Integrin-mediated modulation of mast cell degranulation

Integrine-gemedieerde modulatie van mestcel degranulatie

(met een samenvatting in het Nederlands)

Proefschrift

Ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. dr. H.O.Voorma ingevolge het
besluit van het College van Promoties
in het openbaar te verdedigen
op vrijdag 14 april 2000 des namiddags te 12.45 uur

door

René Houtman

geboren op 28 mei 1970 te Vlaardingen

Promotor: Prof. dr. F.P. Nijkamp

Co-promotor: Dr. A.S. Koster

Afdeling Farmacologie & Pathofysiologie, Faculteit Farmacie, Universiteit Utrecht, Sorbonnelaan 16, 3584 CA, Utrecht

The study presented in this thesis was performed at the Department of Pharmacology & Pathophysiology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Pharmacy, Utrecht University, Utrecht, The Netherlands and was supported by GlaxoWellcome, The Netherlands

ISBN: 90-393-2359-3

Cover design: Martijn Pieck/Maarten van Baggum
Printing: Print Partners Ipskamp, Amsterdam

General introduction

Allergy affects the everyday life of many people. This is illustrated by estimations that almost half the population of the western world is atopic for one or more environmental 'allergens' and that 155 million individuals suffer from asthma, an allergic disease, worldwide[10,69,70,106].

The word '*allergy*' for '*deviation from the original state*' was introduced at the beginning of the 20th century and originally described a variety of harmful immune reactions to vaccination. To differentiate the familial syndrome of asthma and hay fever from these mixed reactions the term '*atopy*' for 'strange *disease*' was used[i].

The earliest report describing clinical phenomena of an allergic response was already written before 925 AD by Rhazes[21]. Since then, the foundations of allergy have started to evolve slowly, with the description of the pathology of 'catarrus aestivus' or hay fever (1819), discovery of a causative link with pollen grains (1873) and a transferable tissue sensitizing serum factor (1921), which was later identified as IgE (1976)[69]. However, not before the last three decades science has begun to understand the molecular basis of this immune deviation.

Allergic response

Allergy is the result of a humoral response of the immune system to an allergen, an antigen that due to particular characteristics (reviewed by Corry *et al.*[31]) is able to skew an immune reponse. Humoral immune responses can be divided in two phases. During the first phase, recognition of the antigen by the immune system leads to the production of antigen-specific antibodies. During the second phase, activation of an effector mechanism results in elimination of the antigen. Antibodies can be grouped into different classes, based on their Fc domain and each class is recognized by a specific set of receptor isotypes, Fc receptors. Because Fc receptor isotypes are differentially expressed in a cell type-specific manner, the antibody class determines the effector cell type that is activated. In other words, specific antigen recognition is linked to a particular effector mechanism through the class of antibody involved.

Sensitization

Antigens that have entered the body are handled through two separate pathways (figure 1). On one end, antigens are taken up by macrophages and dendritic cells. These highly specialized cells are able to process antigens and present their peptide fragments on the cell surface together with molecules required for lymphocyte activation, and hence are called antigen-presenting cells (APC)[ii]. Presented peptides are specifically recognized through the antigen receptor on 'helper' T lymphocytes (T_H cells). This APC-T_H cell interaction triggers activation of the latter cell and subsequent production of a specific T_H cytokine profile. On the other end, native antigens are bound by the antigen receptor on B lymphocytes that is subsequently activated. Costimulation of this B cell through physical interaction with the activated T_H cell and T_H cytokines, promotes maturation into a plasma cell that produces and excretes antigen-specific antibodies[82].

[i] During time, allergy has lost its original broad meaning and currently the terms atopy and allergy are used interchangeably *(see also Holgate[69]).*

[ii] For abbreviations see page 101

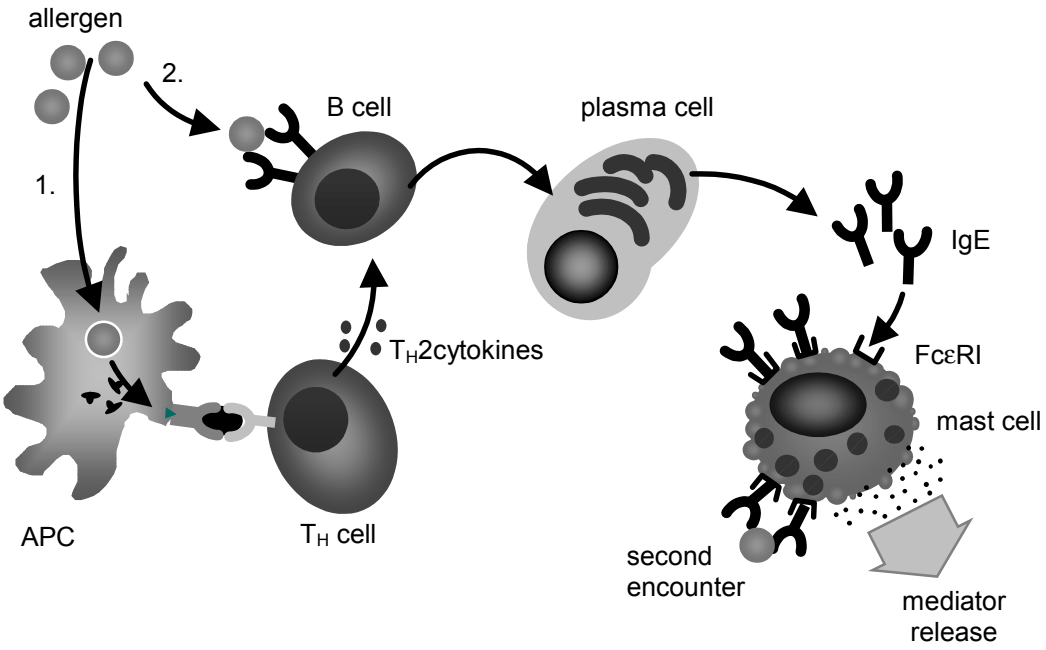


Figure 1. Schematic representation of an allergic response. *During the sensitization phase,* ***1****. allergen is taken up, processed and presented by an antigen-presenting cell (APC) to an allergen-specific helper T lymphocyte (T_H cell), and this cell is subsequently stimulated to produce a T_H2 cytokine pattern;* ***2.*** *native allergen is specifically recognized by a B lymphocyte (B cell), which are subsequently activated. Costimulation of the activated B cell with T_H2 cytokines drives this cell through maturation into an IgE-producing plasma cell. Allergen-specific IgE binds to a high-affinity Fc receptor (FcεRI), expressed on mast cells. Renewed encounter of a sensitized mast cell with allergen triggers release of inflammatory mediators.*

Activation of a T_H cell by an allergen triggers the production of a so-called T_H2 cytokine pattern. Of this cytokine subset predominantly interleukin (IL)-4 and IL–13 are responsible for B cell maturation into a plasma cell that produces Ig*E* antibodies[31]. Formation of allergen-specific IgE is the key event in the development of allergy. Through binding to a high-affinity Fc receptor (FcεRI), IgE links the specific recognition of antigen to the mast cell effector compartment. Binding of IgE to FcεRI on mast cells does not evoke a response but primes these cells for subsequent encounter with the allergen and is hence referred to as sensitization.

Effector phase

Mast cells play a major role during the effector phase of an allergic response. Activation of sensitized mast cells by multivalent allergen triggers release of inflammatory mediators from mast cell granules. These mediators, for example histamine, cause constriction of the bronchial airways, vasodilation, vascular permeability, mucus secretion and other responses that induce clinical phenomena associated with allergy[59]. These cells were described in the 1870s by Paul Ehrlich and termed '*Mastzellen*', for '*well-fed cells'*, because of their cytoplasm, stuffed with prominent granules[42] (figure 2). Mast cells are found in all tissues throughout the body, particularly associated with blood vessels and nerves and in proximity to surfaces that interface the external environment, such as skin, intestins and lungs[105].

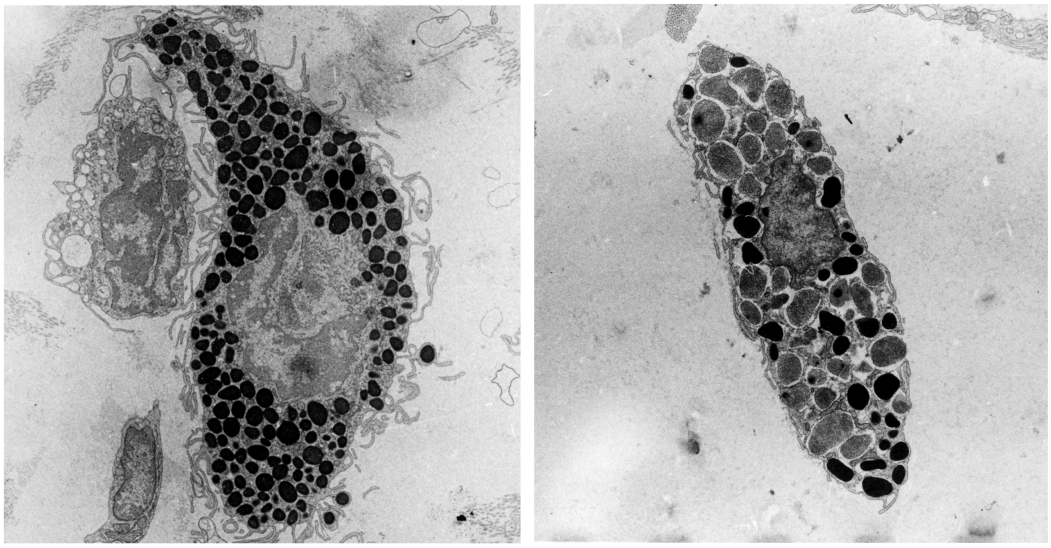


Figure 2. The mast cell. *Pulmonary mast cells visualized by electron microscopy. The electron dense granules are clearly distinctive.* ***A.*** *unstimulated and* ***B.*** *partially degranulated due to stimulation with allergen (courtesy of Dr. Frank A. Redegeld).*

FcεRI

The major receptor for mast cell activation leading to degranulation is FcεRI[88,107,161]. Based on similarities in structural features and signaling targets, FcεRI has been placed in the multichain immune recognition receptor (MIRR) family together with the B and T cell antigen receptor and several IgG Fc receptor isotypes (figure 3). The FcεRI multimeric complex consist of an α-chain, which binds IgE, a β-chain, which plays a role in membrane stabilization and serves as a signal amplifier, and two γ-chains, which form the connection between the receptor complex and the intracellular signaling events leading to degranulation. The β- and γ-subunits contain conserved immunoreceptor

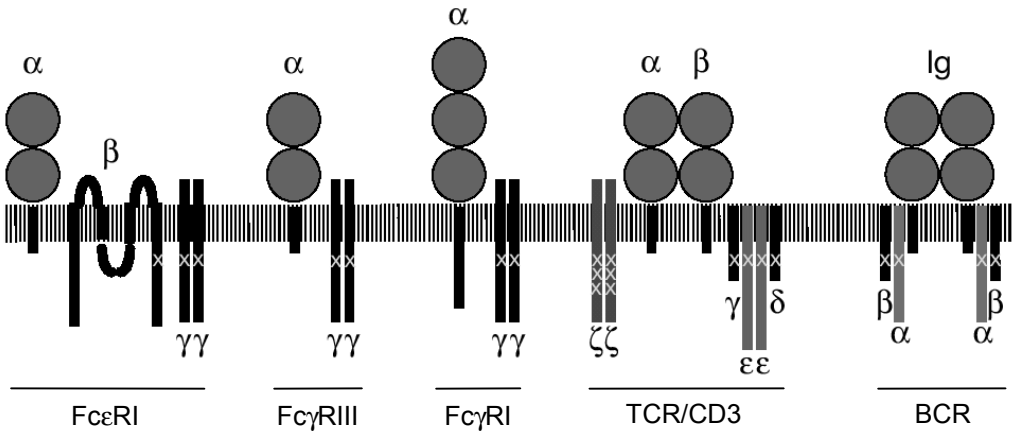


Figure 3. ITAM-containing immunoreceptors. *The tetrameric FcεRI is shown in the context of other members of the MIRR family. Grey circles represent immunoglobulin domains. ITAM motifs are shown as crosses. TCR, T-cell antigen receptor; BCR: B cell antigen receptor; Ig: immunoglobulin; ITAM: immunoreceptor tyrosine-based activation motif. (Adapted from Nature)*[161]

tyrosine-based activation motifs (ITAMs). The tyrosine residues in the ITAM consensus sequence (D/E-XX-YXXL-X_{7-11}-YXXL-L/I) conserved among Fc receptors, serve as phosphoacceptors through which the receptor subunits can interact with downstream signaling proteins.
Stimulation of a sensitized mast cell through binding of multivalent antigen to receptor-bound IgE induces clustering of FcεRI. This initiates a chain of phosphate transfer events within the receptor environment (figure 4A). The first events of the signaling cascade involve phosphorylation of the tyrosine residues of the FcεRI-β and -γ ITAMs by protein tyrosine kinase (PTK) Lyn. Phospho-ITAMs serve as docking sites for proteins that contain a Src-homology-2 (SH2) domain. Subtle variations between FcεRI-β and -γ ITAMs result in affinity for different SH2-containing proteins. Phosphorylation of FcεRI-β ITAM leads to the recruitment of additional Lyn to the receptor complex and binding to the phospho-ITAM enhances the enzymatic activity of this PTK. Phosphorylation of the FcεRI-γ ITAM subsequently leads to the recruitment and activation of cytosolic Syk, a PTK of the ZAP-70 PTK family. Activation of Syk has been shown to be sufficient as well as obligatory for induction of mast cell degranulation[32,132].
A subsequent series of signaling events, involving activation of various adapter molecules that will not be discussed here (reviewed in Turner *et al.*[161]), lead to membrane recruitment of cytosolic phospholipase Cγ1. This enzyme catalyses the formation inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol DAG from membrane phospholipids (figure 4B). IP_3 binds and opens the IP_3 receptor calcium channels on the surface of the intracellular calcium stores, which triggers the release of calcium. To establish sustained elevation of the cytosolic Ca^{2+} concentration, required for degranulation, an influx of calcium from the surrounding environment is initiated by opening of 'store-operated' calcium channels in the plasma membrane. Elevation of the cytosolic Ca^{2+} concentration leads to mast cell degranulation, and although a role for calcium-binding calmodulin and calmodulin-dependent kinase have been established[25,168] the signaling events between calcium influx and exocytosis of granular contents are not very well analyzed in mast cells.

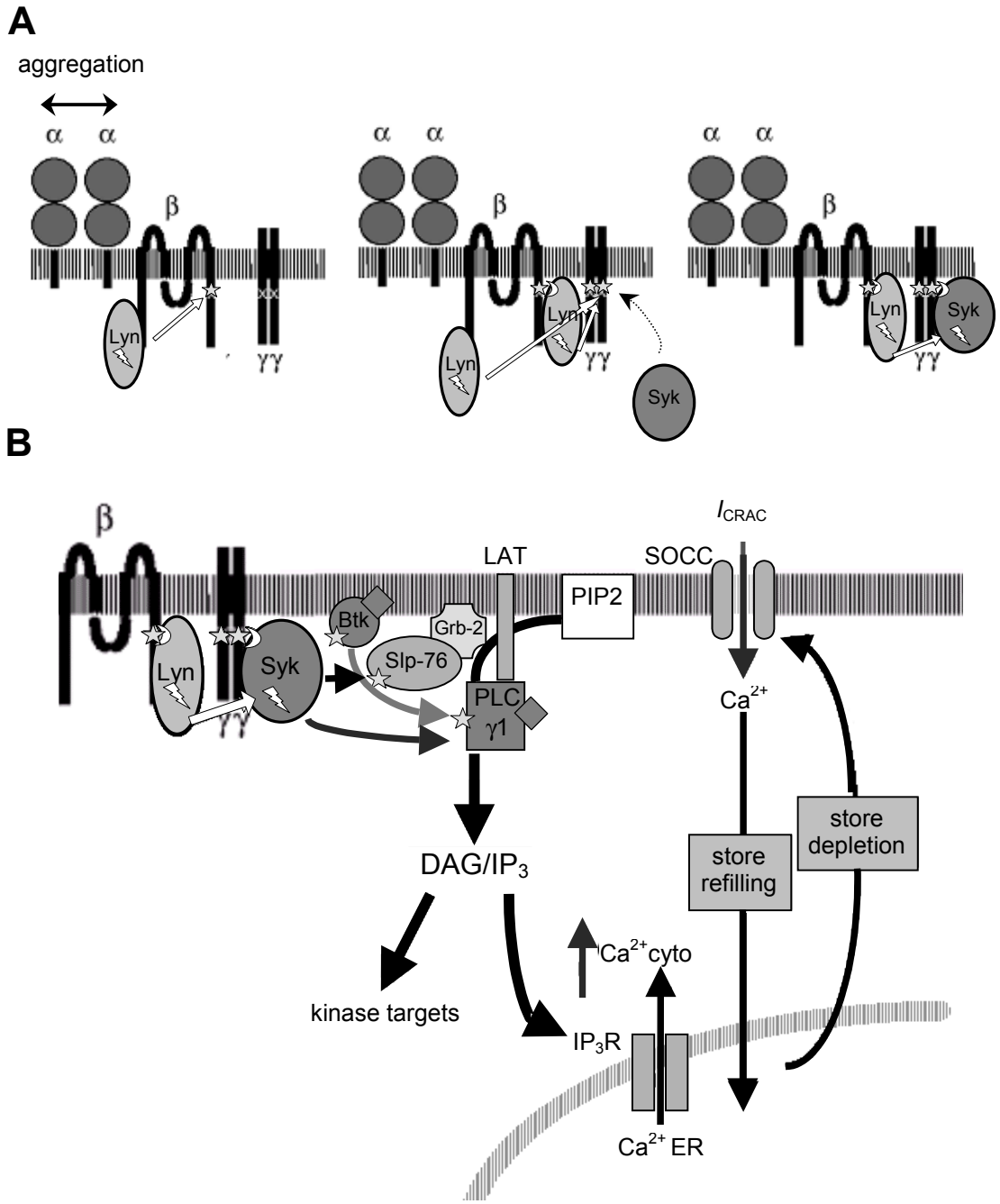


***Figure 4. FcεRI activation induces complex networks of signaling events**. **A, Early events in FcεRI signaling**. FcεRI α-chains are aggregated by crosslinking of bound IgE by multivalent antigen. Association of the receptor complex with activated Lyn induces phosphorylation of the β- and γ-chain ITAMs (indicated by ★), the latter is then able to recruit Syk, which is in turn activated by Lyn. Subsequent actions of Lyn and Syk, include the activation of PI(3)Kinase to produce PtdIns(3,4,5)P_3 (PIP_3) (not shown in this cartoon), phosphorylation of the Bruton's kinase (Btk) and phosphorylation of the membrane-localized adapter protein LAT. **B, FcεRI control of [$Ca^{2+}{}_i$].** Btk, which is localized at the membrane by binding to PIP_3, contributes to activation of PLCγ1, which in turn has been brought to the membrane by the phosphorylated LAT adapter. PLCγ1 acts on membrane inositol phospholipids to generate Ins(1,4,5)P_3 (IP_3) and DAG. The intracellular targets of the latter include PKC isoforms, whereas IP_3 binds to the IP_3 receptors (IP_3R) on the surface of the endoplasmic reticulum (ER) calcium stores, leading to store depletion and elevation of cytoplasmic calcium levels. Depletion of ER calcium stores is coupled to opening of plasma membrane channels for calcium influx; the calcium release activated current (I_{CRAC}) is mediated by these store-operated calcium channels (SOCC), and is responsible for sustained elevations in cytosolic calcium and refilling of ER stores. (Adapted from Nature)*[161]

2

Modulation of mast cell function

Factors associated with development of allergy

The mere fact that mast cells exist suggests that, although they can cause serious damage, their presence has provided an evolutionary benefit. Indeed, a physiological role for mast cells in various processes such as wound healing, angiogenesis, host defense against parasites, and innate immunity has been reported[1,6,51,52]. During the last century the incidence of atopy has increased rapidly to epidemic proportions. This strongly suggests that external factors play an important role in the development of allergic diseases, causing mast cells to do harm instead of good.

