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Role of mast cell-derived mediators for leukocyte/endothelium-interactions and microvascular mechanisms in inflammation and in anaphylaxis

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ABSTRACT

The overall objective of this thesis was to study the roles of mast cell-derived mediators for leukocyte/endothelium interactions and microvascular mechanisms in inflammation and in anaphylaxis, using mast cell-deficient Ws/Ws rats and their wild-type +/+ littermates.

The efflux of endogenous histamine and edema formation evoked by subplantar injection of compound 48/80 in rat hindpaws was dose-dependent in +/+ rats, and was essentially lacking in Ws/Ws rats. These findings suggest a critical role of histamine as trigger of edema formation in vivo.

Spontaneous leukocyte rolling was studied by intravital microscopy of venules in rat mesentery and cremaster muscle. Rolling and rolling velocity in mast cell-deficient animals exactly matched that seen in wild-type animals. Challenge with 48/80 markedly increased leukocyte adhesion and emigration in +/+ animals, whereas in animals lacking mast cells adherent and extravascular leukocytes remained very few and did not increase after challenge with 48/80. The data strongly suggest that the presence or activation of mast cells has no bearing on spontaneous leukocyte rolling.

Experimental surgical skin flaps in mast cell-deficient rats accumulated little histamine and leukocytes and exhibited little tissue necrosis, whereas flaps in wild-type rats were associated with marked accumulation of histamine and leukocytes and showed substantial tissue loss. Antihistamine treatment in the wild-type rats halved leukocyte accumulation and increased flap survival. The data suggest a role for histamine in skin wound healing.

Ovalbumin-sensitized rats, both Ws/Ws and +/+ but not sham sensitized animals, exhibited hypotensive responses after intravenous antigen challenge. There was no mast cell activation in the mesentery of senzitised +/+ rats after intravenous antigen challenge. Hypotension induced by intravenous injection of dextran or compound 48/80 occurred in +/+ rats, but not in Ws/Ws rats, and was inhibited by pre-treatment with histamine receptor antagonists. The data indicate that the hypotensive response induced by antigen-mediated anaphylaxis is independent of mast cell activation.

Plasma exudation and influx of leukocytes in air pouches after immune complex reaction were markedly lower in Ws/Ws rats than in +/+ rats. Leukocyte rolling, adhesion and transmigration in the mesentery were all substantially increased 6 h after immune complex reaction in +/+ rats but not in Ws/Ws rats. Leukocytes adhered under flow conditions in markedly larger numbers to endothelial cell monolayers stimulated with peritoneal fluid (collected 6 h after immune complex reaction) from +/+ rats than from Ws/Ws rats. The data indicate a dominant role of mast cell-derived mediators in the leukocyte response to an IgG immune complex reaction in rat skin and mesentery.

In summary, the data presented in this thesis show distinctive roles of mast cells in inflammation and anaphylaxis. As studied in the rat, mast cells are of critical importance in local tissue response to immune complex activation and surgical trauma, and are likely important in wound healing. Systemic anaphylaxis to allergen is not critically dependent on mast cells. Mast cell-deficient rats should be most useful in further delineating physiological and pathophysiological roles of mast cells.

Key words: anaphylaxis; histamine; intravital microscopy; leukocyte; mast cell-deficient rat (Ws/Ws); microdialysis

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LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their Roman numerals.

I. **Yancai Guo Y**, Takatoshi Mochizuki, Eiichi Morii , Yukihiko Kitamura, Kazutaka Maeyama. (1997)
Role of mast cell histamine in the formation of rat paw edema: A microdialysis study.
European Journal of Pharmacology 331:237-243

II. **Yancai Guo**, Lennart Lindbom & Per Hedqvist. (2000)
Spontaneous leukocyte rolling in rat and mouse microvessels is independent of mast cell activity.
Inflammation Research 49:325-329

III. **Yancai Guo**, Leif Törkvist, Johan Raud & Per Hedqvist. (2003)
Increased tissue survival in experimental skin flaps in mast cell deficient rats.
Manuscript

IV. **Yancai Guo**, Per Hedqvist, Lars E. Gustafsson (2001)
Absence of mast cell involvement in active systemic anaphylaxis in rats.
European Journal of Pharmacology 430: 305-310

V. **Yancai Guo,** Xun Xie, Lennart Lindbom, Lars E. Gustafsson, Per Hedqvist (2003)
IgG Immune Complex-Induced Reaction in Skin and Peritoneum is Attenuated in Mast Cell Deficient (Ws/Ws) Rats.
Manuscript

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ABBREVIATIONS

The main abbreviations used in this thesis:

ADH	leukocyte adhesion
BAEC	bovine aorta endothelial cells
CIM	cimetidine
CPR	chlorpheniramine
C48/80	compound 48/80
CTMC	connective tissue mast cells
EMG	leukocyte emigration
FITC	fluorescein isothiocyanate
H_1	histamine$_1$-receptor
H_2	histamine $_2$-receptor
H_3	histamine $_3$-receptor
HPLC	high performance liquid chromatography
Ig	Immunogloblin (*e.g.* IgE, IgG)
IL	interleukin (*e.g.* IL-1 ß)
LTC_4	leukotriene C_4
MAP	mean arterial pressure
MMC	mucosal mast cells
MNL	mononuclear leukocyte
MPO	myeloperoxidase
OA/OVA	Ovalbumin
PAF	platelet activating factor
PBS	phosphate-buffered saline
PMNL	polymorphonuclear leukocyte(s)
RLC	rolling leukocyte flux
SCF	stem cell factor
TNFα	tumor necrosis factor alpha
Ws/Ws	White-spotting, mast cell-deficient rats
Ws/+	half mast cell-deficient
+/+	Wild-type

INTRODUCTION

Mast cells are regarded as critical effector cells in inflammatory reactions and physiological processes. A variety of mediators derived from mast cells are released in response to immune stimulation and IgE receptor (FcεRI) aggregation as well as through stimulation by biochemical and other signals. Convincing evidence for the importance of mast cells in specific immunologic or pathological responses in vivo has been difficult to obtain, since other cell types produce many mast cell-associated mediators. It is therefore difficult to predict the net effect of mast cell activation. An optimal model for evaluation of the biological function of mast cells is a system in which one anatomic site contains mast cells and a second similar site does not, thereby making it possible to identify differences in the expression of biological responses between these two sites. Mast cell-deficient rats, "white spotting" or Ws/Ws, recently discovered by Niwa *et al.* (1991), together with their wild type (+/+) littermates as controls, provide a useful model to study the role of mast cells.

GENERAL BACKGROUND

Mast cells

Discovery of the mast cell

During the 19th century many of the pioneer researchers noticed the presence of connective tissue cells lying in a perivascular habitat. It wasn't until much later that these cells were identified as mast cells when metachromatic dyes showed them to contain metachromatic cytoplasmic granules. Waldeyer observed these cells in 1875 and mistook them for plasma cells. It was, as discussed by Raud (1989), Paul Ehrlich (1877, 1878) who renamed Waldeyer's plasma cells "Mastzellen" i.e. mast cells, when he demonstrated that these cells have cytoplasmic granules which stained red or violet with the blue aniline dye, dahlia. Since that time extensive studies of the mast cells over the decades have accumulated a vast amount of knowledge of their distribution and potential functions.

Morphology of the mast cell

The morphological shapes of mast cells vary according to their habitat. In loose connective tissue they appear rounded but in close apposition to blood vessels they are

elongated or ovoid, while in connection with dermal fibers they are spindle shaped, stellate or filiform. Mast cells, which are freely suspended in peritoneal fluids in the rat are round. They have a mean diameter of 12.6-13.5 μm (Benditt, 1957; Bloom, 1960a, 1974). Fixed peritoneal cells have a mean diameter of 12.8 μm (Bloom, 1960b).

