliseerd in huid, luchtwegen en maagdarmkanaal. Er bestaat interactie tussen deze twee celtypes en dit is uitgebreid bestudeerd. Zo bezit het zenuwuiteinde verschillende recptoren voor mediatoren die vrijgezet worden vanuit de mestcel. Stimulatie van deze receptoren leidt tot activatie van het zenuwuiteinde. Hierdoor worden neuropeptides en andere neuronale mediatoren vrijgezet waar de mestcel specifieke receptoren voor bezit. Dit leidt tot activatie van de mestcel.
De resultaten beschreven in hoofdstuk 2 t/m 4 van dit proefschrift hebben

aangetoond dat zowel de mestcel als de sensorische zenuw van belang is bij het ontstaan van de overgevoeligheidsreactie in het colon. Vermindering van de functie van één van deze celtypes door mestcel-deficiëntie (hoofdstuk 2), behandeling met antilichamen tegen TNFα (hoofdstuk 3) of behandeling met de NK_1 receptor antagonist (hoofdstuk 4) leidt tot de verhindering van het ontstaan van karateristieken van deze reactie. Deze resultaten suggereren een mestcel-zenuw interactie in dit overgevoeligheidsmodel in het colon.

De rol van lichte keten in zenuwactivatie

Mestcellen en zenuwen kunnen dus gevonden worden in nabijheid van elkaar en aangezien de activatie van mestcellen in overgevoeligheidsreacties gemedieerd wordt door lichte ketens werd de rol van lichte ketens op zenuwen onderzocht (hoofdstuk 6). Sensorische zenuwcellen werden uit de muis gehaald en gekweekt zodat ze weer nieuwe zenuwbanen gingen groeien. Met behulp van visuele bindingsstudies is aangetoond dat lichte ketens specifiek kunnen binden aan deze gekweekte zenuwcellen. Vervolgens werd onderzocht of activatie van lichte ketens met antigenen kan leiden tot stimulatie van neuronen. Hiervoor werd gebruik gemaakt van confocale lasermicroscopie waarmee calcium concentraties in de cel gemeten kunnen worden. Verhoging