Epidemiology studies indicate that atopic disorders have their origin in childhood and are associated with lifestyle[30]. For example, hygiene in the Western society has been indicated as an important factor[83]. Besides environmental factors, the development of asthma appears to have a genetic basis[30]. Most of these factors are explanatory for the skewing towards IgE production during a humoral immune response. However they can not explain the observation that many people who are atopic by skin tests, do not have symptoms of an allergic disease. Furthermore, there is no explanation for the observation that equivalent levels of hypersensitivity to the same allergens in different subjects results in skin disease (atopic dermatitis) in some subjects, in nasal inflammation (rhinitis) in others, and a multi-organ disease in still another population[70]. Overall, these observations suggest that in the target organ the function of a sensitized mast cell is modulated by factors, which are specific for the local microenvironment.

Mast cells and their microenvironment

Mast cells do not form a homogeneous population and morphologic differences between mast cells found at different anatomical locations can be observed[50]. In the rodent system mast cells are classified into connective tissue-type (CTMC)[i] and mucosal (MMC) mast cells. In addition to differences in morphological and histochemical characteristics, rodent MMC and CTMC appear to differ in many other aspects of natural history, biochemistry and function (table 1)[4,17,105,108,172]. Regulatory effects of various microenvironmental components on mast cell function have been demonstrated. For example, cytokines such as IL-3[40,] soluble stem cell factor[29,85,151,156], interaction with fibroblasts via membrane-bound stem cell factor[121] and neuronal cells[18,154], and integrin-mediated cellular adhesion to extracellular matrix-components fibronectin and laminin enhance degranulation[62,64,131,167,177]. The number of factors reported to affect the functional repertoire of mast cells *in vitro* increases rapidly, and at least some of these factors may prove to play an important role in the modulation of mast cell activity *in vivo*.

In all tissues throughout the body, resident mast cells are surrounded by extracellular matrix-components. Very late antigen-5 (VLA-5) has been identified as a key receptor for adhesion of mast cells to fibronectin[45,87,175,177]. The observation that VLA-5-blocking antibodies strongly suppress passive

[i] For abbreviations see page 101

	Connective tissue-type mast cell (CTMC)	Mucosal mast cell (MMC)
Size	10-20 μm	5-10 μm
Formaldehyde fixation	Resistent	Sensitive
Staining	Safranin	Alcian blue
T-cell dependence in development	No	Yes
Protease content	MMCP-3, -4, -5, -6, -7	MMCP-1, -2
Proteoglycans/	Heparin/	Chondroitin sulfate di B/
Molecular mass	750-1000 kDa	100-150 kDa
Histamine	10-20 pg/cell	1 pg/cell
Serotinine	1-2 pg/cell	< 0.5 pg/cell
Prostaglandin D_2	+	+
Leukotriene C_4	-	++
Activated by		
FcεRI aggregation	Yes	Yes
Compound 48/80	Yes	No
Substance P	Yes	No
Inhibited by sodium cromoglycate	Yes	No

Table 1. Rodent mast cell characteristics

β-subunit isoform	α-subunit isoform	ligand
1	1	collagens, laminin
	2	collagens, laminin
	3	fibronectin, laminin, collagens
	4	fibronectin (V25), VCAM-1
	5	fibronectin
	6	laminin
	7	laminin
	8	?
	V	vitronectin, fibronectin
2	1	ICAM-1, ICAM-2
	m	C3b (complement), fibrinogen, ICAM-1
	x	fibrinogen, C3b
3	IIb	fibrinogen, fibronectin, von Willebrand F.
	v	vitronectin, von Willebrand F., fibronectin, thrombospondin, osteospondin, collagen
4	6	laminin?
5	v	laminin
6	v	fibronectin
7	4	fibronectin (V25)
8	IEL	?
	v	?

Table 2. The integrin receptor family. *(Adapted from Cell)*[76]

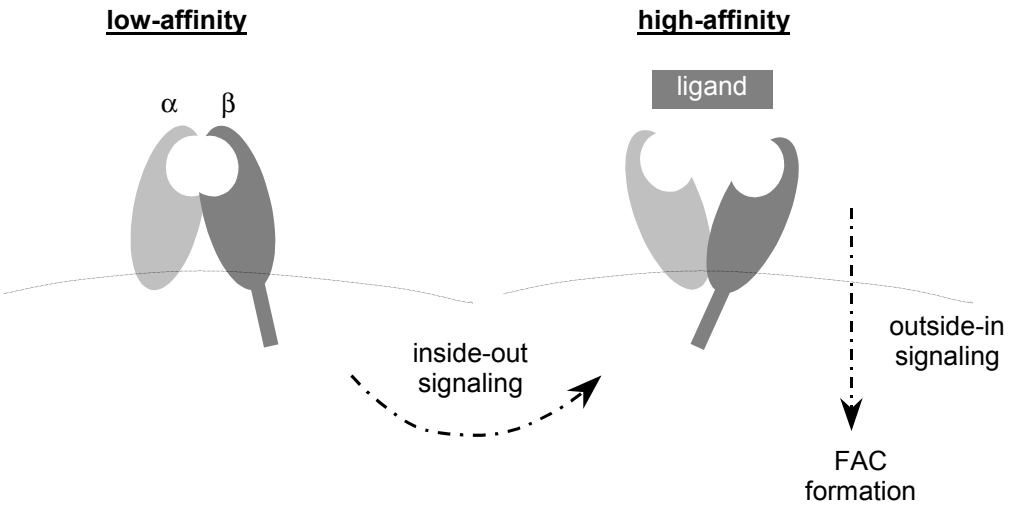


Figure 1. Integrin affinity modulation. *Unstimulated integrin αβ-heterodimers exist in a low-affinity conformation on the plasma membrane. Through inside-out signaling the conformation of the receptor is converted to a high-affinity state, which enables ligand binding. Upon ligand binding integrins are clustered, which initiates outside-in signaling and subsequent focal adhesion complex (FAC) formation.*

cutaneous anaphylaxis, indicates that this receptor is a potent modulator of mast cell function[131,177]. The mechanism by which VLA-5 modulates mast cell function is not completely clear, however could be of interest for the development of future therapy against allergy.

Adhesion via VLA-5

VLA-5 is a member of the integrin family. Integrins are heterodimeric membrane receptors that mediate cell-cell and cell-extracellular matrix interactions, and play a role in various physiological processes[139]. Currently, eight β- and fourteen α-chain isotypes have been identified. Dimerization of a particular α- and β-chain results in affinity for a particular ligand (table 2). VLA-5 consists of an α_5 and β_1 chain and binds the arginin-glycin-aspartic acid (RGD) sequence in fibronectin. On resting cells, integrins exist in a low-affinity conformation and, in order to induce adhesion, a conformational change to a high-affinity state is required. Various mediators such as interleukins[79,141], chemokines[99,171], and growth factors such as stem cell factor[13,36,167] have been shown to induce VLA-5-mediated adhesion to fibronectin. The mechanism through which each of these mediators modulate integrin affinity involves intracellular signaling events and is hence referred to as '*inside-out*' signaling (figure 1)[44,46,55,74,93]. In addition, divalent cations Mn^{2+} and Mg^{2+} have been shown to directly induce a high-affinity state of VLA-5, and this effect can be antagonized by Ca^{2+} ions[116-118]. The extracellular domain of the VLA-5 α-chain contains several cation-binding motifs that are highly homologous with the calcium-binding EF-hand motif of calmodulin[160]. Binding of Mn^{2+} to α_5 'EF-hand-like' domains induces a conformational change of the integrin, enabling fibronectin-binding. A calmodulin EF-hand loop is formed by thirteen amino acids of which five play a role in coordinating the binding of Ca^{2+} by means of their negatively charged side chain (figure 2A). In α_5 EF-hand-like motifs, the coordinating glutamic acid of the calmodulin EF-hand is replaced by an uncharged, therefore nonfunctional, valine. It has been postulated that in the EF-hand like domain located in the α_5 ligand-binding region, the coordinating function of glutamic acid is taken over by the negatively

charged side chain of aspartic acid of the RGD ligand sequence. In this view, high-affinity binding is the result of change of integrin conformation, cooperative

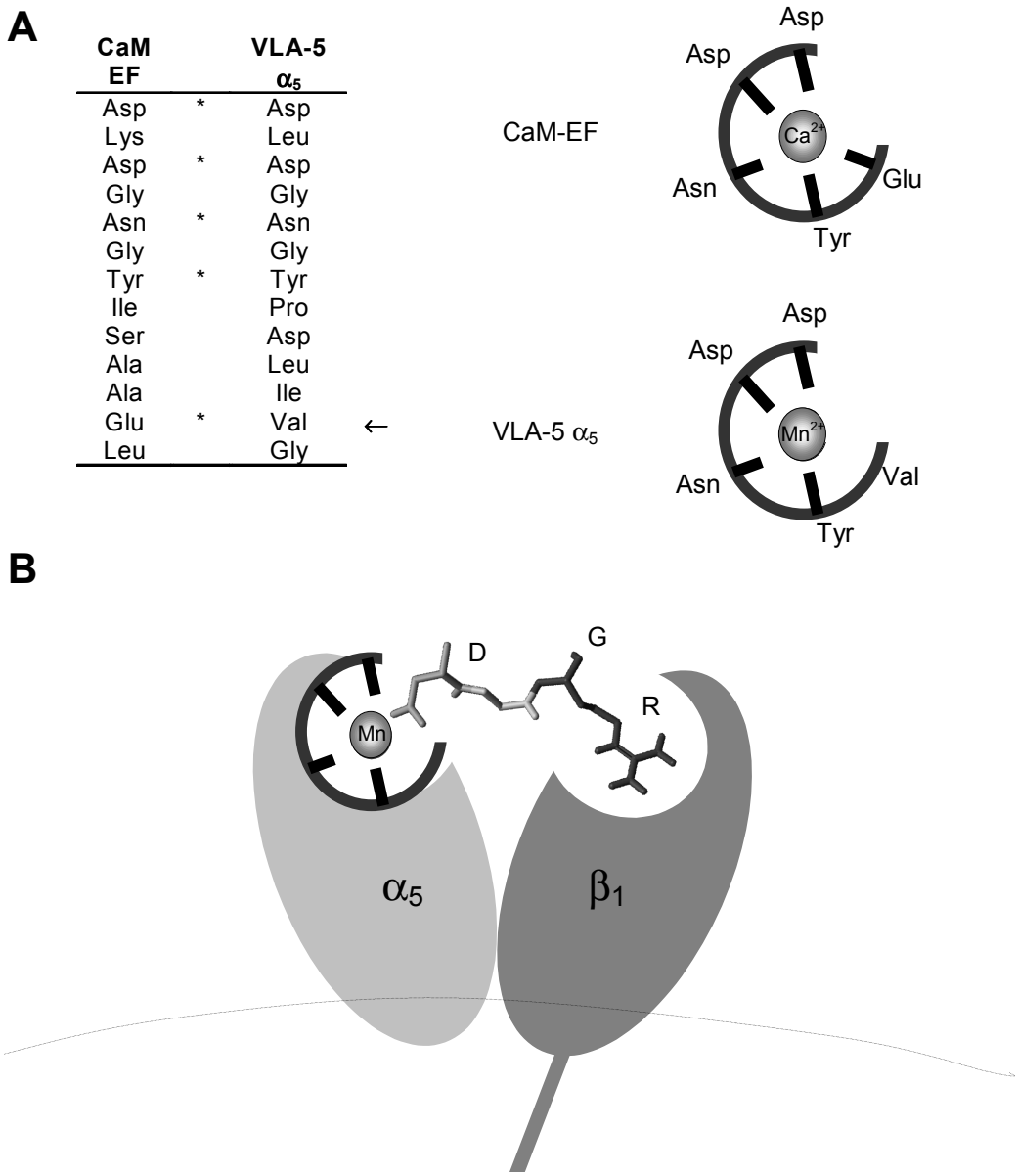


Figure 2. Mechanism for Mn^{2+}-induced adhesion of VLA-5 to fibronectin. A. *The amino acid sequence of the calmodulin (CaM) EF-hand and cation-binding motif of VLA-5 α_5 and coordination amino acids (*) are shown. Due to a glutamic acid (Glu)-valine (Val) substitution (←), the loop that forms the calcium-binding domain of VLA-5 α_5 contains four cation-coordination amino acids compared to five in the EF-hand loop of calmodulin.* ***B.*** *Due to Mn^{2+}-binding to cation-binding motifs in VLA-5 α_5 this integrin is converted to a high-affinity conformation. Mn^{2+}-binding is cooperatively coordinated by four amino acids of a cation-binding domain in the ligand-binding region of VLA-5 α_5 and aspartic acid (Asp) of the arginin-glycin-aspartic acid (RGD) sequence of fibronectin.*

coordination of Mn^{2+} by the integrin and the ligand and stabilization of this complex by the cation itself (figure 2B)[7].

Binding of VLA-5 to fibronectin and subsequent clustering of the integrins triggers '*outside-in*' signaling, involving tyrosine phosphorylation and influx of calcium[58]. Subsequent recruitment of a range of proteins from the cytosol to the cytoplasmic tails of the integrins leads to the formation of complex protein

structures, or focal adhesion complexes, at the cell-fibronectin interface. Focal adhesion complexes have been indicated as hotspots of cellular signaling and play a role in cytoskeleton reorganization of the cell[22,33,100].

Formation of signaling complexes: SMAC and rafts

There are several possible mechanisms by which VLA-5 could modulate the FcεRI signaling cascade. Increasing data suggests that many of the most important cellular functions, such as signal transduction, are carried out by modules made up of different species of interacting molecules[3,68]. In mast cells these supramolecular activation complexes (SMACs) could be assembled from VLA-5, FcεRI and focal adhesion complexes. Such supramolecular assemblies could provide a local membrane environment for efficient signal transduction and complex stability through attachment to the cytoskeleton components. Increased stability and lifetime within a signaling aggregate enhances the biological potency of an individual FcεRI according to the concept of *'kinetic proofreading'*[71,104,159], which postulates that a cascade of intracellular events unfold as long as the initiating event is maintained.

Besides SMACs, so-called rafts have been shown to play an important role in the initiation of the signal transduction cascade of MIRRs. These detergent-resistant microdomains in the plasma membrane that are formed due to lateral interaction of lipids and proteins and are enriched in sphingolipids, cholesterol, glycosylphosphatidyl-linked glycoproteins and Src PTKs such as Lyn (figure 3A)[66,81,146]. They are distributed in a patchy manner over the plasma membrane of various cell types. It was previously demonstrated that interaction between antigen presenting cells and T cells, leads to membrane reorganization and aggregation of antigen-specific T cell receptors (like FcεRI, a member of the MIRR family) and costimulatory molecules with rafts[77,166]. Subsequent SMAC assembly of local T cell receptor components and raft-associated intracellular signaling molecules[114], into a so-called 'immunological synapse', triggers T cell activation[61].