Life span of mast cells

Researchers, in the early 30's reported that mature mast cells exhibited little or no mitotic activity. This notion remained until the 60's when mitotic activity was reported in mast cells in young rats, meaning that mast cells are capable of mitosis. Autoradiographic studies confirmed that mast cells in the adult rat took up little tritiated thymidine compared to that in young animals. It was shown (Hunt & Hunt, 1957) that mast cells were able to regenerate and proliferate in the rat peritoneum after being degranulated with injections of compound 48/80. They observed the sequential repopulation of peritoneal tissue by young mast cells, which were fully matured in 17 days. Combs *et al.* (1965), using a combination of autoradiographic and histochemical methods, concluded that young mast cells were able to divide. The ability disappears as maturation takes place so that when the mast cell is fully developed the ability to divide may be completely lost. Padawer (1974) suggested that rat mast cells are at least capable of living for 10 months when he found that thorium, which was incorporated into mast cell granules steadily decreased to less than 1% by 8-10 months. The fact that thorium with granules persisted in some mast cells for at least 10 months strongly suggested that some mast cells live for at least that length of time or for the whole life span of the animal (Combs *et al.,* 1965). Recent research has shown that mast cells originate from bone marrow, migrate in the blood stream and then lodge in other tissues where they proliferate, differentiate and mature (Kitamura *et al.,* 1989). Mast cell progenitors and development is stimulated by stem cell factor (SCF), utilizing the receptor c-kit (Galli & Hammel, 1994).

Heterogeneity of mast cells

Historically, the classification of rodent mast cell subtypes has been based on phenotypical differences between connective tissue mast cells (CTMC), particularly in the skin and peritoneal cavity, and mucosal mast cells (MMC), principally in the intestinal lamina propria and in the airways mucosa. As shown in Table 1, there are phenotypical differences between these two populations, including size and histamine content as well as proteoglycan and neutral protease composition, the latter determining their staining characteristics (Pearce *et al.,* 1984; Bienenstock *et al.,* 1985; Shanahan *et*

al., 1985; Aldenborg & Enerback, 1988; Metcalfe *et al.,* 1997). Furthermore, these subtypes show differences in function, including responsiveness to various secretagogues, and inhibition by drugs.

Cloning and protein analysis experiments show the presence of more than 20 proteases in mast cells. The functional importance of these proteases remains to be determined (Lützelschwab *et al.,* 1997).

TABLE 1. *Rodent mast cell characteristics*

Characteristics	Peritoneal Cavity Mast Cell (Connective tissue mast cell)	Intestinal Mucosal Mast Cell (Mucosal mast cell)
Size	10-20 μm	5-10 μm
Formaldehyde fixation	Resistant	Sensitive
Staining	Safranin	Alcian blue
T-cell dependent in development	No	Yes
Protease content	RMCPI (a chymase)	RMCP II (a chymase)
Proteoglycans molecular mass	Heparin 759-1,000 kDa	Chondroitin sulfate di B 100-150 kDa
Histamine	10-20 pg/cell	1 pg/cell
5-Hydroxytryptamine	1-2 pg/cell	<0.5 pg/cell
Prostaglandin D_2	+	+
Leukotriene C_4	-	++
Activated by		
$Fc_\varepsilon RI$ aggregation	Yes	Yes
Compound 48/80	Yes	No
Mastoparan	Yes	No
Substance P	Yes	No
Inhibited by sodium cromoglycate	Yes	No

Activation of mast cells

Mast cell activation may be initiated upon interaction of a multivalent antigen with its specific IgE antibody attached to the cell membrane via its high-affinity receptor, $Fc_\varepsilon RI$. Cross-linkage of IgE by the interaction of allergen with specific determinants on the Fab portion of the molecule brings the receptors into juxtaposition and initiates mast cell activation and mediator release (Razin *et al.,* 1995; Metcalfe *et al.,* 1997). Mast cells may also be activated by nonimmunologic stimuli induced by substances such as neuropeptides, compound 48/80, dextran, calcium ionophores, complement components, and certain drugs such as opiates (Joos & Pauwels, 1993; Horsmanheimo *et al.,* 1996; Hua *et al.,* 1996). In addition, mast cell-cytotropic IgG antibodies may

mediate anaphylactic reactions and mast cell mediator release, both in man and in animals (Becker & Austen, 1976; Moore & Dannenberg, 1993). Morphologically, degranulation produced by immunologic and nonimmunologic stimulation appears similar. However, biochemical processes that lead to mediator release may differ.

Mediators of mast cells

Mast cell-dependent mediators may be categorized into three groups: preformed secretory granule-associated mediators, lipid-derived mediators, and cytokines (Table 2). Upon activation, mast cells both release and generate a heterogeneous group of mediators that differ in their potency and biological activities. These mediators are both pleiotropic and redundant; that is, each mediator has more than one function, and mediators may overlap in their biological effects. For instance, histamine alters vasopermeability and induces mucus secretion, properties it shares with leukotriene C_4.

TABLE 2. *Mast cell mediators*

Granule-associated mediators	Lipid-derived mediators	Cytokines and chemokines
Histamine	Leukotriene B_4	TNF-α
Serotonin (major amine in	Leukotriene C_4	IL-4
rodent CTMC)	Prostaglandin D_2	IL-5
Heparin		IL-6
Chymase		IL-8
Tryptase		
Carboxypeptidase		
Cathepsin G-like protease		
TNF-α + others		

The most important vasoactive mediators that are stored in mast cell and basophil granules are histamine in human, and histamine together with 5-hydroxytryptamine in rodents. Histamine is stored in mast cells largely complex-bound to mucopolysaccharide (glycosaminoglycans) such as heparin. Histamine has diverse functions including primary, local dilation of small vessels; widespread arteriolar dilatation; local increased vascular permeability by contracting endothelial cells; the

contraction of nonvascular smooth muscle; chemotaxis for eosinophils, and blocking T lymphocyte function. A number of different cells of the body have receptors for histamine. These can be of three types: H_1, H_2, and H_3. The H_1 receptors mediate acute vascular effects together with smooth muscle constriction in the bronchi (histamine act as a "spasmogen") and the stimulation of eosinophil chemotaxis. In contrast, the H_2 receptors mediate a number of anti-inflammatory effects, including the inhibition of eosinophil chemotaxis, but cause vasodilatation. The H_3 receptor is mainly involved in the control of histamine release by different histamine producing cells. Many cells have the ability to produce histamine such as macrophages (Takamatsu & Nakano, 1994), neutrophils (Shiraishi *et al.,* 2000) and T lymphocytes (Aoi *et al.,* 1989).

Some typical H_1 antagonists include diphenhydramine, chlorpheniramine and tripelennamine; and H_2 antagonists include metiamide, cimetidine, ranitidine and famotidine. H_3 antagonists have been developed with potential application in enhancing alertness by elevating histamine release in the central nervous system. Thioperamide is a prototypical H_3 antagonist that can potentiate the release of histamine and reverse the action of R-a-methylhistamine at the autoreceptor (Watling, 1998).

5-hydroxytryptamine (5-HT) is formed by the hydroxylation and decarboxylation of tryptophan and found in large amounts in rodent connective tissue mast cells (Griesbacher *et al*., 1998). Mast cell release of 5-HT induces rat tracheal contraction (Ikawati *et al.,* 2000), and causes intense vasoconstriction and increased venular permeability (Rioux *et al.,* 1985). However, the effects of serotonin on leukocyte recruitment have not been examined, and it is not known whether this vasoactive amine has similar effects on leukocyte rolling as histamine.