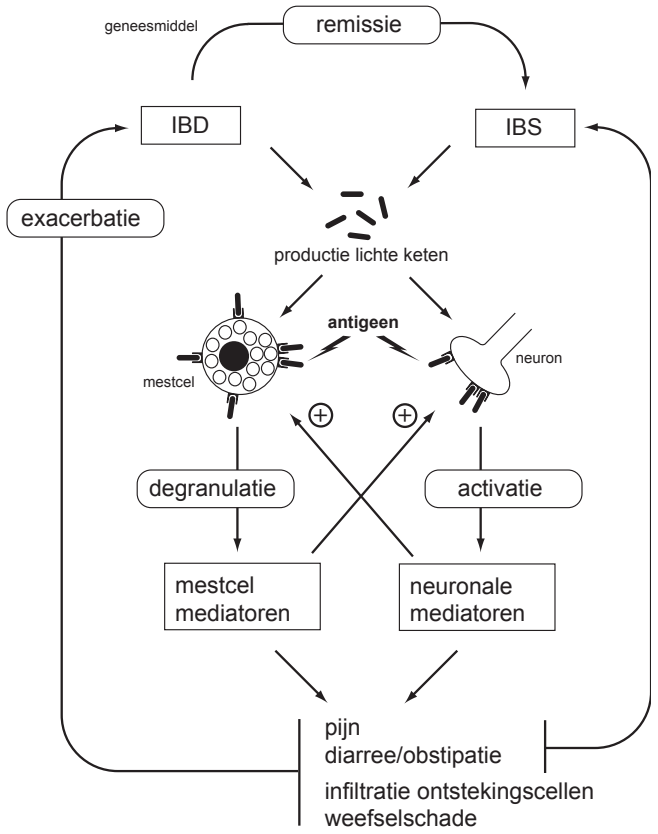


Figuur 1 *Rol voor lichte ketens in mestcel en zenuw activatie.*

van calcium in de cel is een indicatie dat er activatie optreedt. In hoofdstuk 6 wordt beschreven dat antigeen stimulatie van lichte ketens die gebonden zijn aan zenuwcellen leidt tot een langzame stijging van calcium concentraties in de cel.
Deze resultaten geven aan dat lichte ketens ook betrokken zijn bij antigeen-specifieke zenuwactivatie of –modulatie. Het effect van lichte ketens op zenuwen biedt nieuwe perspectieven voor het bestuderen van overgevoeligheidsreacties en kan een nieuwe fysiologische rol voor lichte ketens illustreren. Dit proces is schematisch weergegeven in figuur 1.

Klinische relevantie voor lichte keten

In het bloed van patienten die lijden aan IBD kunnen verhoogde levels van lichte ketens worden aangetoond. Dit is beschreven in hoofdstuk 7. Lichte ketens kunnen ook worden aangetoond in het darmweefsel van patienten die lijden aan de ziekte van Crohn of colitis ulcerosa.
IBD is gerelateerd aan het prikkelbare darmsyndroom, in het engels irritable



Figuur 2 *Hypothese voor een rol voor lichte ketens in de relatie tussen IBD en IBS*

bowel syndrome (IBS) genoemd. IBS is een milde darmaandoening die gepaard gaat met buikpijnen en diarree of obstipatie, maar zelden tot nooit met ontsteking van het darmweefsel. Bij IBS patienten worden ook verhoogde aantallen mestcellen en zenuwactivatie gevonden. In hoofdstuk 7 is aangetoond dat ook bij IBS patienten verhoogde concentraties lichte keten in het bloed kunnen worden gevonden.
Van IBD patienten die in remissie (vermindering van de ziekte) gaan is bekend dat ze vaak IBS-achtige symptomen overhouden. Tegenovergesteld kunnen IBS patienten een chronische ontsteking in de darm ontwikkelen. Daarom wordt een hypothese voorgesteld waarin lichte ketens een rol spelen bij de relatie tussen IBD en IBS. Deze hypothese is weeregeven in figuur 2.
De concentratie lichte ketens in het bloed van IBD patienten is ongeveer 2 tot 4 keer zo hoog als bij patienten die aan IBS leiden. De hoogte van de lichte keten concentraties in het bloed zou een indicatie kunnen zijn voor het exacerbatie (opleving van de ziekte) van IBD of de ontwikkeling van IBS symptomen.

Conclusie

In dit proefschrift is aangetoond dat mestcellen en zenuwen een rol spelen bij het ontstaan van ontstekingsreacties in het colon. Deze reactie wordt gemedieerd door lichte ketens. Het maag-darm-kanaal staat constant bloot aan antigenen als parasieten, bacteriën, voedselcomponenten en lichaamseigen antigenen. Deze antigenen kunnen een overgevoeligheidsreactie veroorzaken die kunnen leiden tot darmaandoeningen als IBD en IBS.
Het muismodel dat beschreven wordt in dit proefschrift kan gebruikt worden voor het verder uitdiepen van de rol van lichte ketens in de mestcel-zenuw interactie in het colon.