One step in the initiation of the early events of FcεRI signaling that remains unclear, is the transition of Lyn from an inactive to an active state. Recent data has suggested a possible role for rafts in this signaling step. Aggregation of FcεRI by binding of multivalent allergen to receptor-bound IgE causes this receptor to associate rapidly with rafts[144]. Subsequent association of FcεRI with active Lyn would induce phosphorylation of the β- and γ-subunits and initiate the FcεRI signal transduction cascade (figure 3B). Disruption of raft integrity by cholesterol depletion[78] impairs FcεRI signaling[143] and furthermore blocking of cholesterol biosynthesis in mast cells inhibits allergen-induced degranulation[37,142].

Recently, a role for rafts in integrin function was demonstrated[89], possibly due to their enrichment for Src-kinases that are important for focal adhesion complex formation[22,33]. Interaction with integrins might therefore also influence the association of rafts with FcεRI.

In summary, complex stabilization through formation of SMACs and enhanced association with rafts could be the molecular basis for VLA-5-mediated modulation of mast cell activation.

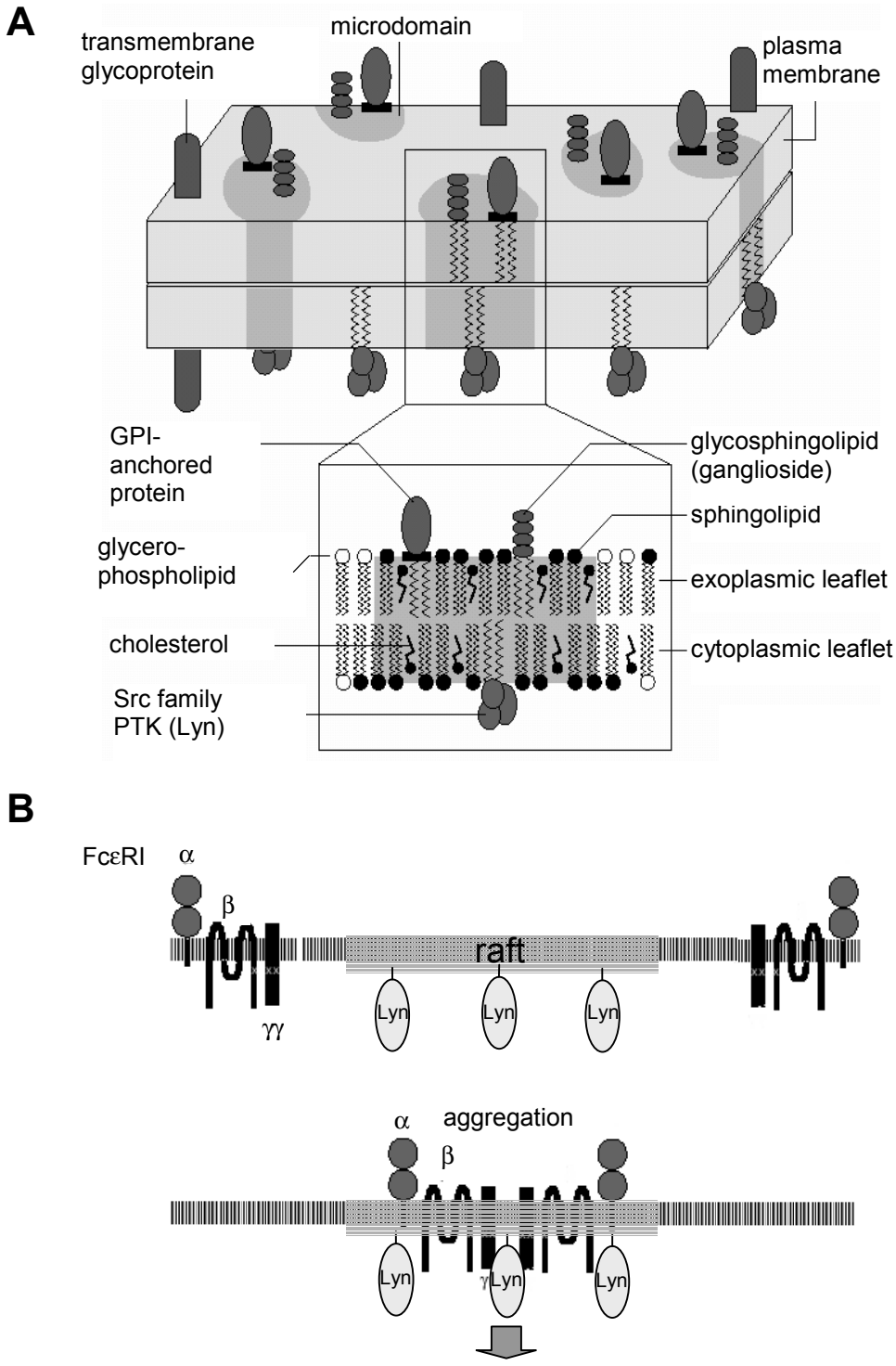


Figure 3. Rafts and FcεRI signaling. A. Organization of the plasma membrane microdomains*. Clusters of liquid-ordered sphingolipids and cholesterol molecules in the exoplasmic leaflet of the plasma membrane constitute 'microdomains' that are dispersed within the fluid mosaic of the membrane glycerophospholipids. Glycosylphos-phatidylinositol(GPI)-anchored proteins also cluster in microdomains but most transmembrane proteins, such as FcεRI, tend to be excluded. Src family PTK Lyn is also found in micro-domains and plays an essential role in FcεRI signaling. (Adapter from Immunology Today)*[77] ***B. FcεRI clustering induces raft association.*** *On the plasma membrane of nonstimulated mast cells FcεRI is not associated with rafts. Upon receptor aggregation by multivalent allergen, FcεRI associates with rafts. Subsequent association of Lyn leads to phosphorylation of FcεRI-β and -γ and triggers the signal transduction cascade leading to degranulation.*

Scope of this thesis

The investigations presented in this thesis are focussed on the mechanism by which VLA-5-mediated adhesion to fibronectin modulates mast cell degranulation.
The experimental setup that is used for the main part of our investigations is introduced and characterized in chapter 4. We use mast cells that are derived from murine bone marrow, which are stimulated with Mn^{2+} to convert VLA-5 into a high-affinity state and thereby induce adhesion to fibronectin. The effects of Mn^{2+}-induced adhesion on mast cell degranulation are presented in chapter 6, and the underlying mechanisms are investigated in more detail in chapter 7. From the obtained data, we formulate a model in which the extent of high-affinity conversion of VLA-5 and the subsequent adhesion to fibronectin correlates with the sensitivity of mast cells for allergen. In chapter 5 we explore the possibility to block VLA-5 with peptides that should theoretically bind to the cation-binding domains the α-subunit of this integrin. Finally, the role for the cholesterol-enriched membrane micromains, rafts, in regulation of mast cell degranulation is investigated (chapter 3).

3

Maintenance of sensitivity for antigenic stimulation in rat basophilic leukemia cells by serum cholesterol

(Submitted for publication)

René Houtman, Merethe Enstad[†], Frank A. Redegeld, Andries S. Koster and Frans P. Nijkamp

[†]Department of Pharmacology, Institute of Pharmacy, University of Oslo, Norway

Abstract

Mast cells play an important role in hypersensitivity type I responses. Antigenic challenge of mast cells results in clustering of the high-affinity receptor for IgE (FcεRI), which leads to tyrosine phosphorylation of the receptor β- and γ-subunits and protein tyrosine kinases Lyn and Syk and eventually release of inflammatory mediators. In this study we show that cholesterol from fetal calf serum is important for the rat mast cell line, RBL-2H3, for sustained susceptibility towards antigenic challenge. Serum deprivation at 37°C prior to antigenic stimulation results in a reversible decrease of β-hexosaminidase release, an effect that becomes more prominent with length of the serum deprivation interval. Serum deprivation does not affect the viability or IgE-binding capacity of the cells. Cholesterol is a vital component of membrane rafts, which have been shown previously to play a role in the initiation of proper tyrosine phosphorylation following FcεRI clustering. Indeed we find impaired tyrosine phosphorylation at an early stage of the FcεRI signal transduction cascade in deprived cells. Although the degree of tyrosine phosphorylation of FcεRI-β is unaltered, tyrosine phosphorylation of FcεRI-γ, Lyn and Syk, located downstream in the signaling cascade, is impaired.
In conclusion, our results demonstrate that absence of cholesterol due to serum deprivation, prior to allergic challenge, affects the FcεRI signaling cascade at an early stage and thereby attenuates degranulation.

Introduction

Mast cells are localized in virtually all vascularized tissues and play an important role in inflammation and hypersensitivity type I responses[147]. Cross-linking of the high-affinity receptor for IgE (FcεRI)[i] leads to tyrosine phosphorylation, phosphoinositol hydrolysis, elevation of cytoplasmic calcium levels and eventually release of various inflammatory mediators from mast cell granules[59,161].

An increasing number of reports suggest regulatory, mostly enhancing, effects on mast cells by various micro-environmental factors, possibly by modulation of the FcεRI signal transduction cascade. For example, integrin-mediated cellular adhesion to extracellular matrix components such as fibronectin and laminin[62,64,131,167,177], humoral factors such as IL3[40], soluble stem cell factor (SCF)[29,85,151,156], and interaction with fibroblasts via membrane-bound SCF[121] have been reported to enhance degranulation. The number of factors reported to affect the functional repertoire of mast cells *in vitro* increases rapidly, and at least some of these factors may prove to play an important role in the modulation of mast cell activity *in vivo*.

Fetal calf serum (FCS) is a widely used but poorly characterized reagent for cell culture and contains a broad spectrum of proteins, lipids, and other factors. Serum deprivation has been reported to affect various cell types at the level of gene expression[80,162], synthesis and activity of proteins[16,145], and phospholipid content[23,149]. Furthermore, serum deprivation has been shown to induce cellular differentiation, cell cycle-arrest[24,169] and impaired cell function[140].

In this study, an inhibiting effect of serum deprivation on IgE-mediated degranulation of rat mast cell line RBL-2H3 is shown. An estimation of the molecular size based on FCS dialysis using a range of MWCO dialysis membranes, and cholesterol addition studies, suggest that cholesterol may be a critical component of serum for the sustained degranulation capacity of RBL-2H3 cells.

Materials & Methods

Cells

RBL-2H3 cells were cultured in 75 or 162 cm^2 flasks at 37°C under humidified air, containing 5 % CO_2, in K-medium: RPMI 1640 supplemented with 10 %(vol/vol) heat-inactivated FCS, 1 mg/ml gentamycin (Life Technologies B.V., Breda, The Netherlands), 100 U/ml penicillin/streptomycin, 40 mM L-glutamine (Sigma Aldrich NV/SA, Axel, The Netherlands) and 0.2 M HEPES. Cells were recultured 2-3 times per week, by treatment with 0.05 % trypsin/0.53 mM EDTA (Life Technologies B.V., Breda, The Netherlands) and transferred to new flasks.

Treatment

Cells were incubated in suspension at a density of $1x10^6$/ml, for indicated time intervals at indicated temperatures in Tyrode's buffer: 0.1 M Tyrode salts (Life Technologies BV, Breda, The Netherlands), 100 mM HEPES/ 0.1 % $NaHCO_3$, pH 7.2, supplemented with

[i] For abbreviations see page 101

0.1 % BSA (Sigma Aldrich NV/SA, Axel, The Netherlands) alone (deprivation) or supplemented with 10 %(vol/vol) FCS (control, Life Technologies BV, Breda, The Netherlands) or cholesterol (polyoxyethanyl-cholesteryl sebacate, Sigma Aldrich NV/SA, Axel, The Netherlands) or FCS dialysis fraction (see below). Subsequently, cells were washed and resuspended in Tyrode's/BSA. Cholesterol concentrations indicated were calculated as 23 % of a polyoxyethanyl-cholesteryl sebacate concentration, according to supplier information sheet.

Dialysis of FCS

One volume of undiluted FCS (Life Technologies B.V., Breda, The Netherlands) ("inside fraction") was dialyzed for 24 hrs at 4°C against four volumes of Tyrode's buffer ("outside fraction") using Spectra/Por® dispodialysers (Spectrum Europe B.V., Breda, The Netherlands) of MWCO 5000, 3500, 2000, 1000, 500 and 100 Da. The residual inside fraction was separated from the outside fraction and dialyzed for an additional 24 hrs at 4°C against 1 L of Tyrode's buffer. Obtained inside and outside fractions were diluted in Tyrode's/BSA in such way that the concentration of the serum factor in our fractions corresponded to that in a 10 %(vol/vol) dilution of complete FCS in Tyrode's/BSA.

β-Hexosaminidase release

Cells were harvested from culture flasks by treatment with trypsin/EDTA, resuspended in K-medium and incubated for 60 minutes at 37°C at a density of $1x10^6$/ml with supernatant of anti-dinitrophenyl (DNP)-specific IgE-producing hybridoma 26.82 (a kind gift from dr. J. Rivera, NIH, Bethesda, USA)[98]. Non-bound IgE was removed by washing and cells were resuspended in Tyrode's/BSA.
After treatment (as described above), anti-DNP IgE-sensitized cells were seeded on a 96-well plate (Costar Europe Ltd., Badhoevedorp, The Netherlands), at a density of $5x10^4$/well, and stimulated for 30 minutes at 37°C with DNP-HSA (Sigma Aldrich NV/SA, Axel, The Netherlands). Supernatants of activated cells were collected and samples of $5x10^4$ cells were lyzed using 0.1 % NP-40 (Omnilabo International, Breda, The Netherlands) in order to quantify the total granular β-hexosaminidase activity present in the cells. β-Hexosaminidase activity was determined as described previously[84]. In short: samples were incubated for 60 minutes with 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (4-MUG, Sigma Aldrich NV/SA, Axel, The Netherlands) and the reaction was stopped with 0.2 M glycin buffer (pH 10.7). 4-MUG hydrolysis was determined by fluorimetric measurement (λ_{ex}: 360nm/λ_{em}: 452nm) using a Millipore Cytofluor 2350. The percentage of degranulation was calculated as follows: 100% x $fluorescence_{supernatant}/fluorescence_{cell\ lysate}$. All results were corrected for spontaneous β-hexosaminidase release from cells incubated with reaction buffer only (always <3%).

Viability

To determine membrane integrity, cells were incubated 10 minutes at 37°C with 10 μM calcein AM (Molecular Probes Europe B.V., Leiden, The Netherlands) and washed. Cytoplasmic fluorescence was quantified by flow cytometric analysis using FACScan and PC-lysis software (Becton Dickinson, Leiden, The Netherlands).
To determine mitochondrial activity, cells were incubated for 3 hrs at 37°C, with 50 μM MTT (Sigma Aldrich NV/SA, Axel, The Netherlands). The reaction was stopped by cell lysis using acidified isopropanol. Formazan formation was quantified by measuring OD_{550nm} using a Biorad Benchmark microplate reader.

Membrane expression of FcεRI

Cells were incubated for 30 minutes at 4°C with FITC-conjugated[103] purified 26.82 IgE in FACS buffer (0.1 % BSA, 0.1 % sodium azide in PBS), washed and resuspended in FACS buffer. Fluorescence was analyzed by flow cytometry using FACScan, and PC-lysis software, unstained cells were used as negative control.

Western blot analysis of protein tyrosine phosphorylation

Anti-DNP IgE-sensitized cells were triggered for indicated time intervals at 37°C with 100 ng/ml DNP-HSA, or with buffer only as a negative control. Tyrosine phosphorylation was stopped by cell lysis using TNT-PLAA: 0.5 % NP-40, 0.15 M NaCl, 50 mM Tris, 1 mM EDTA, 1 mM sodium orthovanadate (Janssen Chimica; Tilburg, The Netherlands), 10 mM tetrasodium pyrophosphate, 0.5 mM sodiumfluoride, 1 mM PMSF (Sigma Aldrich NV/SA, Axel, The Netherlands), 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml antipain, (ICN Biochemicals BV, Zoetermeer, The Netherlands), pH 7.4. After 30 minutes incubation on ice the NP-40-insoluble fraction was removed by centrifugation. Supernatants were precleared by 30 minutes incubation with 5 %(vol/vol) prot-G-Sepharose beads, (Pharmacia Biotechnologie, Roosendaal, The Netherlands). Subsequently, supernatants were immunoprecipitated using antibodies against phosphotyrosine (PY-20), Lyn (Ab 44), Syk (C-20) (Santa Cruz Biotechnology Inc, Santa Cruz, USA), FcεRI-β (JRK, a kind gift from dr. J. Rivera, NIH, Bethesda, USA), or FcεRI-γ (a generous gift from dr. M. -H. Jouvin, Boston, USA) and 5 %(vol/vol) prot-G-Sepharose beads. Beads were washed 4 times with TNT-PLAA and resuspended in SDS-sample buffer: 3 % SDS, 7.5 %(vol/vol) glycerol, 0.05 M Tris and 0.5 mg/ml bromophenol blue.
Samples were boiled for 3 minutes with 10 mM DTT (Sigma Aldrich NV/SA, Axel, The Netherlands), separated on 12.5 % bis-/acrylamide gel (Bio-Rad Laboratories BV, Veenendaal, The Netherlands) and blotted on PVDF membrane (Costar Europe Ltd., Badhoevedorp, The Netherlands). Phosphorylation of blotted proteins was visualized using HRP-conjugated PY-20 (Santa Cruz Biotechnology Inc, Santa Cruz, USA), ECL chemiluminescence (Amersham Nederland B.V., Den Bosch, The Netherlands), and exposure to KODAK X-OMAT-AR film. Films were scanned and analyzed on Bio-Rad GS-670 and Molecular Analyst software (Bio-Rad Laboratories BV, Veenendaal, The Netherlands).