Functional roles of mast cells

Although many cells are involved in the allergic cascade, the mast cell is the initial effector cell in allergic diseases by virtue of its resident tissue location and capacity to rapidly respond with release of potent chemical mediators. Through the release and production of these proinflammatory molecules, mast cells set in motion a series of events that results in both immediate and late-phase allergic responses. The consequences of mediator release usually occur within minutes, thus the term immediate hypersensitivity. The mediators interact with surrounding tissues and elicit allergic inflammation, the manifestations of which are determined by the local

environment. Many, but not all, hypersensitivity reactions result in a second reaction, the late-phase reaction. The finding of the association of mast cells with various diseases has provided hints to their function. Mast cells were supposed to be involved in atherosclerosis of large vessels. This suspicion was based on the observation that macrophage phagocytosis of low density lipoproteins (LDL) was enhanced by mast cell products. The cholesterol of ingested LDL is esterified and remains at the site of atheroma (Kovanen, 1993, 1995). Mast cells are present in large numbers during the active process of wound healing. It appears that mast cells have stored cytokines which are stimulatory to fibroblasts and serine proteases, which help remodel extracellular matrix during the healing process (Hebda *et al.,* 1993). However, Cordeiro *et al.* (2000) suggest that ischemia-reperfusion injury in a rat skin flap could be prevented by inhibition of the mast cell mediator histamine. There is also considerable evidence to show that mast cells play an active role in inflammation by virtue of the fact that they make and release cytokines under certain conditions (Galli *et al.,* 1993). Secretions of IL-4, IL-5 and TNF-alpha are associated with chronic rhinitis (Bradding *et al.,* 1993), asthma and other allergic reactions. Experimental studies show that there is communication between mast cells, microvascular endothelium, and neuronal axons which involve histamine, tumor necrosis factor (TNF) and other mast cell mediators. The release of histamine, TNF-alpha and other cytokines promotes plasma leakage, recruitment of polymorphonuclear leukocytes and other effector cells in the inflammatory process (Wershil *et al.,* 1991; Akerstrom & Lisander, 1994; Zhang *et al.,* 1995a; Kubes & Gaboury, 1996; Iuvone *et al.,* 1999; Biedermann *et al.,* 2000).

The inflammatory response

Inflammation is a defense reaction against tissue damage or injury, characterized by redness, heat, swelling, and pain. The primary objective of inflammation is to localize and eradicate the irritant and repair the surrounding tissue. For the survival of the host, inflammation is a necessary and beneficial process. The inflammatory response involves three major stages: first, dilation of capillaries to increase blood flow; second, microvascular structural changes and escape of plasma proteins from the bloodstream; and third, leukocyte transmigration through endothelium and accumulation at the site of injury.

The leukocyte adhesion cascade is a sequence of adhesion and activation events that ends with extravasation of the leukocyte. Each of the steps of the adhesion cascade,

rolling, slow rolling, firm adhesion, transmigration appears to be necessary for effective leukocyte recruitment, because blocking any of them will severely reduce leukocyte accumulation in the tissue. These steps are not phases of inflammation, but represent the sequence of events from the perspective of a single leukocyte. At any given moment, capture, rolling, slow rolling, firm adhesion and transmigration all happen in parallel, involving different leukocytes in the same microvessels.

Anaphylaxis

Anaphylaxis is a potentially fatal allergic reaction to a foreign agent, such as insect venom, vaccines, drugs etc. This allergic response involves direct or indirect activation of mast cells and basophils (Kemp & Lockey, 2002). There are two forms of anaphylaxis: anaphylactic reaction and anaphylactoid reaction.

Anaphylactic reactions are immune-mediated, that is, when a human or an animal is exposed to an antigenic agent, called an antigen, for the first time, the body may produce antibodies to that agent, which bind to the mast cells. At renewed exposure to the same agent, at a later time, these antibodies activate the mast cells to produce an inflammatory response.

Anaphylactoid reactions are identical to anaphylactic reactions in terms of clinical symptoms, however, anaphylactoid reactions are the result of non-immunological factors which directly activate the mast cells. As such, a single exposure to the foreign agent may result in clinical symptoms. In both conditions, activation of mast cells results in a number of physiological changes affecting respiration and circulation which may present a life-threatening emergency, such as the loss of fluid from blood vessels causing sudden drop in blood pressure (Holgate & Church, 1993; Novick, 1994; Lawlor *et al*., 1995).

Mast cell-deficient white spotting (W_s/W_s) rats

Genetically mast cell-deficient Ws/Ws rats, when used in comparison with normal rats, are useful and well-suited for the investigation of biological roles of mast cells. A spontaneous mutation (Ws/+) was identified by Kitamura in a BN/fMai rat colony, and the heterozygous rats were bred with female rats of the Donryu strain to obtain viable Ws/Ws rats (Niwa *et al*., 1991). Ws/Ws rats have a 12-base deletion in the c-kit gene, coding for the stem cell factor receptor (Tsujimura *et al.,* 1991). The deletion is in the tyrosine kinase domain of the protein, leading to loss of receptor function, results in a lack of mast cells. The genotypes were identified by their fur color, which in the

homozygous mutants changes to white with black eyes, denoted as white spot pattern (Ws). The non-mutated animals are black, denoted as +/+.

AIMS OF THIS THESIS

The specific aims were as follows:

1, To establish a microdialysis technique for determination of endogenous histamine concentration changes *in vivo.*

2, To investigate the differences in leukocyte-endothelial interactions caused by surgical preparation using intravital microscopy between mast cell-deficient Ws/Ws rats and their littermates +/+ rats.

3, To investigate the role for mast cells in skin flap survival.

4, To examine mast cell involvement in active anaphylaxis and try to elucidate mast cell-dependent and independent mediators responsible for the hypotensive responses.

5, To study the role of mast cell mediators in IgG immune complex-induced reaction in skin and peritoneum.

MATERIALS AND METHODS

ANIMALS

Mast cell deficient (Ws/Ws) and their congenic littermates +/+ rats were bred, and their offspring as well as Wistar rats, weighing 200-250g, were used throughout the study. In paper II, mast cell-deficient WBB6F1 male W/W^{v} mice and their congenic wild type littermates (The Jackson Laboratory), weighing 20-30g, were used.

Ws/Ws rats breeding

How to distinguish Ws/Ws, Ws/+ and +/+ rats:

---Ws/Ws, white fur, black eyes, giving white-spot (Ws) appearance.

---Ws/+, light gray or brown fur, black eyes.

---+/+, almost black or dark brown fur, black eyes.

How to breed Ws/Ws rats

---In principle brother and sister should not be hybridized!

---Hybridization of homozygous(Ws/Ws) and heterozygous(Ws/+).

---The best combination is (Ws/Ws) male and (Ws/+) female. The reverse will give fewer pups.

Note to the breeding

---Both male and female rats (in healthy condition) are hybridized after 9 weeks of age.

---The period of hybridization should be 2-4 weeks (one male and one female rats are kept in the same cage).

---Recognizing the pregnancy when the weight of the female rat increases 20-30g per week (or swelling of the lower abdomen is found). The pregnant rats should be immediately separated.

---As the mother is very nervous at the time of delivery, she is apt to eat her pups. Therefore, do not clean the cages or measure the weight when delivery is approaching.

---In the case both the parents are homozygous (Ws/Ws), the pups are pale, small and thin. Many of them die of severe anemia after 10-25 days.

Cull distinctly the small and thin pups and those with low body temperature.