Abbreviations

Ab	antibody
ACSF	artificial cerebrospinal fluid
BMMC	bone marrow-derived mast cell
$[Ca^{2+}]_i$	intracellular calcium concentration
CAE	chloro-acetate esterase
CAM	cell adhesion molecule
CD	Crohn's disease
CGRP	calcitonin-gene related peptide
CNS	central nervous system
dex	dexamethasone
DNFB	2,4 dinitrofluorobenzene
DNP-HSA	dinitro phenyl – human serum albumin
DNS	dinitro sulfonic acid
DSS	dextran sodium sulfate
DTH	delayed-type hypersentivity
ENS	enteric nervous system
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
ICC	interstitial cells of Cajal
Ig	immunoglobulin
IgLC	immunoglobulin-free light chain
IL	interleukin
mMCP-1	mouse mast cell protease-1
NANC	non-adrenergic non-cholinergic
NGF	nerve growth factor
NK1	neurokinin-1 receptor
NKA/B	neurokinin A/B
PAR	protease activated receptor
PGP	protein gene product
PO	peroxidase
PSA	picryl sulfonic acid
RF	relative fluorescence
THP	Tamm-Horsfall protein
TNBS	trinitrobenzene sulfonic acid
TNFα	tumor necrosis factor-α
TNP	trinitro phenyl
UC	ulcerative colitis
VIP	vasoactive intestinal polypeptide

Curriculum Vitae

Anneke Rijnierse werd 17 december 1978 geboren te Leidschendam. Na het behalen van het VWO diploma aan de scholengemeenschap Sint Ursula in Horn in 1996 begon zij aan de studie Farmacie aan de Universiteit Utrecht. In de doctoraalfase van de studie werd een onderzoeksstage voltooid aan het William Harvey Research Institute in Londen. Onderwerp was cardiovasculaire complicaties bij type 2 diabetes als gevolg van overgewicht. In november 2001 studeerde zij af. In diezelfde maand begon ze als assistent-in-opleiding bij de vakgroep Farmacologie & Pathofysiologie van de faculteit Farmaceutische Wetenschappen aan de Universiteit Utrecht. Onder leiding van Frans Nijkamp, Aletta Kraneveld en Andries Koster werd daar gewerkt aan het onderzoeksproject 'Mast cells and nerves in the development of inflammatory bowel disease'. De resultaten van dit onderzoek staan beschreven in dit proefschrift.

List of Publications

Full papers

Anneke Rijnierse, Andries S. Koster, Frans P. Nijkamp, Aletta D. Kraneveld. Critical role for mast cells in the pathogenesis of 2,4-dinitrobenzene-induced murine colonic hypersensitivity reaction. *J Immunol. 2006 Apr; 176: 4375-4384.*

Anneke Rijnierse, Kim M.F. van Zijl, Andries S. Koster, Frans P. Nijkamp, Aletta D. Kraneveld. Beneficial effect of neurokinin-1 receptor antagonism in the development of hapten-induced colitis in mice. *Submitted.*

Anneke Rijnierse, Andries S. Koster, Frans P. Nijkamp, Aletta D. Kraneveld. TNFα is crucial for the development of mast-cell dependent colitis in mice. *Submitted.*

Anneke Rijnierse, Frank A. Redegeld, Maurice W. van der Heijden, Bart R. Blokhuis, Anje A. te Velde, Inge Pronk, Daan W. Hommes, Frans P. Nijkamp, Andries S. Koster, Aletta D. Kraneveld. Putative role for immunoglobulin-free light chain in inflammatory bowel disease. *In preparation*.

Anneke Rijnierse, Alfons B.A. Kroese, Frank A.M Redegeld, Maurice W. van der Heijden, Bart R.J. Blokhuis, Andries S. Koster, Jean-Pierre Timmermans, Frans P. Nijkamp, Aletta D. Kraneveld. Immunoglobulin-free light chains mediate the activation of mouse dorsal root ganglion neurons by antigens. *In preparation*.

Abstracts

Anneke Rijnierse, Frank A. Redegeld, Maurice W van der Heijden, Bart R Blokhuis, Frans P. Nijkamp and Aletta D. Kraneveld. Immunoglobulin Free Light Chain: a New Immunological Factor in IBD. *Gastroenterology, 2005 Apr; 128(4): supplement 2.*

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Dankwoord

het boek is vol
ik ben tevree
rest mij nog te zeggen:

everyone who came into my life
for a *reason*
for a *season*
or to stay *forever*
thank you for everything!

You could not have come at a better time
Not if you tried
Luka Bloom - Riverside (1990)

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