Results

Effect of serum deprivation on IgE-mediated β-hexosaminidase release by RBL-2H3

In our experiments using RBL-2H3 cells in a model for mast cell degranulation, we observed that incubation of our cells at 37°C in Tyrode's buffer (hereafter referred to as deprivation) prior to antigenic stimulation leads to decreased β-hexosaminidase release. This phenomenon becomes more prominent with increased length of serum deprivation. As is shown in figure 1A, cells that were incubated in Tyrode's buffer for 15, 30, 60 or 90 minutes at 37°C show decreased degranulation. Maximal degranulation is decreased more than 50% after 90 minutes incubation. As a control we performed the same incubation of cells however in buffer supplemented with 10 %(vol/vol) FCS (comparable to culturing conditions). As is shown in figure 1B, decrease of mediator release could not be observed when cells were incubated in the presence of serum. We conclude that

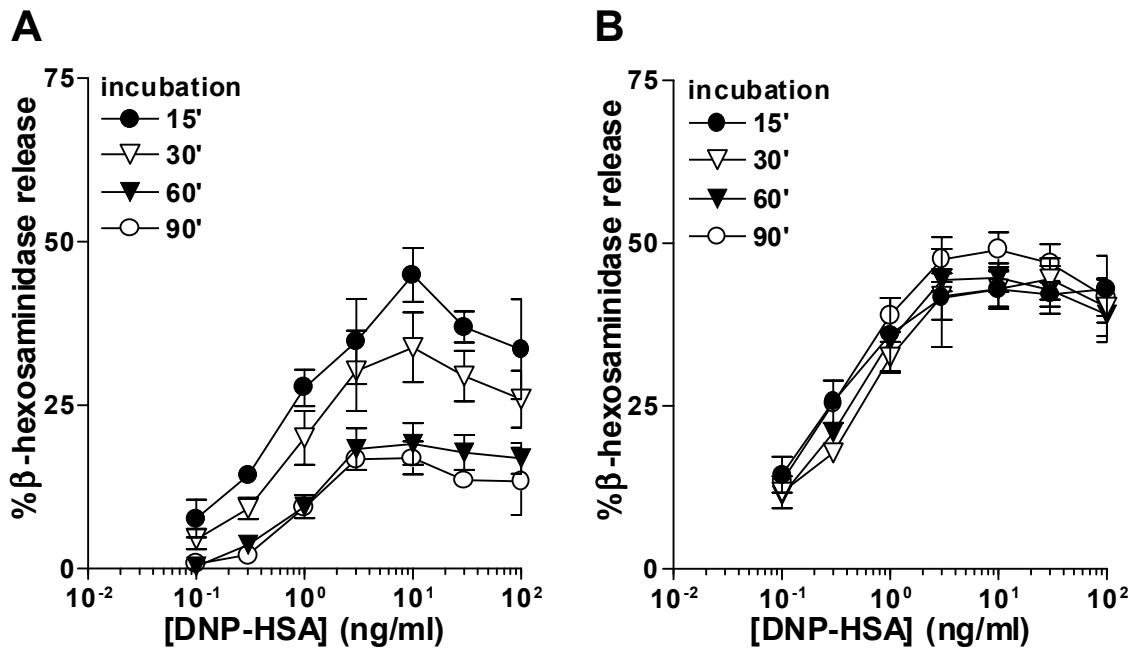


Figure 1. Effect of preincubation in absence or presence of FCS on IgE-induced β-hexosaminidase release of RBL-2H3 cells. *Anti-DNP IgE-sensitized cells were incubated at 37°C in Tyrodes buffer without (**A**) or with (**B**) 10 %(vol/vol) FCS for indicated time periods and stimulated with DNP-HSA. After 30 minutes β-hexosaminidase release was measured. Mean ± s.e.m. of a representative experiment of triplicates are shown.*

serum deprivation leads to decreased IgE-mediated degranulation in a time-dependent manner.

Effect of serum deprivation-temperature on β-hexosaminidase release

Our cells show decreased degranulation after serum deprivation performed at 37°C, and we were interested if this effect was temperature-dependent. To investigate this we compared IgE-mediated degranulation of cells deprived for 90 minutes at 4°C, or at 37°C and cells incubated for 90 minutes at 37°C in the presence of 10 %(vol/vol) FCS (control cells). As is shown in figure 2, serum deprivation at 37°C leads to decreased degranulation, but cells deprived at 4°C

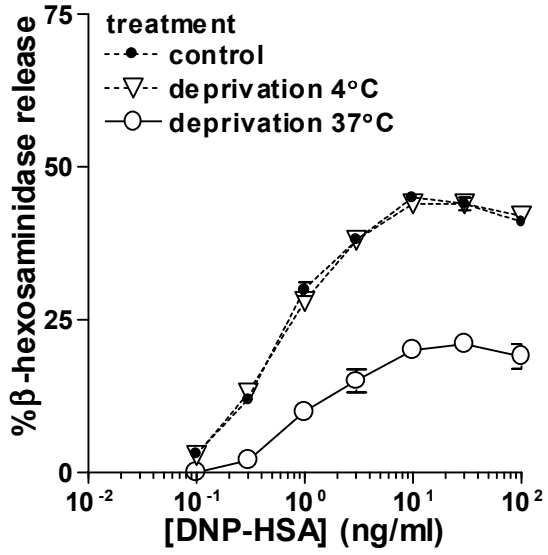


Figure 2. Effect of serum deprivation temperature on β-hexosaminidase release of RBL-2H3 cells. *Anti-DNP IgE-sensitized cells were incubated for 90 minutes at 37°C (deprivation 37°C) or 4°C (deprivation 4°C) in Tyrodes buffer or at 37°C in Tyrodes buffer with 10 %(vol/vol) FCS (control), followed by stimulation with DNP-HSA. After 30 minutes β-hexosaminidase release was measured. Mean ± s.e.m. of a representative of three independent experiments of quadruplicates are shown. (In several cases error bars are covered by symbols).*

show levels of β-hexosaminidase release that are comparable to control cells. We conclude that serum deprivation leads to decreased IgE-mediated degranulation in RBL-2H3 cells via a mechanism that takes place at a physiological temperature of 37°C, but not at 4°C.

Effect of addition of FCS after serum deprivation on β-hexosaminidase release

Decreased degranulation after serum deprivation was shown to become more pronounced with time and we were interested if this effect is reversible. To investigate this we compared IgE-mediated β-hexosaminidase release of deprived cells, cells that after serum deprivation were incubated for an additional 90 minutes in buffer supplemented with 10 %(vol/vol) FCS, and control cells. Figure 3 shows that the decrease of degranulation observed after serum deprivation can be completely reversed by administration of FCS to deprived cells, because degranulation of these cells is comparable to that of control cells. We conclude that the effect of serum deprivation on degranulation can be reversed by administration of serum to deprived cells.

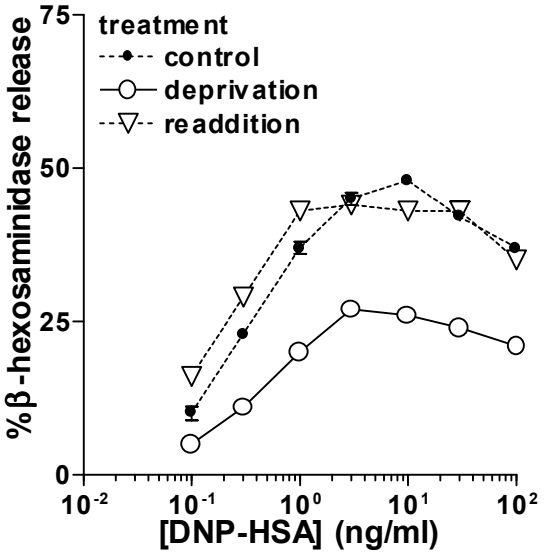


Figure 3. Effect of readdition of FCS on β-hexosaminidase release of deprived RBL-2H3 cells. *Anti-DNP IgE-sensitized cells were incubated for 90 minutes at 37°C in Tyrodes buffer with (control) or without 10 %(vol/vol) FCS (deprivation). One group of deprived cells was incubated additionally for 90 minutes at 37°C with 10 %(vol/vol) FCS (readdition), followed by stimulation with DNP-HSA. After 30 minutes β-hexosaminidase release was measured. Mean ± s.e.m. of a representative experiment of quadruplicates are shown (in all cases error bars are covered by symbols).*

Determination of the approximate size of the serum component

To elucidate the identity of the serum component responsible for the observed phenomena, our goal was to achieve an estimation of its size. For this purpose samples of FCS were dialyzed using a range of dialysis membranes of different pore size. Via this approach, dialysis of FCS with MWCO 5000 membrane results in an inside fraction containing serum components larger than 5000 Da and an outside fraction containing all serum components with a molecular weight below 5000 Da. Decrease of pore size will eventually lead to a loss of the factor of interest from the outside fraction. As is shown in figure 4, cells incubated with the outside fractions of MWCO higher than 1000 Da retain optimal sensitivity towards antigenic challenge (comparable to cells incubated with 10 %(vol/vol) FCS, data not shown) indicating that the serum component of interest has a molecular weight below 1000 Da. Using dialysis membranes with MWCO <1000 Da the critical serum component was largely retained in the inside and depleted from the

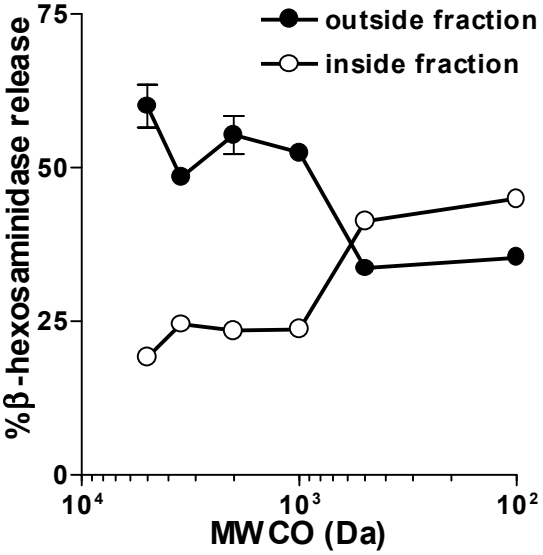


Figure 4. Effect of addition of FCS-dialysis samples during serum deprivation on β-hexosaminidase release of RBL-2H3 cells. *Different samples of 10 %(vol/vol) FCS were dialyzed with filters of various pore size, resulting in an inside and an outside fraction (see materials & methods). Prior to antigenic stimulation, anti-DNP IgE-sensitized cells were incubated for 90 minutes at 37°C with the resulting fractions. Subsequently cells were stimulated with 30 ng/ml DNP-HSA and after 30 minutes β-hexosaminidase release was measured. Mean ± s.e.m. of a representative experiment of quadruplicates are shown. (In several cases, error bars are covered by symbols used)*

outside fraction. From these results we conclude that the critical serum factor has a molecular weight below 1000 Da.

Effect of replacement of FCS by cholesterol during serum deprivation on β-hexosaminidase release

Based on the estimation of a molecular size below 1000 Da combined with data from literature, several candidate agents were tested. All agents tested failed to prevent decreased degranulation when replacing FCS during serum deprivation (data not shown), except for cholesterol. As is shown in figure 5, the loss of sensitivity towards antigenic challenge could be prevented by addition of cholesterol in a dose-dependent manner. Degranulation comparable to control cell was achieved when a concentration of 12 μg/ml polyoxyethanyl-cholesteryl sebacate was used. Cells incubated with a higher concentration of cholesterol showed even increased degranulation, compared to control cells. From these data we conclude that decreased degranulation by serum deprivation can be prevented by addition of cholesterol.

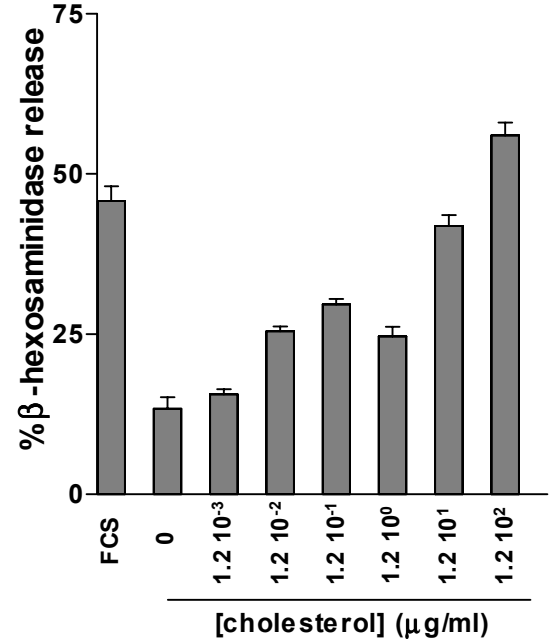


Figure 5. Effect of replacement of FCS by cholesterol during serum deprivation on β-hexosaminidase release of RBL-2H3 cells. *Prior to antigenic stimulation, anti-DNP IgE-sensitized cells were incubated for 90 minutes at 37°C in Tyrodes buffer with 10 %(vol/vol) FCS or various concentrations of cholesterol. Subsequently cells were stimulated with 30 ng/ml DNP-HSA. After 30 minutes β-hexosaminidase release was measured. Mean ± s.e.m. of a representative of three independent experiments of quadruplicates are shown. The cholesterol concentration was calculated as indicated in materials and methods.*

Effect of serum deprivation on cell viability

One explanation for the observed phenomena of serum deprivation could be that the condition of the cells is affected thereby influencing their responsiveness towards antigenic challenges. To exclude this possibility we compared deprived and control cells in several tests which are indicative for cell viability.

The ability of cells to retain cytoplasmic components is impaired in cells with decreased viability due to reduced membrane integrity. Cells were loaded with calcein AM, which is cleaved to fluorescent calcein by cytoplasmic esterases[96], and cytoplasmic fluorescence was determined as a measure for viability.

Decreased cell viability correlates directly with loss of mitochondrial activity of the cell. Therefore, we analyzed the cleavage of MTT into formazan by the succinate-tetrasodium reductase system, which belongs to the respiratory chain of mitochondria[115], as a second parameter for the viability of deprived cells. Table I shows that cell membrane integrity as well as mitochondrial activity is comparable in deprived and control cells. This indicates that cell viability is not affected by serum deprivation and therefore cannot be the reason for the observed effect on degranulation.

Effect of serum deprivation on IgE binding capacity of the cells

To investigate if serum deprivation leads to either decreased FcεRI membrane expression or decreased affinity for IgE, the binding of FITC-conjugated IgE to control and deprived cells was compared via flow cytometry. In figure 6 histograms of FITC conjugated IgE binding to control and deprived cells are shown. No difference in fluorescence between control and deprived cells can be observed indicating that the ability of RBL-2H3 to bind IgE is not affected by serum deprivation

	mitochondrial activity	membrane integrity
control	1.24 ± 0.04	422 ± 1
deprivation	1.22 ± 0.07	419 ± 8

Table 1. Comparison of viability of deprived and control cells. *RBL-2H3 cells were incubated for 90 minutes at 37°C in the presence or absence of 10 %(vol/vol) FCS and subsequently analysed for mitochondrial activity (OD550) and membrane integrity (cyoplasmic fluorescence). Mean arbitrary units ± s.e.m. of a respresentative experiment performed in quadruplicate are shown.*

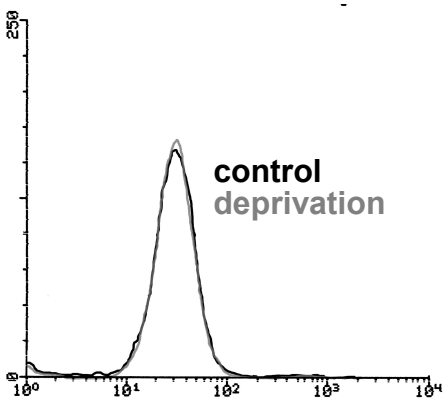


Figure 6. Effect of serum deprivation on IgE binding capacity of RBL-2H3. *Control and deprived cells were incubated for 1 hr at 37ºC with FITC-conjugated IgE. Cells were washed to remove unbound IgE-FITC and analyzed by FACScan. Nonstained cells were used as negative control. A representative example of four independent experiments is shown.*

Effect of serum deprivation on FcεRI-mediated tyrosine phosphorylation

To test the effect of serum deprivation on FcεRI signal transduction, overall protein tyrosine phosphorylation patterns were obtained after antigenic stimulation of control and deprived cells for various periods of time followed by immunoprecipitation with anti-phosphotyrosine Ab (PY20) and Western blot analysis using the same antibody. As is shown in figure 7, maximal tyrosine phosphorylation of the majority of proteins is reached within 2 minutes after stimulation in both control and deprived cells. Importantly, tyrosine phosphorylation of the majority of proteins is impaired in deprived cells (right panel) compared to control cells (left panel). Because phosphorylation of a large number of proteins is affected it appears to be likely that serum deprivation affects the FcεRI signaling pathway at an early stage.