---In the case of hybridization between heterozygous (Ws/+) and homozygous (Ws/Ws), the ratio of Ws/+ pups to Ws/Ws pups is 1:1. But as Ws/+ pups survive and gain weight better than Ws/Ws, some Ws/+ pups should be culled to keep the ratio 1:1.

MICRODIALYSIS

The rats were anaesthetized with urethane (1.2 g/kg, i.p.) and placed in a special device. The body temperature was kept at 37°C with a warming light. Either of the hind paws was fixed tightly on a platform, and a dummy needle covered with the guide tubing for the microdialysis probe was inserted into the subcutaneous space of the plantar area (paper I) or dorsal skin (paper III). After the dummy needle was removed from the tubing, the microdialysis probe (CMA/20, membrane length 4 mm; Carnegie Medicine, Stockholm, Sweden) was inserted through the tubing into the tissue. Then, the tubing was torn apart to expose the membrane surface to the subcutaneous tissue. The area was perfused with saline at a flow rate of 2 μl/min. Under these conditions, the recovery of histamine from the surrounding fluid in the dialysate (relative recovery) was estimated by an in vitro perfusion test, in which the probe was placed in a test tube containing a 10 μM standard solution of histamine and was perfused at 37°C. The dialysate was collected every 20 min for up to 8 h, and was kept at -20°C until histamine determination.

INTRAVITAL MICROSCOPY

The rat mesentery preparation was performed according to a previous description (Yamaki *et al.,* 1998). Laparotomy was performed by a midline incision, and a segment of the ileum was exteriorized from the peritoneal cavity. The cremaster muscle was prepared as previously described (Baez, 1973). Briefly, a midline incision of skin and fascia was made over the ventral aspect of the right scrotum and the incised tissues were retracted to expose the cremaster muscle sack. The cremaster muscle was then incised and the testis was pushed back into the peritoneal cavity and the epididymis to the side of the preparation. Both preparations were performed on a heated transparent pedestal to allow microscopic observation of the mesenteric and cremaster muscle microcirculation. The exposed tissue was superfused with a thermostated (37°C)

bicarbonate-buffered saline solution (composition in mM: NaCl 132, KCl 4.7, $CaCl_2$ 2.0, $MgSO_4$ 1.2, $NaHCO_3$ 18) equilibrated with 5% CO_2 in nitrogen to maintain physiological pH. Observations of the mesenteric and cremaster muscle microcirculation were made using a Leitz Orthoplan microscope equipped with water immersion lenses (x25, NA 0.6; x55, NA 0.8). The microscopic image was televised (Panasonic WV-1550, Panasonic WV-1900 low light camera) and recorded on video tape (Panasonic NV-F 100 S-VHS) via a time/date generator (Panasonic WJ-810) for subsequent off-line analysis. After positioning under the microscope, a 20-30 min stabilization period preceded quantitative measurements. Rolling leukocyte flux was determined off line by counting the number of cells passing a reference point in two minutes, starting one min before the indicated time point. The number of firmly adherent leukocytes (stationary for > 60 s) was counted in 200 µm long venule segments and expressed as the number of adherent cells per 200 µm. Leukocyte emigration was quantified by counting the number of extravascular cells within a tissue area of 200 x 300 µm (0.06 mm^2).

DETERMINATION OF HISTAMINE BY HPLC

Histamine content in the dialysate and the tissue homogenate in rat paw skin was determined by an HPLC-fluorometry technique established by Yamatodani *et al.* (1985). Each 40 µl of dialysate was diluted 5 times with 5 mM Na_2EDTA, and 50 µl of the sample was injected directly into a column packed with cation exchanger, TSKgel SP2SW (150×6 mm i.d.; Tosoh, Tokyo, Japan). The histamine, eluted with 0.25 M potassium phosphate at a flow rate of 0.6 ml/min, was post-labeled with *o*-phthalaldehyde and detected fluorometrically in an F1080 fluorometer (Hitachi, Tokyo, Japan) using excitation and emission wavelengths of 360 and 450 nm, respectively. Paw skins were washed with cold saline, blotted with filter paper, weighed, and promptly homogenized in 1 ml of 3% perchloric acid with a polytron homogenizer operated at maximal speed in an ice bath. The homogenate was centrifuged at 10000×*g* for 10 min at 4°C, and 50 µl of the supernatant was subjected to HPLC-fluorometry.

SKIN FLAP

A standardized 2 x 7 cm (W x L) cranially based dorsal midline surgical flap with the base at the lower scapular angle was aseptically raised and sutured back as previously described (Törkvist *et al*., 1997). Six days after surgery, the animals were killed with CO_2 and decapitation, whereafter the percentage of flap survival was estimated using computer-assisted planimetry of schematic drawings of surviving and necrotic flap areas. In all animals, a clear demarcation line separated vital and necrotic skin areas.

ACTIVE SYSTEMIC ANAPHYLAXIS AND CARDIOVASCULAR MEASUREMENTS

Rats were sensitized by a single intraperitoneal injection of 10 µg of ovalbumin and 10 mg of aluminum hydroxide (Alhydrogel, Superfos Biosector, Frederikssund, Denmark) as adjuvant in saline. Thirteen days after sensitization, animals were anaesthetized and challenged by intravenous injection of ovalbumin (1 mg ml^{-1} at 1 mg kg^{-1}) in saline. Tracheal cannulation was made to facilitate spontaneous breathing in the anaesthesia. The carotid artery and jugular vein were cannulated, and catheters inserted were used for systemic blood pressure recording. The systolic (sBP) and diastolic (dBP) blood pressures and heart rate were measured by a pressure transducer connected to a Polygraph (Grass Instrument Co, Quincy, MA, USA). Mean arterial pressure (MAP) was calculated as MAP = dBP + (sBP-dBP)/3.

AIR POUCH MODEL

Rats were injected with 10 ml of sterile air subcutaneously into the dorsal skin, under light ether anesthesia, to make an air pouch in the shape of an ellipsoid or oval, as described before (Konno-Amano, 1989). After three days, the air pouch was reinflated with 5 ml sterile air. Experiments were performed six days after the initial air injection.

REVERSE PASSIVE ARTHUS REACTIONS

Rabbit IgG anti-chicken egg albumin (Sigma, St. Louis, MO, USA) 60 µg in 500 µl was injected into the preformed air pouch or peritoneum for passive sensitization. Forty-eight hours later, 1 ml of chicken egg albumin (Sigma, St. Louis, MO, USA 20 mg/kg in PBS) was locally administrated.

BOVINE AORTA ENDOTHELIAL CELLS

Bovine aorta endothelial cells (BAEC) were harvested according to the method of Booyse *et al.* (1975) with a few modifications. Briefly, the lumen of the bovine aorta or umbilical vein was incubated with a 0.25% collagenase solution for 10 min, and detached endothelial cells washed out with PBS. Harvested cells were pelleted and resuspended in culture medium consisting of a 1:1 mixture of M-199 and RPMI-1640 and supplemented with 20% heat-inactivated FCS, penicillin (100 U/ml) and streptomycin (100 µg/ml), seeded in tissue culture flasks and incubated under standard conditions. Endothelial cells of the second passage or third passage were used in perfusion assays.

ISOLATION OF HUMAN PMNL

Leukocyte-rich plasma, obtained from human whole blood by dextran (Macrodex) sedimendation, was carefully layered onto Ficoll-Paque and centrifuged at 400 x g for 30 min. The PMNL-containing pellet was resuspended and washed twice (400 g, 7 min) in ice cold PBS. The PMNL were resuspended in culture medium at a final concentration of 1 x 10^6 cells ml^{-1}. Purity of PMNL was > 98% and viability by Trypan blue exclusion was > 95%.