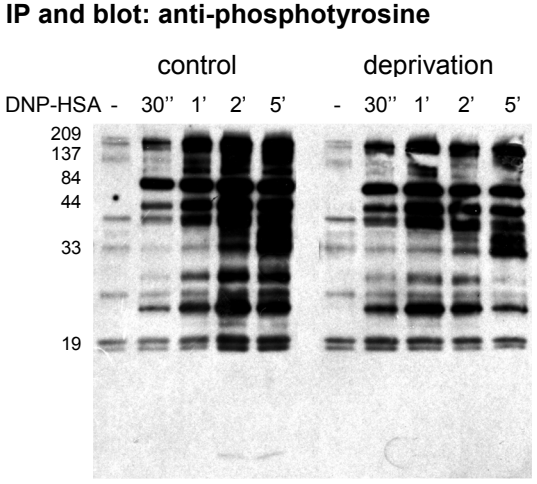


Figure 7. Effect of serum deprivation on tyrosine-phosphorylation in RBL-2H3 cells. *Anti-DNP IgE-sensitized cells were incubated for 90 minutes at 37°C in Tyrodes buffer with (control) or without 10 %(vol/vol) FCS (deprivation). Cells were stimulated for indicated time periods with 100 ng/ml DNP-HSA and lyzed after indicated time periods. Cell lysates were immunoprecipitated using anti-phosphotyrosine antibody PY-20, and immunoprecipitates were analyzed by Western-blotting using HRP-conjugated PY-20 and chemiluminescence.*

Effect of serum deprivation on tyrosine phosphorylation of FcεRI-β, Lyn, FcεRI-γ, and Syk

In order to be more accurate on the locus within the FcεRI pathway affected by serum deprivation, we decided to study tyrosine phosphorylation of FcεRI-β, Lyn, FcεRI-γ, and Syk, which have been established as the first sequential steps of FcεRI signaling following receptor clustering by multivalent antigen[14]. Immunoprecipitates using Abs against each of these proteins separately were analyzed for tyrosine phosphorylation via Western blot analysis using PY20. As is shown in figure 8A, in deprived as well as control cells maximal tyrosine phosphorylation of FcεRI-β, Lyn, FcεRI-γ and Syk is reached within 2 minutes after stimulation. Importantly, the degree of phosphorylation of each of these proteins in control and deprived cells is not comparable. Densitometric analysis of phosphorylation signals at 2 minutes after stimulation, in figure 8B, shows that tyrosine phosphorylation of FcεRI-β is largely unaffected. However when comparing the degree of phosphorylation of Lyn, FcεRI-γ and Syk in deprived cells and control cells we observe an increased reduction in deprived cells of 40, 65 and 90% respectively. It should be noted that, although no tyrosine phosphorylation of FcεRI-γ is optically visible, densitometric analysis showed increase of density after background subtraction of the film. Measured densities

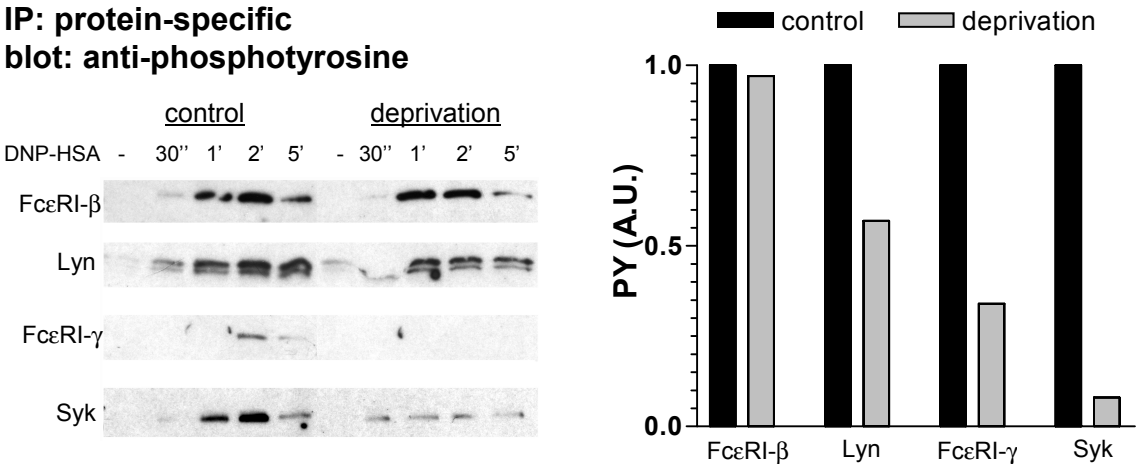


Figure 8. Effect of serum deprivation of tyrosine phosphorylation of FcεRI-β, FcεRI-γ, Lyn and Syk in RBL-2H3 cells. *(**A.**) Anti-DNP IgE-sensitized cells were incubated for 90 minutes at 37°C in Tyrodes buffer with **(control)** or without 10 %(vol/vol) FCS **(deprivation)**. Cells were stimulated for indicated time periods with 100 ng/ml DNP-HSA and lyzed after indicated time periods. Cell lysates were immunoprecipitated with antibodies against FcεRI-β, FcεRI-γ, Lyn or Syk, and immunoprecipitates were analyzed by Western-blotting using HRP-conjugated PY-20 and chemiluminescence. **(B)** Obtained signals on film from cells stimulated for 2 minutes were quantified using a Bio-Rad GS-670 densitometer. Tyrosine phosphorylation of each investigated protein was compared to that in control cells. Arbitraty units tyrosine phosphorylation (A.U. PY) were obtained by dividing the OD_{PY} of each protein by the OD_{PY} of that protein in control cells.*

were used, after local background subtraction, to calculate arbitrary units of tyrosine phosphorylation: $density_{deprived\ cells}/density_{control\ cells}$. These data indicate that serum deprivation decreases degranulation by affecting the signaling events that occur immediately after FcεRI clustering.

Discussion

It is well-established that the functional repertoire of the mast cell is influenced by various factors from its microenvironment[40,131]. In this report, we demonstrate that continuous presence of serum is needed for RBL-2H3 to maintain sensitivity towards antigenic challenges, and that cholesterol is most likely the factor responsible for this effect.

Our data on membrane integrity and mitochondrial activity indicate that the viability of deprived cells is comparable to control cells and therefore reduced viability can not be an explanation for the phenomenon observed here. Furthermore we found that the ability of deprived cells to bind IgE is comparable to control cells, and therefore decreased degranulation is not due to an altered FcεRI receptor affinity for IgE or amount of receptors expressed on the membrane.

We tested lysophosphatidic acid (max. conc.: 10 μM), ADP, ATP (max. conc.: 1 mM) and glutathione (oxidized and reduced, max. conc. 3 mg/L) as possible candidates, but all fail to prevent decreased degranulation after deprivation (data not shown).

On the other hand, the replacement of serum by cholesterol does prevent decrease of degranulation, and several arguments support our conclusion that cholesterol plays a key role in the observed phenomena. First, the molecular size of cholesterol, 386.7, corresponds with our conclusions drawn from the

dialysis experiments stating that the serum factor should be smaller than 1000 Da. Second, we found in our experiments that cells incubated with 12 μg/ml cholesterol (23% of 50 μg/ml polyoxyethanyl-cholesteryl sebacate, according to the manufacturers datasheet) during deprivation showed degranulation comparable to cells incubated with complete FCS. This concentration lies in the same order of magnitude as the concentration of cholesterol found in FCS (22-38 μg/ml in 10 %(vol/vol) suspension according to supplier's information).

Importantly, cholesterol has been shown to be an important component of detergent-resistant plasma membrane structures[20] also known as rafts, which have been shown to play a critical role in FcεRI signaling[47-49,143,144,152]. Our data show that serum deprivation affects degranulation in a time- and temperature-dependent manner. It is tempting to speculate that, if normal physiologic cell metabolism results in depletion of the endogenous cholesterol pool and cells fail to replenish this pool due to lack of a cholesterol source this leads to decreased efficiency of raft formation.

FcεRI aggregation induces a shift in Lyn distribution towards rafts[47,143,144]. We find that tyrosine phosphorylation of Lyn is impaired in serum deprived cells and decreased Lyn recruitment and phosphorylation could be the result of impaired formation of functional Lyn-containing membrane microdomains. Availability of Lyn to the early FcεRI signaling complexes may be rate-limiting for FcεRI induced degranulation[158,173]. We find that activation of "downstream" components of the FcεRI signaling cascade is progressively less effective in deprived cells, which is consistent with the concept of "kinetic proofreading"[71,120], which also applies for FcεRI signaling[159]. This concept states that a cascade of intracellular events unfold as long as the initiating event is maintained. It has been postulated that the biological potency of an individual FcεRI receptor is determined by its lifetime within a signaling aggregate, and this lifetime could be affected by attenuated raft formation in deprived cells.

Previous reports have shown that Syk tyrosine phosphorylation and induction of its kinase activity is essential for mast cell degranulation[32,132]. The decreased degranulation observed in this study could be easily explained as a direct consequence of decreased Syk activation in deprived cells.

Membrane cholesterol levels appear to play a role in the activation of RBL-2H3 cells. To maintain these levels cells can also use cholesterol-precursors for *de novo* synthesis of cholesterol. Interestingly, inhibition of cholesterol biosynthesis by lovastatin was shown to reduce FcεRI-induced tyrosine phosporylation and blocks serotonin release of RBL-2H3[37,142]. We can not exclude the possibility that the serum component, which upon serum withdrawal leads to the observed phenomena, is a cholesterol-precursor instead of cholesterol. However, since cells can use the uptake of complete cholesterol to maintain their cellular levels this option seems unlikely.

In conclusion, we show in this study that serum deprivation results in decreased degranulation in RBL-2H3. We propose that this decrease is the result of the absence of cholesterol thereby disturbing the formation of membrane rafts and attenuation of the coupling between cross-linking of the receptor and the pathway leading to mediator release from mast cell granules. These results provide us with possible new insights into the fine-tuning of mast cell degranulation by microenvironmental factors.

4

Development and characterization of an *in vitro* murine model to study modulation of FcεRI-induced mast cell degranulation through VLA-5-mediated adhesion to fibronectin

(Submitted for publication)

René Houtman, Khalil Karimi, Frank A. Redegeld, Andries S. Koster and Frans P. Nijkamp

Abstract

Mast cells play an important role in allergic responses. Release of inflammatory mediators from mast cell granules cells, triggered by antigenic stimulation, can be modulated by adhesion to fibronectin via integrin very late antigen-5 (VLA-5). Investigation of the molecular basis of this modulation requires an *in vitro* model system in which adhesion and degranulation can be controlled independently. Here, a novel model that meets these criteria, is presented and characterized. We show that adhesion of mast cells derived from murine bone marrow to fibronectin can be induced by Mn^{2+}-stimulation and that this adhesion is mediated via VLA-5. The mechanism involved can be divided into two stages. During the first stage, a high-affinity conformation of VLA-5 is induced by Mn^{2+} and adhesion to fibronectin is enabled. This effect can be antagonized by extracellular Ca^{2+}. During the second stage, which is temperature-sensitive and can be attenuated by kinase and phosphatase inhibitors, intracellular signaling strengthens the cell-substrate bond. After attachment of the cells to fibronectin is established, we found that adhesion is no longer affected by the presence of extracellular Ca^{2+}. This makes adhered cells available for degranulation experiments for which influx of Ca^{2+} is critical. By using polystyrene beads as carrier for immobilized fibronectin, this setup can also be applied for biochemical analysis of adhered cells when larger amounts of cells are needed. In conclusion, we present a novel *in vitro* model that can be used to investigated the molecular basis of VLA-5-mediated modulation of allergen-induced mast cell degranulation.

Introduction

Mast cells play an important role in allergic responses[147]. First encounter with an allergen activates the immune system and results in the production of allergen-specific IgE, which binds to the high-affinity receptor for IgE (FcεRI)[i] expressed on mast cells. Second encounter with the allergen induces clustering of FcεRI[161] and triggers a signal transduction cascade leading to degranulation of various inflammatory mediators, which cause clinical phenomena associated with allergy[12,14,59].

Mast cells are localized in all vascularized tissues and an increasing number of reports suggest that the function of these cells is affected by their microenvironment consisting of neighboring cells, extracellular matrix and various humoral factors[39,56,134,155]. Understanding of the mechanism by which microenvironmental factors modulate mast cell degranulation is potentially useful for the development of future therapy against allergy.

Adhesion to extracellular matrix component fibronectin has been shown to enhance FcεRI-induced degranulation of RBL-2H3 mast cells[62,64,131,155]. Although it was shown that this adhesion is mainly mediated by $\alpha_5\beta_1$ integrin very late antigen-5 (VLA-5) and the arginine-glycine-aspartic acid (RGD) sequence of fibronectin[150,177], the mechanism by which FcεRI signaling is influenced is not yet understood in detail.

In order to be able to study the molecular basis of VLA-5-mediated modulation of FcεRI signaling, an *in vitro* model system is required of which VLA-5-mediated adhesion and FcεRI-induced degranulation can be controlled independently.

RBL-2H3 cells, a widely used model for mast cells, adhere to various surfaces without assembling specialized adhesion structures at the cell-substrate interface[8,124] and are therefore, in our opinion, not a suitable model to study the effect of adhesion on degranulation. We prefer to use mast cells cultured from mouse bone marrow (BMMC)[84,133] because these cells do not show nonspecific adhesion. Although it has been shown that VLA-5 is expressed on BMMC, these cells do not adhere spontaneously to fibronectin[45,177]. It has been demonstrated that on resting cells, integrins exist in a low-affinity conformation and that inside-out signaling is needed for the conversion of these receptors into a high-affinity state[44,46,55,74,93]. This suggests that stimulation of BMMC is necessary in order to induce VLA-5-mediated adhesion to fibronectin. Indeed it was shown that stimulation of BMMC with phorbol myristate acetate (PMA) induces VLA-5-mediated adhesion to fibronectin, via a phosphatidyl inositol 3-kinase-dependent pathway[87]. Besides PMA[41], various integrin-stimulating agents are known such as ionomycin[67], stem cell factor[13,36] and antigenic stimulation[87]. However, these stimuli directly modulate the FcεRI signaling cascade leading to degranulation and we therefore prefer not to use them in our protocols[15,157].

Additonally, it was demonstrated in K562 erythroleukemia cells, that the affinity of VLA-5 for fibronectin can also be modulated by cations. Through binding of Mn^{2+} to cation-binding motifs in the extracellular domain of the VLA-5 α-subunit, a conformational change of the integrin and thereby high-affinity for fibronectin

[i] For abbreviations see page 101

is induced. This effect is antagonized by Ca^{2+} [116,118]. Additionally, integrin-ligand binding is stabilized by the cooperative coordination and binding of Mn^{2+} by the negatively charged amino acid residues of the cation binding domain localized in the ligand binding area of VLA-5 and the negatively charged aspartic acid of the RGD-sequence in fibronectin[7]. Soluble RGD peptide has been shown to block mast cell adhesion to fibronectin[36,155,175]. Binding to fibronectin induces clustering of VLA-5 which triggers the activation of tyrosine kinases and phosphatases, and subsequent formation of focal adhesion complexes at the site of integrin clustering which strengthens the cell-fibronectin interaction[22,58].
In this report we use cations to regulate VLA-5-mediated adhesion of BMMC to fibronectin. The mechanism involved is characterized and the potential to apply this protocol as a new model to study VLA-5-mediated modulation of FcεRI-induced degranulation of mast cells is evaluated.

Materials & Methods

Cells

BMMC were obtained as described previously[84]. Briefly, bone marrow from femurs of Balb/c mice was flushed and cells were cultured at a density of 2 x 10^5/ml in complete RPMI (RPMI1640 medium containing 4 mM L-glutamine, 5 x 10^{-5} M β-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.1 mM nonessential amino acids) supplemented with 20 %(vol/vol) culture supernatant from Balb/c splenocytes which were stimulated for 7-days with pokeweed mitogen (Sigma Aldrich NV/SA, Axel, The Netherlands) in complete RPMI. Medium was refreshed once per week.

Reagents

VLA-5 blocking antibody BMA5 was a kind gift from Dr B.M.C. Chan, Transplantation and Immunobiology group, Univ. Western Ontario, Canada[45]. RGD-peptide and phenylarsene oxide (PAO) were purchased from Sigma Aldrich NV/SA, Axel, The Netherlands. Genistein was purchased from Sanvertech B.V. Heerhugowaard, The Netherlands.

Immobilization of fibronectin

NUNC maxisorp 96 well plates were coated for 3 hours at 37°C with 2.5 mg/ml human fibronectin (CLB, Amsterdam) in PBS, 200 μl/well, washed 3 times with 200 μl/well PBS, and used for adhesion experiments.
Latex beads (average diameter 11.9 μm, Sigma Aldrich NV/SA, Axel, The Netherlands) were incubated for 1 hour at room temperature with 2.5 mg/ml human fibronectin in PBS, under constant rotation, followed by three times washing with an excess volume of PBS and used for fluorescence microscopy experiments.

Loading of cells with 4-(and-5)-carboxyfluorescin diacetate

The cytoplasm of the BMMC was stained with the fluorescent product of 4-(and-5)-carboxyfluorescin diacetate (4(5)CFDA) hydrolyzed by cytoplasmic esterases. Cells

were washed twice with FCS-free culture medium and incubated for 25 minutes at 37°C with 10 mM 4(5)CFDA. Subsequently, cells were washed twice with complete culture medium and prepared for adhesion.

Adhesion of BMMC to fibronectin

4-6 week old BMMC were washed once with PBS, once with 1 mM EDTA in PBS at 37°C, and once with tris-buffered saline (TBS, 2.9 g/L Tris-Cl, 4 g/L NaCl, 0.2 g/L KCl, 0.4 g/L glucose, 0.1 % BSA) and resuspended at a density of 5 x 10^5 cells/ml TBS.
For adhesion to fibronectin-coated 96 well plate, per well 100 µl of cell suspension was incubated with 100 µl of the indicated agent in TBS for 30 minutes on ice, followed by an incubation at 37°C. After the indicated incubation interval, nonadhered cells were removed by three times washing with 200 µl/well PBS.
For adhesion to beads, 4(5)CFDA-loaded cells were prepared for adhesion as described and resuspended at a density of 1 x 10^7 per ml. Next, cells were stimulated with 0.2 mM $MnCl_2$, mixed with a pellet of fibronectin-coated beads (beads:cells ratio = 1:1) and centrifuged to pellet the cell/bead mixture. The cell/bead pellet was incubated at 37°C for 5 minutes, gently resuspended and incubated for an additional 25 minutes at 37°C. Cell/bead complexes were fixed with paraformaldehyde (final concentration of 1%) and mounted on object slides using Vectashield Mounting medium (Molecular Probes Europe B.V., Leiden, The Netherlands) and cover slides. Samples were examined microscopically using a Nikon Eclipse E600 with Nomarski optics and epifluorescence (filters: λ_{ex}: 465-95/ λ_{em}: 515-55), and recorded digitally using an Apogee KX2 camera (Lambert Instruments B.V.) and processed with Adobe Photoshop 4.0.

Quantification of adhesion

Adhesion was quantified using Cyquant proliferation assay kit (Molecular Probes Europe B.V., Leiden, The Netherlands) according to the manufacturer's protocol. In short, plates were frozen overnight at -20°C, thawed and incubated for 60 minutes under continuous agitation at room temperature with a DNA-specific fluorescent dye and lysing reagent. Fluorescence was measured at $\lambda_{ex.}$: 485nm/$\lambda_{em.}$: 530nm using a Millipore Cytofluor 2350 and adhesion was calculated as the residual fluorescence as percentage of input fluorescence.

Results

Cation-induced modulation of adhesion of BMMC to fibronectin

To test if cation-induced modulation of VLA-5 affinity for fibronectin[116] can be extrapolated to BMMC, we investigated the effect of Mn^{2+} on adhesion of BMMC to fibronectin. Extracellular Ca^{2+} was removed by EDTA-treatment of the cells. Next, cells were incubated with various concentrations of $MnCl_2$ on a fibronectin-coated 96 well plate for 30 minutes on ice to synchronize adhesion. Additionally, cells were incubated for 30 minutes at 37°C and nonadhered cells were removed by washing. Adhered cells were lysed, DNA was fluorescently stained and quantified as a measure for the amount of residual cells and % adhesion was calculated. Figure 1A shows that Mn^{2+} dose-dependently induces adhesion of BMMC to fibronectin, reaching maximal adhesion at 0.2 mM $MnCl_2$. Subsequent experiments were performed as described above unless otherwise

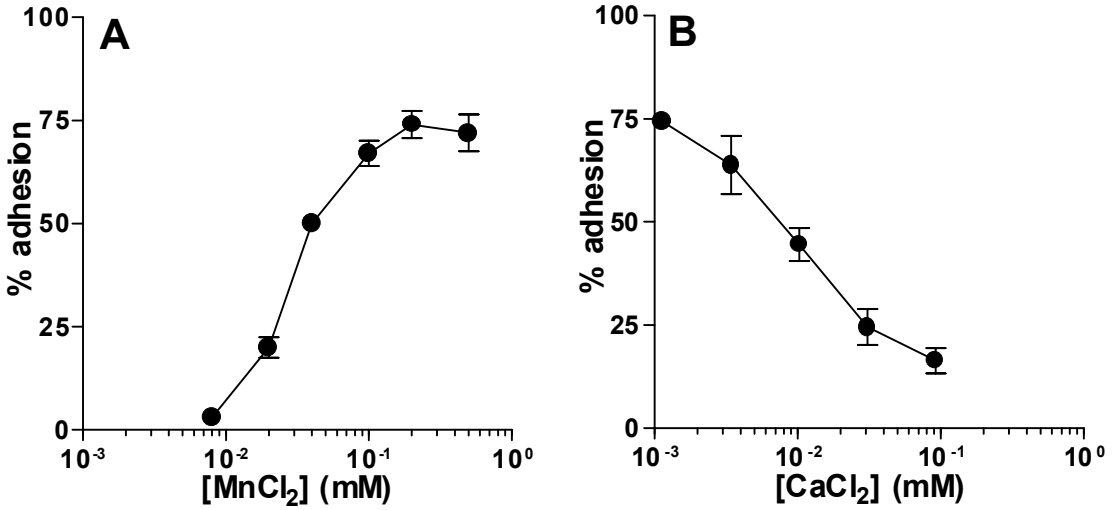


Figure 1. Effect of $MnCl_2$ on the adhesion of BMMC to fibronectin. *BMMC were washed with 1 mM EDTA and incubated on fibronectin-coated 96 well plate **(A)** with (0-0.5 mM) $MnCl_2$ or **(B)** with 0.2 mM $MnCl_2$ and (0-0.1 mM) $CaCl_2$ in TBS for 30 minutes on ice, followed by 30 minutes incubation at 37°C. Nonadhered cells were removed by washing, adhered cells were lyzed and quantified by fluorescent staining of DNA. % Adhesion was calculated from residual fluorescence compared to input fluorescence. (In several case error bars are covered by symbols used).*

indicated. Adhesion to non-coated surfaces was never observed (data not shown).

To investigate if Ca^{2+} can antagonize Mn^{2+}-induced adhesion, EDTA-treated BMMC were incubated on immobilized fibronectin with 0.2 mM $MnCl_2$ and various concentrations of $CaCl_2$. Figure 1B shows that Ca^{2+} dose dependently inhibits Mn^{2+}-induced adhesion of BMMC to fibronectin.

Effect of VLA-5 blocking on Mn^{2+}-induced adhesion

To confirm that adhesion of BMMC to fibronectin is mediated by VLA-5 we tested the effect of VLA-5-blocking antibody, BMA5, and the effect of soluble RGD on Mn^{2+}-induced adhesion. BMMC were prepared for adhesion, stimulated

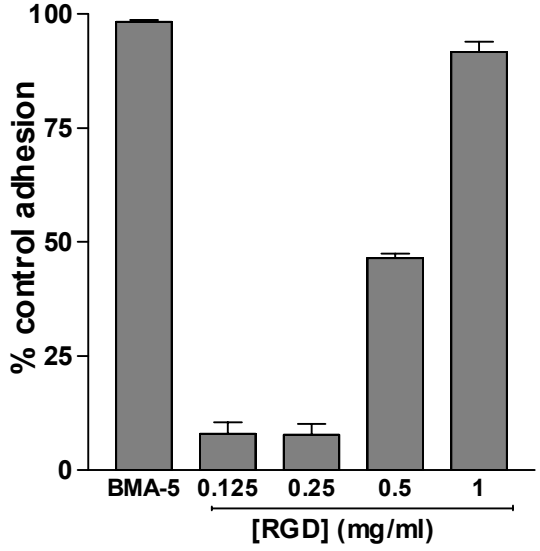


Figure 2. Effect of BMA5 and RGD-peptide on adhesion of BMMC to fibronectin. *BMMC prepared for adhesion as described, were incubated with 15 μg/ml BMA5 for 60 minutes on ice before, or various concentrations of RGD-peptide during the incubation on a fibronectin-coated 96 well plate, 30 minutes on ice and 30 minutes at 37°C. Nonadhered cells were removed by washing and adhesion was quantified. % Control adhesion was calculated from the absolute adhesion of $MnCl_2$-stimulated cells treated with BMA5 or RGD compared to cells stimulated with $MnCl_2$ alone.*

with $MnCl_2$ and incubated with either BMA5 1 hour before, or various concentrations of RGD-peptide during the incubation on immobilized fibronectin. The percentage of inhibtion was calculated from the absolute adhesion of $MnCl_2$-stimulated cells treated with RGD or BMA5, compared to the absolute adhesion of cells stimulated with $MnCl_2$ alone. As is shown in figure 2, both BMA-5 and RGD are able to almost completely inhibit $MnCl_2$-induced adhesion of BMMC to fibronectin indicating a role for VLA-5.

Effect of a subphysiological temperature on adhesion of BMMC to fibronectin

In vivo integrin adhesion involves intracellular signaling events that take place under strict physiological conditions. To investigate if Mn^{2+}-induced adhesion of BMMC to fibronectin also involves intracellular signaling, we tested the effect of lowering of the temperature to a nonphysiological level on adhesion.

$MnCl_2$-stimulated BMMC were incubated on immobilized fibronectin on ice for 30 minutes. Additionally, one group of cells was transferred to 37°C while a second group of cells was kept on ice. As is shown in figure 3, Mn^{2+}-induced adhesion on ice is severely impaired compared to adhesion at 37°C.

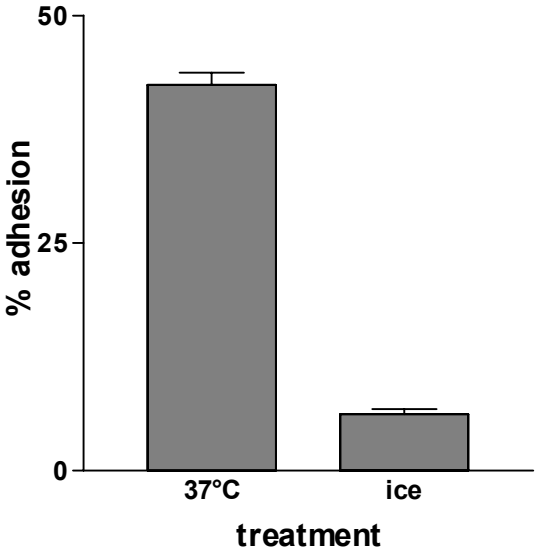


Figure 3. Comparison of adhesion of BMMC incubated on ice vs. 37°C. *BMMC were prepared for adhesion as described and incubated on fibronectin-coated 96 well plate for 60 minutes on ice* ***(ice)*** *or for 30 minutes on ice followed by 30 minutes at 37°C* ***(37°C)****. Additionally, nonadhered cells were removed by washing and % adhesion was quantified.*

Role for protein tyrosine kinases in Mn^{2+}-induced adhesion of BMMC to fibronectin

The observation that $MnCl_2$-induced adhesion is temperature-sensitive suggests intracellular signaling, probably through induction of intracellular enzyme activity during this process. Phosphorylation of tyrosine residues of various proteins by protein tyrosine kinases (PTK) has been shown to be a prominent characteristic for integrin-mediated adhesion[100,139]. To determine a possible role for PTK, we tested the effect of PTK inhibitor, genistein, in our setup.

BMMC were stimulated for adhesion and and incubated with various concentrations of genistein on immobilized fibronectin. Figure 4 shows that genistein dose-dependently inhibits adhesion of BMMC to fibronectin, indicating a role for PTKs.

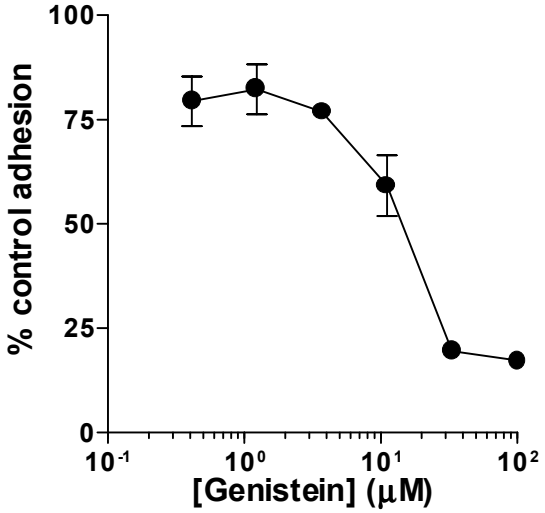


Figure 4. Effect of genistein on adhesion of BMMC to fibronectin. *BMMC were prepared for adhesion and incubated on a fibronectin-coated 96 well plate with 0.2 mM $MnCl_2$ and various concentrations of genistein, for 30 minutes on ice and 30 minutes at 37°C. Nonadhered cells were removed by washing, adhesion was quantified and % control adhesion was quantified*.

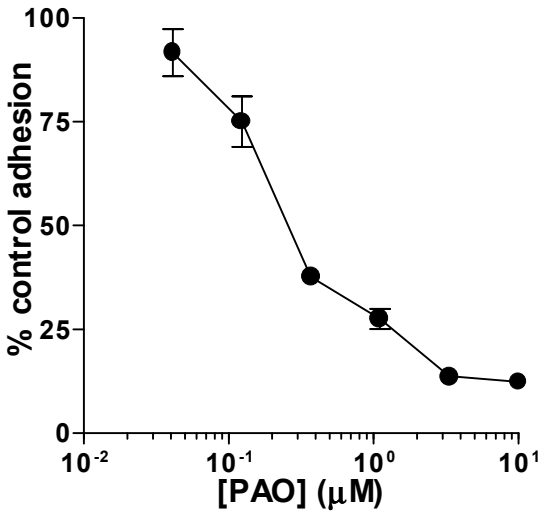


Figure 5. Effect of PAO on adhesion of BMMC to fibronectin. *BMMC were prepared for adhesion and incubated on a fibronectin-coated 96 well plate with 0.2 mM $MnCl_2$ and various concentrations of PAO, for 30 minutes on ice and 30 minutes at 37°C. Nonadhered cells were removed by washing and adhesion was quantified.*

Role for protein phosphatases in Mn^{2+}-induced adhesion of BMMC to fibronectin

In cell signaling involving tyrosine phosphorylation, the action of kinases is often counteracted by phosphatases[75]. To study if dephosphorylation of proteins by phosphatases plays a role in Mn^{2+}-induced adhesion, we tested the effect of phosphatase inhibitor, phenylarsene oxide (PAO), in our setup.
BMMC, prepared for adhesion, were stimulated with Mn^{2+} and incubated with various concentrations of PAO on immobilized fibronectin. As is shown in figure 5, PAO dose-dependently attenuates Mn^{2+}-induced adhesion of our cells to fibronectin, which shows that phosphatases participate in adhesion of BMMC to fibronectin.

Effect of Ca^{2+} on sustained adhesion of BMMC to fibronectin

Influx of extracellular Ca^{2+} was shown to be critical for mast cell degranulation[11], however our data show that the presence of Ca^{2+} has an inhibiting effect on the induction of adhesion of BMMC to fibronectin. We tested if administration of Ca^{2+}, in a concentration that is normally used in protocols for degranulation experiments, to already adhered BMMC leads to deadhesion of these cells.
Three groups of BMMC were prepared for adhesion and stimulated with $MnCl_2$ on immobilized fibronectin as described above. After 30 minutes of incubation at 37°C nonadhered cells of the first group (30'Mn) were removed by washing, to the second group (30'Mn/30'Ca) Ca^{2+}-supplemented buffer was added, and the third group of cells (60'Mn) was left untreated. After an additional incubation of 30 minutes at 37°C, the nonadhered cells of the second and third group were removed by washing and the adhesion of all three groups was determined. As is shown in figure 6, Mn^{2+}-induced adhesion after 60 minutes (60'Mn) was

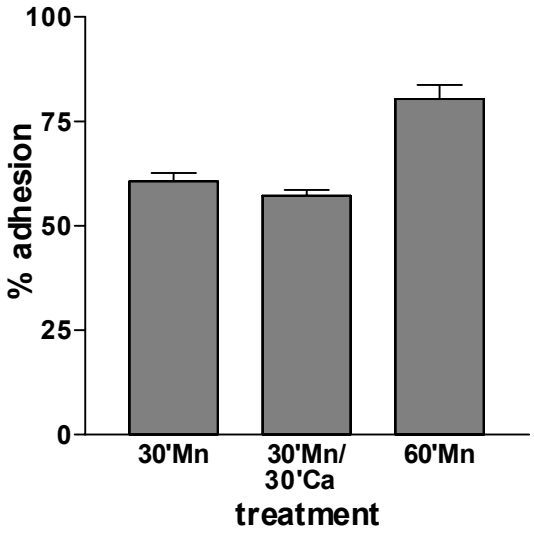


Figure 6 Effect of Ca^{2+} on adhered cells. *BMMC were prepared for adhesion and incubated with 0.2 mM $MnCl_2$ on a fibronectin-coated 96 well plate for 30 minutes on ice and subsequently at 37°C. After 30 minutes, of the first group (30'Mn) nonadhered cells were removed by washing, to a second group (30'Mn/30'Ca) Ca^{2+}-supplemented buffer (final concentration: 1.8 mM) was added and the third group was left untreated. After 60 minutes nonadhered cells of the second and third group were removed by washing and % adhesion of all three groups was determined.*

increased compared to 30 minutes incubation (30'Mn), which indicates that from 30 to 60 minutes after the start of adhesion *de novo* adhesion still takes place. The adhesion of the group that was treated with Ca^{2+} after 30 minutes (30'Mn/30'Ca) is comparable to the adhesion after 30 minutes (first group). This observation suggests "freezing" of the adhesion process in which adhered cells are unaffected and remain adhered, while *de novo* adhesion is prevented by the presence of Ca^{2+}.

Adhesion of BMMC to fibronectin-coated beads

For practical reasons we explored the possibility to modify our protocol into a setup in which polystyrene beads instead of 96 well plate are used as a carrier of immobilized fibronectin.