PERFUSION FLOW CHAMBER

Perfusions under steady flow were performed in a modified form of transparent parallel plate perfusion chamber, as previously described (Patel, 1999). This microchamber has a slit height of 0.2 mm and width of 2 mm. The chamber contains a circular plug on which a coverslip (18 mm x 18 mm) with confluent BAEC was mounted. PMN suspended at 0.8-1.0 x 10^6 cells/ml in RPMI medium plus 1% FCS, were perfused through the chamber via a syringe pump for 4 min at 50 µl/min at room temperature and a constant shear stress of 1.5 dyn/cm^2. Accumulated cells were visualized at 100x and 200x magnifications using bright field optics and recorded onto videotape via a camera (Panasonic, Japan) attached to an inverted microscope (Panasonic, Japan), as described previously (Guo *et al.,* 2000).

MEASUREMENT OF PLASMA EXUDATION IN AIR POUCH

Plasma exudation was measured with the aid of fluorescein-labeled BSA (F-BSA) as a tracer. At a suitable time after challenge, rats were injected intravenously with 10

mg of F-BSA dissolved in 0.2 ml saline. Thirty min later pouch fluid was collected and centrifuged. The supernatant was diluted fivefold with PBS and then its fluorescence intensity was read at 530 nm (excitation at 480 nm) in a plate reader. Total fluorescence intensity for the entire volume of the pouch fluid was calculated and expressed in terms of percent of the amount of F-BSA injected, and used as an index of the plasma exudation.

MAST CELL STAINING IN SKIN AND ON-LINE ACTIVATION IN RAT MESENTERY

Paw and dorsal skin pieces of +/+ and Ws/Ws rats were removed, fixed in Carnoy's solution and embedded in paraffin. Paraffin sections were cut 4 μm thick and stained with alcian blue. Mast cells in the skin were examined under a light microscope.

Ruthenium Red was diluted in bicarbonate-buffered saline solution to yield a final concentration of 0.001% for mast cell staining (Shepherd and Duling, 1995; Kubes, 1999). Observation of mast cell activation (Ruthenium Red staining) in mesentery was made using a Leitz Orthoplan microscope equipped with a water immersion lens (x55, NA 0.8). Ruthenium Red (0.001%) was superfused for at least 20 min prior to treatment and throughout the experiment.

REAGENTS

Ovalbumin (Albumin, Chicken Egg), dextran of 162,000 average molecular weight (Dextran-162), compound 48/80, histamine, chlorpheniramine and cimetidine were obtained from Sigma, St. Louis, MO, USA, and human recombinant TNF-α and IL-1β from R&D systems Inc. Minneapolis. Macrodex was obtained from Apoteksbolaget, Stockholm, Sweden.

STATISTICAL ANALYSIS

All data are expressed as means ± S.E.M. Two-way analysis of variance, followed by Scheffe test, was used to determine if the difference between two groups at a selected time point was statistically significant. A probability value of less than 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

MAST CELL ACTIVATION AND *IN VIVO* HISTAMINE RELEASE

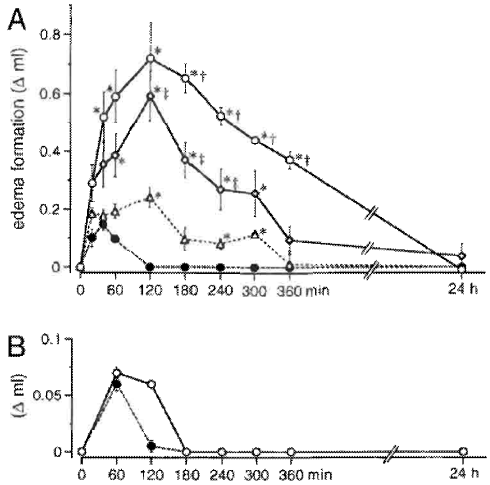
Microdialysis is a sampling technique which makes it possible to measure substances in the extracellular water space such as in animal skin in vivo. A microdialysis probe, i.e. a tubular semipermeable membrane connected to afferent and efferent tubings, is placed in the dermis and perfused. Substances diffusing from extracellular space through the pores of the membrane are collected in the dialysate for further analysis. Using this technique, histamine release from mast cells in rat paw and dorsal skin was addressed in paper I and III.

We determined the endogenous histamine concentration in the subplantar space of rat hind paws and dorsal skin using the in vivo microdialysis technique. The histamine content in dialysates was measured by high performance liquid chromatography-fluorometry. In wild type (+/+) rats, the mere implantation of a probe induced histamine release that reached a peak in 20 min, attaining 1.73 ± 0.15 nmol/ml (fig.1, Paper I) and 459 ± 87 pmol/ml (fig.2, paper III), in paw and in dorsal skin, respectively. In about 2 h histamine output declined to relatively low and stable levels (25.7 ± 0.9 pmol/ml and 21.8 ± 3.5 pmol/ml, in paw and dorsal skin, respectively). In Ws/Ws rats the histamine output (8.8 ± 0.8 pmol/ml and 6.6 ± 1.6 pmol/ml, in paw and dorsal skin, respectively) was low immediately after probe implantation and did not change with time. Because these tissues have complete lack of mast cells, confirmed by histological examination, the low level output of histamine in Ws/Ws animals must be derived from sources other than mast cells, such as basophils and enterochromaffin-like cells. The marked, more than 60-fold (paw) and 20-fold (dorsal skin), increase in histamine release in +/+ rats soon after the implantation, while no change in histamine levels in Ws/Ws rats, indicates that local tissue damage, or adverse stimuli such as pain, may induce release of histamine from tissue mast cells. Similarly, subplantar injection of saline in +/+ rats elicited an 11-fold increase in histamine release (fig. 2, paper I) and a small loss of histamine content in the skin of the treated paw (table 1 in paper I). We also found that a mere prick with the injection needle without saline infusion caused a 2-fold increase in histamine release in +/+ rats (unpublished data). These results suggest that sensory stimuli are involved in activating histamine release from mast cells. Previous reports support the idea of an interaction between mast cells and sensory nerves (C-fibers). Mast cells are in direct

anatomical contact with neuropeptide-containing sensory nerves and electrical stimulation of such nerve fibers may induce mast cell degranulation (Gazelius *et al.*, 1981; Dimitriadou *et al.*, 1991). In addition, neuropeptides such as substance P and neurokinin A, released from sensory nerve terminals, have been suggested to stimulate histamine release from mast cells (Joos & Pauwels, 1993; Horsmanheimo *et al.*, 1996; Hua *et al.*, 1996). Such mechanisms might also underly inflammatory disorders caused by activation in the central nervous system (Harvima *et al.*, 1994). We didn't perform histamine measurement after saline injection in rat dorsal skin. However the lower histamine dialysate level in dorsal skin (0.46 ± 0.87 nmol/ml) than in paw (1.73 ± 0.15 nmol/ml) after probe implantation in our data may suggest some difference in the anatomical structure of sensory nerve innervation and in mast cell distribution between the paws and the dorsal skin.

Subplantar injection of the compound 48/80 caused an up to several hundreds fold increase in histamine output from the injected paw in +/+ rats (fig.2 paper I). Simultaneously, there was at the most a 2-fold increase of histamine in the general circulation, indicating that the released histamine was derived almost exclusively from the paw per se. This view was supported by the observation that compound 48/80 caused substantial reduction in the histamine content in the injected paw but no change in the contralateral paw (table1 in paper I). In paws of Ws/Ws rats, there was neither increase in histamine release nor decrease in histamine content after injection of compound 48/80.