BMMC were activated for adhesion and incubated with fibronectin-coated polystyrene beads for 30 minutes at 37°C. Cell-bead complexes were fixed, and analyzed microscopically. Figure 7A shows a representative example of a BMMC adhered to a fibronectin-coated bead, both visualized by light microscopy. To distinguish between cells and beads, BMMC were loaded with

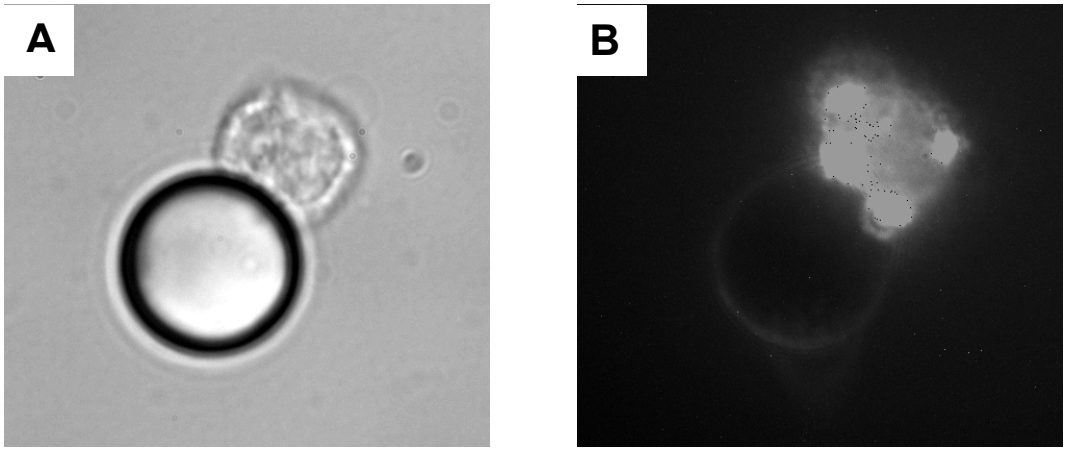


Figure 7. Adhesion of BMMC to FN-coated bead*s. 4(5)CFDA-loaded BMMC were prepared for adhesion, stimulated with 0.2 mM $MnCl_2$ and incubated with fibronectin-coated beads for 30 minutes at 37°C.* ***(A)*** *Example of a BMMC (C) adhered to a latex bead (F) detected by light microscopy and* ***(B)*** *the same example detected by epifluorescence.*

4-(and-5)-carboxyfluorescin, which is cleaved into a fluorescent product by cytoplasmic esterases. Figure 7B shows the same cell-bead complex as shown in figure 7A, visualized by epifluorescence. In this figure the fluorescently stained cytoplasm of the cell is clearly visible, while the contours of the bead can only vaguely be distinguished. Adhesion was absent when unstimulated cells or BSA-coated beads were used (data not shown).

Discussion

The understanding of the mechanism by which fibronectin can modulate mast cell degranulation is potentially useful for the development of future therapy against allergy and therefore topic of our investigations. Detailed study of this phenomenon requires a mast cell model of which adhesion and degranulation can be controlled independently, and in this report the protocols for such a model are presented.

Our data showed that adhesion of BMMC to fibronectin can be modulated by divalent cations. Consistently with other studies, adhesion is induced by Mn^{2+} and is antagonized by Ca^{2+} probably through modulation of the VLA-5 conformation[7,116].

It has been shown previously that VLA-4, another fibronectin-binding integrin, is also expressed on BMMC, however the role for this integrin in adhesion of BMMC to fibronectin decreases during ontogeny and its function is taken over gradually by VLA-5 [45]. In 4-6 weeks old BMMC that we use in our experiments, adhesion to fibronectin is completely VLA-5-mediated. This conclusion is confirmed by our data showing that blocking of VLA-5 function by either soluble RGD or blocking Ab almost completely inhibits adhesion of BMMC to fibronectin.

Binding to immobilized fibronectin induces integrin clustering, tyrosine phosphorylation and eventually cytoskeleton rearrangement and focal adhesion formation. These events are crucial for proper integrin-mediated cell adhesion[22,33,100]. Our observation that Mn^{2+}-induced adhesion is temperature-sensitive is a strong indication that this adhesion can not simply be explained as a cation-stabilized complexation of two proteins, VLA-5 and fibronectin, but involves cellular signaling by enzymes. In addition, we show that inhibition of tyrosine phosphorylation and dephosphorylation which are both needed for proper integrin signaling[58,75], attenuates Mn^{2+}-induced adhesion of our cells to fibronectin.

Consistently with other reports, Ca^{2+}attenuates Mn^{2+}-induced VLA-5-mediated adhesion of BMMC to fibronectin. However, our data show that Ca^{2+} can only prevent the induction of adhesion while the adhesion of cells already connected to fibronectin, remains unaffected. This suggests that adhesion of BMMC to fibronectin, induced by our protocol, can be divided into two stages as was recently suggested [54]. During the first stage, binding of Mn^{2+} to the cation binding domain of VLA-5 induces a conformational change and thereby high-affinity state of this integrin for fibronectin. During the second stage the binding of VLA-5 with fibronectin results in ligand-mediated integrin clustering and initiation of cellular signaling, strengthening the cell-substrate link through formation of focal contacts and cytoskeleton rearrangement[22,28,33]. This stage could be referred to as the outside-in signaling stage and is insensitive to Ca^{2+}.

The observation that BMMC adhered to fibronectin are unaffected by extracellular Ca^{2+}, indicates that our model is suitable for the investigation of the modulation of FcεRI-induced degranulation that depends on influx of extracellular calcium[11].

One could argue that Mn^{2+} might directly influence the FcεRI signaling cascade by interfering with Ca^{2+} signaling. However in our experiments we never observed a significant effect of 0.2 mM $MnCl_2$ on FcεRI-induced degranulation (data not shown). Moreover, in our experiments all cells are treated with Mn^{2+} before exposure to either fibronectin or BSA, and any observed effect is therefore substrate-specific.

The use of a horizontal surface, such as the bottom of a well, as carrier for immobilized fibronectin has some disadvantages. First, for biochemical analysis of this model, a larger amount of cells and therefore larger substrate area for adhesion is needed. Secondly, variation in vertical position of the cells and thereby variation in distance towards the substrate (bottom of the well), introduces heterogeneity in the starting point of adhesion among the cells. To prevent this, synchronization of adhesion by 30 minutes incubation on ice for cell sedimentation is applied. Low temperature rarely is beneficial for biological systems and should therefore, if possible, be prevented. Use of beads as carrier for immobilized fibronectin[26,95,112], provides a solution for the above mentioned problems. The large area-to-volume ratio of beads enables us to work in smaller volumes than when using a 2-dimensional carrier, and also enables us to mix cells with fibronectin-coated beads so that adhesion of all cells is initiated at the same time. We deliberately chose beads of which the size is equal to or higher then that of the cells because it has been shown that smaller beads are subject to phagocytosis instead of being an immobilizer of cells[125].

In conclusion, in this report we introduce a novel model to study modulation of mast cell degranulation through adhesion to fibronectin. We designed a protocol using BMMC of which adhesion to fibronectin can be controlled. FcεRI-induced degranulation of adhered BMMC can be studied under the normal conditions needed for this cell function. By using polystyrene beads as carrier for immobilized fibronectin, all steps of the protocol can be performed at a physiological temperature and besides degranulation, analysis of the function of adhered mast cells can be extrapolated to biochemical methods.

Attenuation of VLA-5 mediated adhesion of bone marrow-derived mast cells to fibronectin by peptides with inverted hydropathy to EF-hands

(Submitted for publication)

René Houtman, J. Edwin Blalock†, Matteo Villain†, Andries S. Koster and Frans P. Nijkamp

†Department of Physiology and Biophysics, Cancer Center, School of Medicine and Dentistry, University of Alabama, Birmingham, USA

Abstract

Release of allergic mediators from mast cells is enhanced by very late antigen-5 (VLA-5)-mediated interaction of these cells with fibronectin. The extracellular part of the VLA-5 α-subunit was previously shown to contain a cation-binding domain, homologous to the EF-hand of calmodulin, which is important for its function. In this report we show that VLA-5-mediated adhesion of bone marrow-derived mast cells can be induced by two different pathways, first by FcεRI-clustering, which dependents on calmodulin activation and extracellular Ca^{2+}, and second by Mn^{2+} stimulation, independent of calmodulin activation and antagonized by Ca^{2+}. To show a role for EF-hands from different proteins in VLA-5-mediated adhesion we used calcium-like peptides (CALP)1 and CALP2, designed to bind to EF-hands based on inverted hydropathy. CALP1 and more potently CALP2 inhibited FcεRI-induced calmodulin-dependent adhesion to fibronectin, in correlation with their affinity for the calmodulin-EF-hand. Interestingly only CALP2 was able to inhibit Mn^{2+}-induced calmodulin-independent adhesion by interfering with an extracellular target, probably VLA-5. We conclude that CALP1 and 2 can inhibit VLA-5-mediated adhesion of mast cells to fibronectin through binding to EF-hands of multiple proteins, and that these peptides can be used as lead compounds for the development of future therapy against allergy.

Introduction

Integrins are a family of heterodimeric cell surface receptors that bind extracellular matrix and cell surface ligands and play a major role in various processes such as inflammation, cell adhesion, migration, proliferation and differentiation[139]. One example is the modulation of mast cell sensitivity for antigenic stimulation through $\alpha_5\beta_1$ integrin very late antigen-5 (VLA-5)[i]-mediated interaction with the extracellular matrix-component fibronectin[62,94,131]. Antigenic stimulation of mast cells by crosslinking of the high-affinity IgE receptor (FcεRI) leads to release of various mediators which cause clinical phenomena associated with an allergic response[138,147]. The observation that VLA-5 can modulate mast cell degranulation makes this receptor a possible target for future therapeutic strategies against allergy and is the topic of the current study.

Integrin ligand-affinity is modulated by a poorly understood mechanism, referred to as inside-out signaling, whereby these receptors are converted from a low- to high-affinity state[44,74,93]. It has been reported that the affinity of VLA-5 for fibronectin can be modulated by cations. Mn^{2+} induces high-affinity of VLA-5 for the Arginine-Glycine-Aspartic acid (RGD) sequence in fibronectin through binding to the cation-binding motif in the extracellular domain of the VLA-5 α-subunit. This effect can be antagonized by Ca^{2+} [116,118]. The cation-binding motif of VLA-5 is highly homologous to the Ca^{2+} binding EF-hand motif of calmodulin (CaM)[160]. An EF-hand loop is formed by thirteen amino acids of which five play a role in coordinating the binding of Ca^{2+} by means of their negatively charged side chain. In the EF-hand-like motif of VLA-5, one of the coordinating amino acids is replaced by an uncharged, nonfunctional, residue and it has been postulated that its coordinating function is taken over by the negatively charged side chain of aspartic acid of the RGD ligand sequence. In this view, high-affinity binding is the result of cooperative coordination of the cation by the integrin and the ligand and stabilization of this complex by the cation itself [7].

In addition to cations, it was recently shown that clustering of FcεRI triggers VLA-5-mediated adhesion of murine bone marrow-derived mast cells (BMMC) to fibronectin[87,175]. Although it has been established that influx of Ca^{2+} [11] and CaM activation[25,168] play a role in the FcεRI signaling cascade leading to degranulation, it is unclear whether they play a role in the FcεRI-induced VLA-5 affinity modulation.

Accumulating evidence suggests that the gross architecture of a peptide or protein is, at least in part, determined by its pattern of hydropathy and that exact inversion of this pattern will result in a second peptide with a complementary surface contour to the first because the hydrophobic effect is involved in reversed orientation (reviewed by Blalock[19]). Supporting this theory, CALP1 (for calcium-like peptide 1) designed as an amino acid sequence complementary to that encoding a primordial EF-hand, was shown to mimic Ca^{2+} inducing CaM activity, binding EDTA, inducing smooth muscle contraction[38] and blocking Ca^{2+} influx through binding to the EF-hands of Ca^{2+} channels or indirectly via CALP/CaM interaction with such channels (Manion *et al. submitted for*

[i] For abbreviations see page 101

publication)[ii]. Another complementary peptide, CALP2, was designed by computer-assisted optimal inversion of the hydropathy pattern of the EF-hand 4 amino acid sequence of CaM[43,102,165]. Due to increased length and optimal hydropathy inversion, the affinity of CALP2 for the EF-hand compared to CALP1 was increased 11-fold as determined by surface plasmon resonance detection. Interestingly, increased affinity by increased reciprocity of the pattern of hydropathy and increased length of the peptide resulted in a change of functional activity. Whereas CALP1 shows biological effects similar to Ca^{2+}, CALP2 acts as an antagonist for CaM[165].

Influx of Ca^{2+} and subsequent CaM activation play a possible role in FcεRI-induced affinity modulation of VLA-5 for fibronectin. In combination with the assumption that based on the pattern of hydropathy the EF-hand-like domain of VLA-5 is a potential binding site for CALP1 and CALP2, this suggests several possible targets for these peptides to interfere with mast cell adhesion. In this report we investigate the ability of CALP1 and CALP2 to inhibit VLA-5-mediated adhesion of BMMC to fibronectin induced either by cationic stimulation or FcεRI clustering.

Materials & Methods

Cells

BMMC were obtained as described previously[84]. Briefly, bone marrow from femurs of Balb/c mice was flushed and cells were cultured at a density of 2×10^5/ml in complete RPMI (RPMI1640 medium containing 4 mM L-glutamine, 5×10^{-5} M β-mercaptoethanol, 1mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.1 mM nonessential amino acids) supplemented with 20% (vol/vol) supernatant from Balb/c splenocytes which were stimulated for 7-days with pokeweed mitogen (Sigma Aldrich NV/SA, Axel, The Netherlands) in complete RPMI. Medium was refreshed once per week. In all graphs mean ± s.e.m. of quadruplicates of a representative experiment are shown.

Design and synthesis of the hydropathically complementary peptides

The design of the eight residue complementary peptide CALP1 (VAITVLVK) was based on the primordial CaM EF hand 4 motif. Selection of the complementary peptide CALP2 (VKFGVGFKVMVF) was carried out using the computer program AMINOMAT® (Tecnogen ScpA, Italy), with an averaging window r=9, a range of inverted hydropathy of 0.8 and considering also eight amino acids of the flanking regions. The program generated 1,417,176 possible sequences, and chose the one with the lowest Q value (0.0068). This value is defined by the formula: $Q= [(a_i+b_i)2/(n-2s)]^{1/2}$, where a_i represents the moving averaged hydropathy assigned to every amino acid of the target peptide, b_i represents the moving averaged hydropathy assigned to every amino acid of each of the complementary peptides generated by the program, s is (r-1)/2 (where r is the number of amino acids considered in the moving window) and n is the number of residues in the target peptide.

The peptides were synthesized using continuous flow solid phase peptide synthesis with Fmoc chemistry, on a PerSeptive Biosystems 9050 Peptide synthesizer. Pre-

[ii] Manion, M. K., Z. Su, M. Villain, and J. E. Blalock. A new type of Ca^{2+} channel blocker that targets Ca2+ sensors and prevents Ca^{2+}-mediated apoptosis. (*submitted for publication)*

activated Opfp amino acids with HoAt and preloaded PEG-PS resin were used. The peptides were purified by RP-HPLC on a Waters Delta Pack C18 300 A (300 x 39 mm I.D.). The purity of the product was checked by RP-HPLC on a Dynamax C18 (300 x 4.8 mm I.D.) column equilibrated at a flow rate of 1 ml/min and eluted with a linear gradient from 5 to 80% CH3CN containing 0.1% TFA, in 40 min. MilliQ water previously treated with Chelex 100 to remove any Ca^{2+} was used in the purification. The identity of the peptides was confirmed by TOF-MALDI MS (U.A.B. Core Facility).

Adhesion assay

For Mn^{2+}-induced adhesion, 4-6 weeks old BMMC were washed with PBS, 1 mM EDTA in PBS and Tris-buffered saline (TBS, 2.9 g/L Tris-Cl, 4 g/L NaCl, 0.2 g/L KCl, 0.4 g/L glucose, 0.1 % BSA) and resuspended at a density of 5 x 10^5 cells/ml TBS. For FcεRI-mediated adhesion, 4-6 week old BMMC were incubated 1 hr at 37°C in complete RPMI supplemented with supernatant of the anti-DNP IgE producing hybridoma, 26.82[98], washed as described above, and cells were resuspended in TBS with or without 1.8 mM $CaCl_2$ as indicated, at a density of 5 x 10^5 cells/ml. NUNC maxisorp 96 wells plates were coated for 3 hrs at 37°C with 200 µl/well 2.5 mg/ml human fibronectin (CLB, Amsterdam) in PBS, followed by 3 times washing with 200 µl/well PBS. 100 µl cells per well were incubated with 50µl of Mn^{2+} as indicated or 50 µl of DNP-HSA (final concentration: 3 µg/ml, or as indicated, Sigma Aldrich NV/SA, Axel, The Netherlands), and 50 µl of CALP or W7 (Sanvertech B.V., Heerhugowaard, The Netherlands) (final concentrations as indicated in graphs). CALP2-biotin (2 x 10^{-4} M) was preincubated with or without streptavidin (SA, 1 x 10^{-4} M, Boehringer Mannheim B.V.) in TBS for 1 hr at room temperature under constant rotation, before application to the cells. Cells were incubated on a fibronectin-coated plate at 37°C and after 60 minutes non-adhered cells were removed by three times washing with 200 µl/well PBS. For functional blocking of VLA-5, cells were incubated with blocking antibody BMA5 (a generous gift from Dr. B.M.C. Chan, Univ. Western Ontario, Canada)[45], one hour before at room temperature, or various concentrations of RGD peptide (Sigma Aldrich NV/SA, Axel, The Netherlands) during incubation on fibronectin. Adhesion was quantified using Cyquant proliferation assay kit (Molecular Probes Europe B.V., Leiden, The Netherlands) according to the manufacturer's protocol. In short, plates were frozen overnight at –20°C, thawed and incubated for 60 minutes under continuous agitation at room temperature with a DNA-specific fluorescent dye and lysing reagent. Fluorescence was measured at $\lambda_{ex.}$: 485 nm / $\lambda_{em.}$: 530 nm using a Millipore Cytofluor 2350 microplate reader and adhesion was calculated as the residual fluorescence as a percentage of input fluorescence. In all graphs mean ± s.e.m. of quadruplicates of a representative experiment are shown.