In conclusion, histamine released from mast cells could be monitored in vivo using microdialysis technique. Genetically mast cell-deficient Ws/Ws rats are useful and well–suited for studying the role of mast cell mediators in inflammatory responses when compared with the results from their wild-type littermates.



Paper I, Figure 3. Time-course of the development of paw edema in +/+ (A) and Ws/Ws (B) rats elicited by subplantar injection of compound 48/80. The edema was expressed as the increased volume (Δ ml, mean±S.E.M.). (A) Compound 48/80, at doses of 0.5 (Δ, n=4), 5 (◇, n=4) and 50 (○, n=5) μg, or saline (●, n=3) was injected at 0 min. (B) Compound 48/80, at a dose of 50 (○, n=3) μg, or saline (●, n=3) was injected at 0 min. * $P<0.05$, compared to saline-treated group. † $P<0.05$, compared to compound 48/80 (0.5 and 5 μg)-treated group. ‡ $P<0.05$, compared to compound 48/80 (0.5μg)-treated group.

CHARACTERISTICS OF MAST CELL ACTIVATION AND LEUKOCYTE RECRUITMENT BY SURGICAL PREPARATION

Upon activation, mast cells release histamine and other inflammatory mediators that can induce leukocyte recruitment. In wild type (+/+) rats, the histamine level was quite high, even just after microdialysis probe implantation in paw and dorsal skin (see above). Leukocyte rolling is a retarded movement of leukocytes that depends on the function of the selectin class of adhesion molecules. In venules of internal organs, rolling is rapidly induced on tissue exposure by unknown mediator(s). Histamine was suggested to cause leukocyte rolling in vivo by inducing endothelial expression of P-selectin (Ley, 1994). The hypothesis in paper II was to address whether spontaneous leukocyte rolling in rat and mouse microvessels was dependent on mast cell activity. Directly after the surgical preparation for intravital microscopy, spontaneous rolling

leukocyte flux and rolling velocity in venules of rat mesentery and mouse cremaster muscle were almost identical in wild-type and mast cell-deficient animals and then remained largely unchanged when followed 30 min (fig.1 in paper II). There was no difference in median venular diameters between wild-type and mast cell-deficient animals (28 ± 4 μm versus 26 ± 3 μm), in rat mesentery, and (31 ± 3 μm, compared with 32 ± 4 μm) in mouse cremaster muscle. However, due to higher blood flow in the mouse cremaster (Xie *et al.,* 1999), both rolling flux and rolling velocity in cremaster venules were significantly higher when compared to mesenteric venules. Systemic leukocyte counts (table 1 in paper II) were the same in wild-type and mast cell-deficient animals. Moreover, we found that virtually all mast cells remained intact after the surgical preparation in rat mesentery and mouse cremaster muscle. Our findings confirm and extend the observations by Tromp (1996) on the lack of role of mast cells in preparation-induced leukocyte rolling in the rabbit mesentery and in murine skeletal muscle (Kanwar *et al.,* 1998). In wild-type animals, pretreatment with compound 48/80 (50 μg intraperitoneally in rats and 5 μg intrascrotally in mice) for 4 h induced mast cell degranulation to 99% and 95% in rat mesentery and mouse cremaster muscle, respectively. This treatment, caused a very marked increase in the number of leukocytes with regard to both adhesion and transmigration, compared with animals pretreated with PBS. In contrast to wild-type animals, there was no increase in the number of adherent or extravascular leukocytes in mast cell-deficient animals challenged with compound 48/80. Considered together, these findings suggest that mast cell activation and the surgical trauma associated with tissue preparation for intravital microscopy are two separate processes with distinct biological characteristics. It may be argued that exteriorization of tissue may be associated with a very low degree of mast cell activation and a degranulation-independent release of lipid mediators from cell membrane phospholipids. For example, it has been reported that leukotriene C_4 (LTC_4) can trigger leukocyte rolling (Kanwar *et al.,* 1995). The similarity between wild-type and mast cell-deficient animals with regard to rolling characteristics following exteriorization did not support mast cell-dependent factors being responsible for spontaneous leukocyte rolling.

The mast cell is a key component in activating and directing leukocytes to extravascular tissues in allergic reactions because of the perivascular localization and capacity to release potent mediators, such as histamine and tumor necrosis factor-α (Gordon & Galli, 1991; Zhang *et al.,* 1992; Guo *et al.,* 1997). Previous studies showed that mast cell activation rapidly increased leukocyte rolling and adhesion (Raud *et al.,*

1988, Thorlacius *et al.,* 1994). The present findings extend these observations by showing that prolonged (4h) treatment with compound 48/80 provokes marked leukocyte adhesion and emigration in the rat mesentery and mouse cremaster muscle in wild type animals. However, the leukocyte response in mast cell-deficient animals is insignificant and not different from that in PBS-treated rats and mice. The conclusion from paper II was that, although mast cells can trigger the recruitment of leukocytes, the induction of spontaneous leukocyte rolling in rat mesentery and the mouse cremaster muscle is independent of mast cell activation and function.

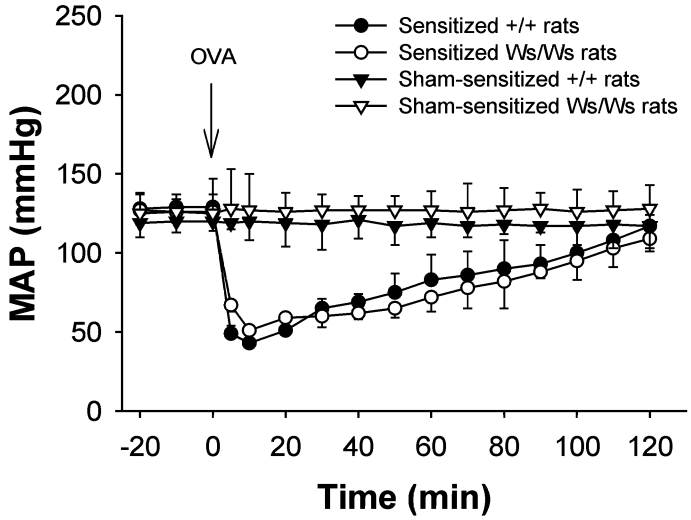
The degree of surgical trauma between preparations of skin flap and of rat mesentery and mouse cremaster muscle for intravital microscopy may differ, and affect the local leukocyte recruitment to a different extent. A previous study showed that the pro-inflammatory potency of cut (skin flap surgery) causes more pronounced leukocyte recruitment than injection of high doses of some inflammatory mediators such as platelet-activating factor (PAF, 10 μm) and zymosan activated serum (ZAS, undiluted) (Törkvist, 1999). As shown in figure 3 of paper III, we found that accumulation of skin flap myeloperoxidase (MPO, units g^{-1}tissue) (reflecting leukocyte recruitment) in the distal part of dorsal skin flaps immediately after surgery was low and virtually the same in mast cell-deficient Ws/Ws rats, wild-type (+/+) littermates and Wistar rats (0.3 ± 0.08, 0.4 ± 0.07 and 0.4 ± 0.09 units g^{-1}, respectively). However, twenty-four hours after surgery, MPO increased to 24.4 ± 10.4 in wild-type (+/+) rats and to 30.7 ± 10.9 in Wistar rats, whereas significantly less MPO, 10.3 ± 2.3 units g^{-1}, accumulated in Ws/Ws rats. The fact that leukocytes did not accumulate to any appreciable extent in skin flaps of our mast cell-deficient rats provides an obvious coupling between presence of mast cells and substantial accumulation of leukocytes in skin flaps of the wild-type (+/+) littermates or normal Wistar rats. Mast cells may both release and synthesize an impressive array of mediators (preformed granule-associated, lipid derived, and cytokines) thought to have a number of important functions, including involvement in host defense, modulation of the immune system, and in tissue repair (Metcalfe *et al.*, 1997). Histamine is a major mediator liberated from degranulating mast cells and has long been associated with inflammatory processes. Thus, it causes endothelial cell contraction and increased vascular permeability (Wu & Baldwin, 1993; Iuvone *et al.*, 1999), upregulation of P-selectin for initiation of leukocyte rolling (Yamaki *et al.*, 1998), and potentiation of chemoattractant-induced leukocyte adhesion (Thorlacius *et al.*, 1995). In Paper III, we show that combined pre-treatment with

histamine H_1 and H_2 antagonists (chlorpheniramine and cimetidine) drastically reduced leukocyte accumulation in wild-type (+/+) littermates and Wistar rats (13.1 ± 5.6, and 15.2 ± 9.6 MPO units g^{-1}, respectively) to a level not far from that seen in the mast cell-deficient animals. The notion that H_1 and H_2 receptors together mediated the leukocyte response to histamine was supported by the observation that challenge with H_1- or H_2-receptor agonists triggered venular accumulation of leukocytes (Yamaki *et al.,* 1998). Further, injection (i.p.) of the mast cell secretagogue 48/80 drastically increased the venular concentration of leukocytes, which was also inhibited by the antihistamines (Thorlacius *et al.*, 1994). This may seem surprising because challenge with 48/80, in addition to rolling, causes significant adhesion of leukocytes (Thorlacius *et al.*, 1994), possibly due to the release of mast cell-derived chemotactic components, such as platelet activating factor and leukocyte-attracting cytokines (Gaboury *et al.*, 1995). Increase in adhesion should thus be expected to decrease rolling. However, because leukocyte rolling is known to be a prerequisite for firm adhesion of leukocytes (Lindbom *et al.*, 1992), it was suggested that free flowing leukocytes in the microcirculation do not respond to mast cell chemoattractants unless they are first stimulated to roll by histamine (Yamaki *et al.*, 1998). This view is supported by the observation that inhibition of 48/80-induced leukocyte rolling by an anti-P-selectin antibody causes a parallel reduction of rolling and firmly adhering leukocytes (Thorlacius *et al.*, 1994). Taken together all these experiments suggest that histamine, either per se or via induction of P-selectin expression, mediates leukocyte rolling which represents a first and crucial step in the sequence of microvascular events that finally result in leukocyte accumulation in inflamed tissues. Our experiments with rat skin flaps strongly support this view and argue for mast cell-derived histamine being of prime importance in this respect. However, it should be pointed out that antihistamine treatment markedly inhibited but did not prevent leukocyte recruitment, and even in mast cell-deficient animals there was some accumulation of leukocytes and tissue necrosis in the skin flaps post surgery.

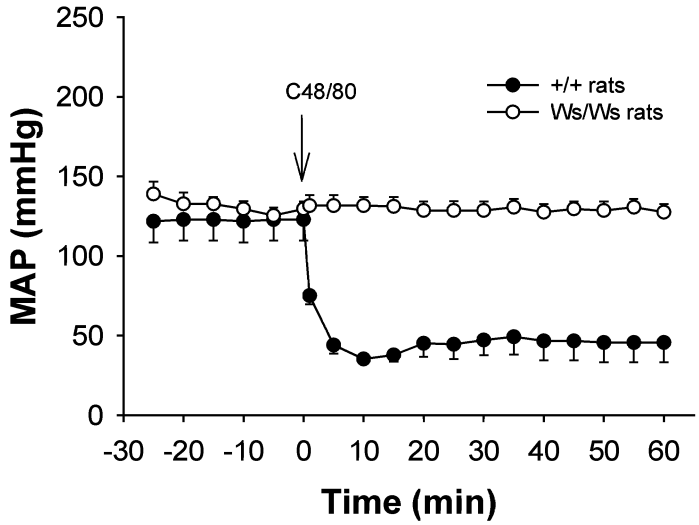
FUNCTIONAL ROLES OF MAST CELLS IN ACTIVE SYSTEMIC ANAPHYLAXIS

Degranulation of sensitized tissue mast cells was believed to be involved in both local and systemic anaphylactic reactions. But since systemic anaphylaxis can be induced in genetically mast cell-deficient mice (Jacoby *et al.*, 1984; Ha and Reed, 1987), the necessary participation of mast cells in this type of anaphylaxis is now being seriously

questioned. To address this question, cardiovascular effects caused by antigen, dextran and compound 48/80 were determined using mast cell-deficient Ws/Ws and their control +/+ littermate rats. Active sensitization was performed by a single intraperitoneal injection of 10 μg of ovalbumin (OA), followed by intravenous injection of OA thirteen days after sensitization. Ten minutes after challenge to the rats, mean arterial pressure fell from a level well above 100 mmHg to 43 mmHg and 51 mmHg in sensitized +/+ and Ws/Ws rats, respectively (fig.1 in paper IV).



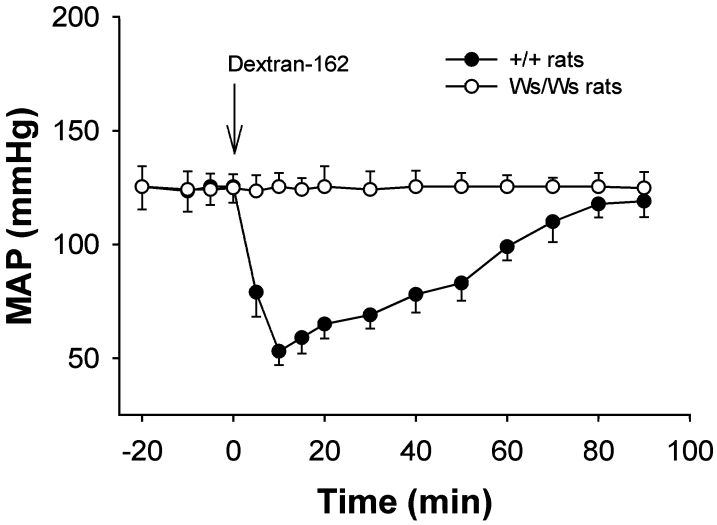
Paper IV, Figure 1. Mean arterial pressure (MAP) of sensitized +/+ (filled circles), mast cell-deficient Ws/Ws (open circles); and sham-sensitized +/+ (filled triangles), mast cell-deficient Ws/Ws (open triangles) rats, receiving ovalbumin (OVA, 1 mg kg^{-1} i.v.) at time 0 (arrow). Data are means ± S.E.M., n = 5.



Paper IV, Figure 2. Effects of compound 48/80 on mean arterial pressure (MAP) in +/+ (filled circles), and mast cell-deficient Ws/Ws (open circles) rats, receiving compound 48/80 (C48/80, 1 mg kg^{-1} i.v.) at time 0 (arrow). Data are means ± S.E.M., *n* = 6.

These results do not support the view that mast cells serve as the critical effector cells in mediating the hypotensive response associated with systemic anaphylaxis. Mast cell degranulation, above controls, could not be detected in examined mesentery from sensitized rats that had just gone into hypotension following intravenous antigen challenge (paper IV). This gives support to conclusions reached by others, who have shown that mast cell-deficient mice can undergo anaphylaxis (Jacoby *et al.*, 1984; Galli and Kitamura, 1987) and that mast cell-competent mice do not require mast cell responses for anaphylaxis (Einbinder *et al.*, 1964; Justus *et al.*, 1990). The observation of numerous degranulating mast cells during topically applied antigen (fig.4 in paper IV) suggests that during anaphylaxis the antigen remained in the vasculature and did not reach the tissue mast cells. This means that intravascular and not extravascular cells or elements are responsible for initiating the events that give rise to the hypotensive response. Pretreatment with the combination of histamine H_1 (chlorpheniramine) and H_2 (cimetidine) receptor antagonists did not exert any inhibiting effect on the

ovalbumin-induced hypotension in sensitized +/+ rats (fig.6 in paper IV). The data suggest that mast cell amines are not involved in this response. In mice, anaphylaxis can be inhibited by anti-granulocyte antibody (Kimura *et al.*, 1997). It is thus possible that mediators released not from mast cells but from other cells including neutrophils, basophils, monocytes/macrophages, platelets and endothelial cells are involved in the reaction. Whether this is a consequence of or occurs in parallel with complement activation (Luskin and Luskin, 1996) remains to be determined.



Paper IV, Figure 3. Mean arterial pressure (MAP) of mast cell containing +/+ (filled circles) and mast cell-deficient Ws/Ws (open circles) rats, receiving dextran (Dextran-162, 6%, 2 ml kg^{-1} i.v.) at time 0 (arrow). Data are means ± S.E.M., $n = 3$.

In contrast to the results from antigen-induced anaphylaxis, hypotensive responses by compound 48/80 or dextran were observed only in +/+ rats. Mast cell activation (Ruthenium Red staining) was observed in rat mesentery 20 min after intravenous administration of compound 48/80 or dextran. The total lack of hypotensive effect in Ws/Ws rats demonstrates that mast cell mediators play a pivotal role in responses to 48/80 or dextran. Pretreatment with the combination of chlorpheniramine and cimetidine, which exhibited a 1000-fold antagonism of the blood pressure lowering effect of exogenous histamine, almost completely blocked hypotension by compound

48/80 or dextran. However, no inhibitory effect on antigen induced hypotensive response was obtained (see above). The results of our experiments agree with a previous study by Kogure (1986) who showed that corticosterone inhibited dextran-induced shock in rats by inhibition of histamine release from mast cells. Our results and those of others thus indicate that mast cell histamine plays a critical role in compound 48/80 and dextran-induced hypotensive responses in rats.

The combination of histamine receptor antagonists thus caused a 1000-fold antagonism of exogenous histamine and blocked hypotension induced by compound 48/80 or dextran. Despite this, the antagonist combination failed to modify the hypotension induced by intravenous allergen in sensitized animals. This further supports that mast cells and histamine are not involved in the hypotensive response in systemic anaphylaxis.

Taken together, the present study suggests that active systemic anaphylaxis can be induced in mast cell-deficient rats. Mediators, not from mast cells but from unknown cells, are responsible for the hypotensive response. However, the vasoactive substances, primarily histamine, released from mast cells, play the main role in the hypotension by compound 48/80 or dextran.

PLASMA EXUDATION AND LEUKOCYTE RECRUITMENT INDUCED BY IgG IMMUNE COMPLEX REACTION

Based on our findings in paper IV, which suggested absence of mast cell involvement in active systemic anaphylaxis, the question arose whether or not mast cells participate in passive IgG immune complex reactions in the rats. Thus, in paper V, effects on leukocyte recruitment and plasma leakage evoked by passive IgG sensitization and challenge with OA were investigated, using genetically mast cell-deficient rats (Ws/Ws) together with their control littermates (+/+). A marked vascular response was induced by passive IgG immune reaction in wild-type rats, whereas only minor changes were evoked in mast cell-deficient Ws/Ws rats. Similarly, leukocytes having emigrated into air pouch as six hours after OA challenge were clearly more aboundant in the wild-type rats, both with respect to polymorphonuclear and mononuclear cells. With intravital microscopy, we found that the leukocyte recruitment, including rolling, adhesion and transmigration, was markedly increased in wild-type +/+ rat mesentery 6h after Arthus reaction when compared with PBS challenge, and also when compared with Arthus reaction in mast cell-deficient Ws/Ws rats. Previous studies have suggested a role of mast cells in immune complex reaction using mast cell-deficient mice (Ramos

et al., 1990; Zhang, 1991, 1995b), and it has been reported that mast cells play an essential role in the early phase of immune complex-mediated leukocyte adhesion and emigration in rat (Kosaka *et al.,* 1997). Our data give considerable support to this view and add that the same obviously holds true for the later phase of immune complex-mediated leukocyte recruitment. Using flow chamber, we confirmed that mast cell-derived mediators caused immune complex-mediated human leukocyte adhesion to BAEC in vitro. The number of firmly adherent leukocytes was seven times larger in BAEC monolayers stimulated with peritoneal fluids from +/+ rats than that from Ws/Ws rats and tenfold that in monolayers treated with PBS. The data clearly suggest a dominant role of mast cells for the Arthus reaction in the rat, using both in vivo and in vitro assay systems. When the reaction is evoked in the peritoneum, one or several messengers stable in transfer experiment and overnight storage, are formed. Whether this involves release of known messengers such as TNF-α and IL-1β, which in combination could induce the response, is presently not known. Our observations, that mast cells are important for the late stages of immune complex activated responses, might be of clinical importance since immune complex evoked reactions often give clinical manifestations of considerable duration (Newkirk, 2002; Stassen *et al.,* 2002).

MAJOR CONCLUSIONS

This thesis provides important insights to better understanding the roles of mast cell-derived mediators in inflammation in distinct tissue sites and in anaphylaxis. This was achieved by direct observations of histamine concentration change, plasma exudation, hypotensive response, and leukocyte recruitment in mast cell-deficient (Ws/Ws) and their control littermates (+/+) rats, using microdialysis, intravital microscopy, a skin flap model, an anaphylaxis model, and measurement of leukocyte-endothelial interaction in flow chambers.

In summary, the findings of this study suggest that:

- the use of a microdialysis method for analysis of histamine in the subplantar tissues is adaptable to further studies on allergic and inflammatory disorders. Genetically mast cell-deficient Ws/Ws rats are useful and well-suited for the investigation of the pathophysiological roles of mast cells.

- although mast cells can trigger the recruitment of leukocytes, the induction of spontaneous leukocyte rolling in venules of rat mesentery and mouse cremaster muscle is independent of mast cell activation and function.

- there are distinct differences in tissue site-specific participation of mast cells between mesentery and dorsal skin surgery preparations. Decreased mast cell activation may increase tissue survival in surgical skin flaps. The protective effect of anti-histamine might be related to the decrease in neutrophil recruitment. The use of mast cell-deficient animals may be of considerable help in the search of additional factors that contribute to leukocyte recruitment.

- active systemic anaphylaxis can be induced in mast cell-deficient rats. Mediators, not from mast cells but from unknown cells, are responsible for the hypotensive response. However, the vasoactive substances, primarily histamine, released from mast cells, play the main role in the hypotension by compound 48/80 or dextran since the combination of histamine receptor antagonists blocked hypotension by compound 48/80 or dextran, whereas, the antagonist combination failed to modify the hypotension induced by intravenous allergen in sensitized animals.

- the substantial differences in plasma leakage; in the number of emigrated leukocytes after Arthus reaction in air pouches; and in leukocyte recruitment, including rolling, adhesion and transmigration in +/+ rat mesentery after Arthus reaction when compared with PBS challenge and also when compared with Arthus reaction in Ws/Ws rats; as well as in the number of firmly adhesion leukocytes in BAEC monolayers stimulated with peritoneal fluids from +/+ rats when compared with that from Ws/Ws rats and that in monolayers treated with PBS clearly suggest a dominant role of mast cells for the Arthus reaction in the rat.

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