Degranulation assay

4-6 Weeks old BMMC were sensitized with anti-DNP IgE as described above, washed and resuspended at 5 x 10^5 cells/ml in Tyrode's buffer (Life Technologies B.V., Breda, The Netherlands) supplemented with 10 mM HEPES, 1 g/L $NaHCO_3$, 0.1 % BSA, pH 7.2). 100 µl of cells per well of a Costar 96 well plate were incubated with 50 µl of DNP-HSA (final concentration: 30 ng/ml) and 50 µl of CALP or with CALP alone as indicated, for 30 minutes at 37°C. Supernatants were analyzed for β-hexosaminidase content as parameter for mast cell degranulation as described previously[84]. In short, 50 µl of supernatant was incubated for 60 minutes at 37°C with 50 µl 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (Sigma Aldrich NV/SA, Axel, The Netherlands) in citrate buffer (0.1 M, pH 4.5), the reaction was stopped by addition of 100 µl of glycine buffer (0.2 M glycine, 0.2 M NaCl, pH 10.7) and fluorescence was measured at $\lambda_{ex.}$:

360nm/$\lambda_{em.}$:460nm using a Millipore Cytofluor 2350 microplate reader. Degranulation was calculated as the amount of β-hexosaminidase activity present in the supernatant as a percentage of the total β-hexosaminidase activity present in the cells, determined in lysates of 5 x 10^4 cells. In all graphs mean ± s.e.m. of quadruplicates of a representative experiment are shown.

Results

Cationic modulation of BMMC adhesion to fibronectin

To investigate if the previously reported cation-mediated modulation of VLA-5 affinity for fibronectin[116] can be extrapolated to BMMC, extracellular Ca^{2+} was removed by EDTA treatment and cells were incubated with various concentrations of $MnCl_2$ on immobilized fibronectin. As is shown in figure 1A,

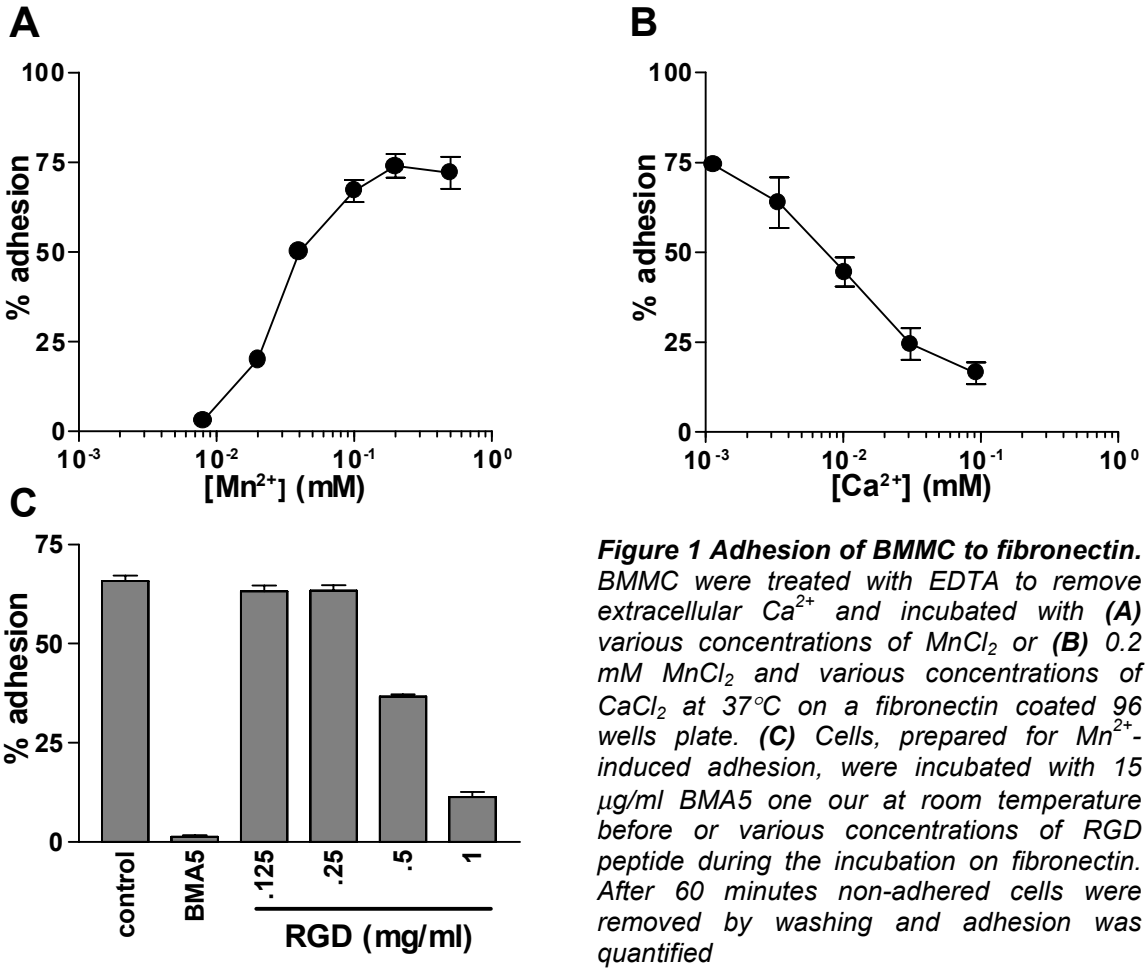


Figure 1 Adhesion of BMMC to fibronectin. *BMMC were treated with EDTA to remove extracellular Ca^{2+} and incubated with **(A)** various concentrations of $MnCl_2$ or **(B)** 0.2 mM $MnCl_2$ and various concentrations of $CaCl_2$ at 37°C on a fibronectin coated 96 wells plate. **(C)** Cells, prepared for Mn^{2+}-induced adhesion, were incubated with 15 μg/ml BMA5 one our at room temperature before or various concentrations of RGD peptide during the incubation on fibronectin. After 60 minutes non-adhered cells were removed by washing and adhesion was quantified*

$MnCl_2$ dose-dependently induces the adhesion of BMMC to fibronectin. Optimal adhesion of approximately 75% of cells is reached at 0.2 mM $MnCl_2$, and this concentration was used in all subsequent experiments. To investigate if Ca^{2+} can antagonize Mn^{2+}-induced adhesion, cells were treated with EDTA and incubated on immobilized fibronectin with $MnCl_2$ and various concentrations of

$CaCl_2$. Figure 1B shows that $CaCl_2$ dose-dependently reduces $MnCl_2$-induced adhesion. Previous data showing that adhesion of BMMC to fibronectin is VLA-5-mediated were confirmed by almost complete inhibition of Mn^{2+}-induced adhesion of our cells to fibronectin by soluble ligand, RGD peptide, and blocking antibody BMA5 as is shown in figure 1C. From these data we conclude that in BMMC, the regulation of adhesion to fibronectin by cations parallels earlier published data reporting that Mn^{2+} induces a conformational change of VLA-5 to high-affinity for fibronectin, via a mechanism that is antagonized by Ca^{2+}.

A role for extracellular Ca^{2+} in FcεRI-induced adhesion of BMMC to fibronectin

To investigate if the presence of extracellular Ca^{2+} is necessary for the induction of adhesion by FcεRI clustering, anti-DNP IgE sensitized BMMC were treated with EDTA and stimulated with DNP-HSA on immobilized fibronectin in $CaCl_2$-supplemented buffer (final concentration: 1.8 mM) or calcium-free buffer. As is shown in figure 2, stimulation of BMMC via FcεRI dose-dependently induces adhesion to fibronectin in buffer supplemented with Ca^{2+}, however the absence of this divalent cation completely abrogates FcεRI-induced adhesion of these cells. From these results we conclude that in addition to FcεRI-induced mast cell degranulation, the presence of extracellular Ca^{2+} is critical for the FcεRI signaling cascade that induces adhesion of BMMC to fibronectin.

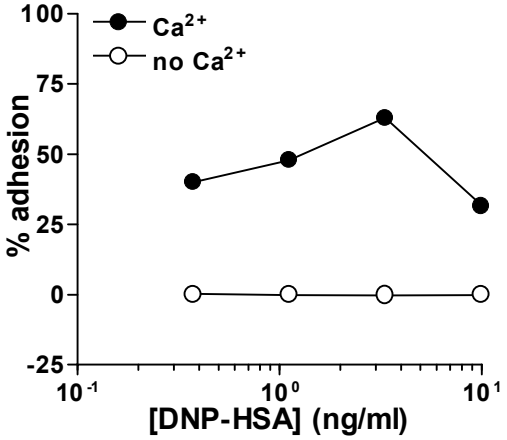


Figure 2 FcεRI-induced adhesion of BMMC to fibronectin in absence and presence of extracellular Ca^{2+}. *Anti-DNP IgE-sensitized BMMC were treated with EDTA to remove extracellular Ca^{2+} and resuspended in buffer without (no Ca^{2+}) or with (Ca^{2+}) $CaCl_2$ (final concentration: 1.8 mM). Cells were incubated with 0.3 ng/ml DNP-HSA at 37°C on a fibronectin coated 96 wells plate. After 60 minutes non-adhered cells were removed by washing and adhesion was quantified. (Error bars are covered by symbols used)*

A role for CaM in FcεRI- or Mn^{2+}-induced adhesion

To study whether CaM plays a role in antigenic or cationic modulation of VLA-5 affinity for fibronectin, we investigated the effect of the CaM inhibitor, W7, on the adhesion induced by either FcεRI clustering or Mn^{2+} stimulation. Anti-DNP IgE sensitized cells were stimulated with DNP-HSA and EDTA-treated cells were stimulated with Mn^{2+} on immobilized fibronectin with various concentrations of W7. As is shown in figure 3, W7 inhibits adhesion of FcεRI-stimulated BMMC, however it has no effect on adhesion of cells stimulated with $MnCl_2$. From these data we conclude that adhesion of BMMC to fibronectin due to modulation of VLA-5 affinity by either FcεRI clustering or Mn^{2+} stimulation is mediated via distinct signaling pathways that are CaM-dependent and –independent, respectively.

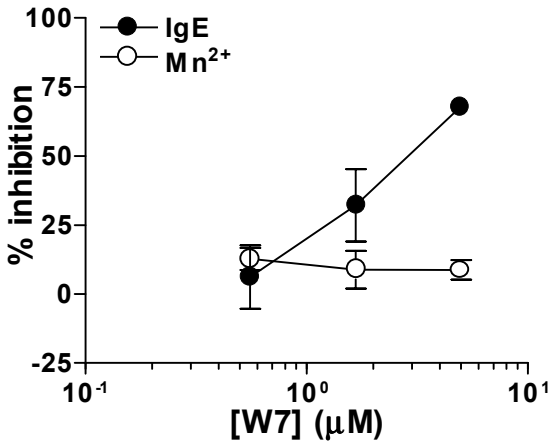


Figure 3 Effect of CaM inhibitor W7 on cation- and antigen-induced adhesion of BMMC to fibronectin*. Anti-DNP IgE sensitized mast cells were stimulated with 3 ng/ml DNP-HSA in $CaCl_2$-supplemented buffer (closed circles), or 0.2 mM $MnCl_2$ in calcium-free buffer (open circles) and incubated with various concentrations of W7 at 37°C on a fibronectin coated 96 wells plate (96W). After 60 minutes non-adhered cells were removed by washing and adhesion was quantified. In several cases error bars are covered by symbols*

The effect of CALP1 and CALP2 on FcεRI and Mn^{2+}-induced adhesion of BMMC to fibronectin

Our data indicate that FcεRI signaling leading to adhesion is Ca^{2+} and CaM dependent and therefore an effect of both CALP1 and 2 on FcεRI-induced adhesion can be expected. Anti-DNP IgE sensitized BMMC were stimulated with DNP-HSA in the presence of various concentrations of either CALP1 or CALP2 on immobilized fibronectin. Indeed, as is shown in figure 4A, we find that both CALP1 and CALP2 are able to dose-dependently inhibit adhesion induced by FcεRI clustering. We observed that CALP2 is a more potent inhibitor than CALP1 (EC_{50}= 3.5 x 10^{-5} and 1 x 10^{-4} M respectively) and that both peptides fail to induce adhesion of our cells to fibronectin (data not shown).

The observation that Mn^{2+}-induced adhesion of BMMC to fibronectin is mediated via a Ca^{2+}- and CaM–independent pathway provides us with the opportunity to investigate if CALP1 and CALP2 are able to interfere with adhesion through binding to a target other than the CaM EF-hand. EDTA-treated BMMC were stimulated with Mn^{2+} and various concentrations of either CALP1 or CALP2 on immobilized fibronectin. As is shown in figure 4B, CALP1 does not have an effect on Mn^{2+}-induced adhesion, however CALP2 dose-

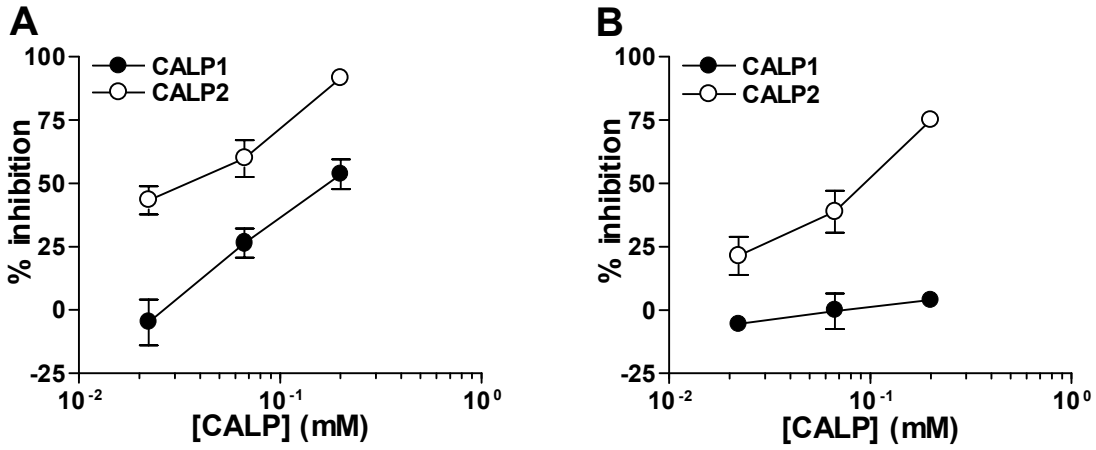


Figure 4. Effect of CALP1 and CALP2 on FcεRI- and Mn^{2+}-induced adhesion of BMMC to fibronectin. *BMMC were stimulated **(A)** via clustering of FcεRI or **(B)** with Mn^{2+} and incubated with various concentrations of CALP1(closed circles) or CALP2 (open circles) on immobilized fibronectin. After 60 minutes non-adhered cells were removed by washing and adhesion was quantified.*

dependently inhibits Mn^{2+}-induced adhesion of our cells to fibronectin, suggesting a target for CALP2 other than CaM because this pathway is CaM-independent.

Effect of prevention of CALP2 cell entry on its ability to inhibit adhesion

Our data show that CALP 2, as well as CALP1, are able to inhibit the Ca^{2+}- and CaM-dependent adhesion, suggesting an intracellular target for these peptides. Only CALP2 is able to attenuate Ca^{2+}- and CaM-independent adhesion, and we suggest that the target for CALP2 is the EF-hand-like domain of VLA-5 which is located extracellularly.

To test for an extra- versus intracellular "non-CaM" target, CALP2 was labeled with N-terminal biotin. This peptide was incubated and complexed with SA to prevent it from entering the cells, and to specifically target extracellular sites. BMMC were stimulated on immobilized fibronectin either via FcεRI or with Mn^{2+} together with free or complexed CALP2-biotin. Figure 5 shows that prevention of cell entry largely abrogates the inhibiting effect of CALP2 on FcεRI-induced adhesion, while the inhibiting effect of this peptide on Mn^{2+}-induced adhesion of BMMC to fibronectin is largely retained. These data show that besides the ability of CALP2 to inhibit adhesion through binding to intracellular targets such as CaM, this peptide can also attenuate adhesion of BMMC to fibronectin by way of binding to extracellular targets.

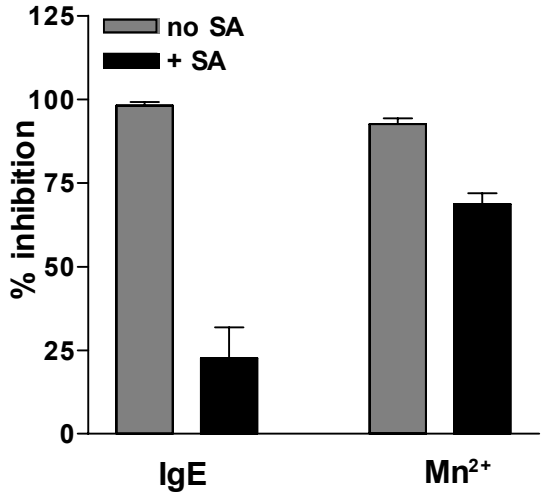


Figure 5. Effect of prevention of cell entry of CALP2 on its adhesion inhibiting potency. *BMMC were stimulated via clustering of FcεRI or with Mn^{2+} and incubated with free CALP2-biotin* ***(no SA)*** *or with CALP2-biotin complexed with streptavidin* ***(SA)*** *on immobilized fibronectin. After 60 minutes non-adhered cells were removed by washing and adhesion was quantified.*

Effect of CALP1 and CALP2 on mast cell degranulation

Our data show that both CALP1 and 2 inhibit FcεRI-induced adhesion of BMMC to fibronectin, which suggest that that these peptides interfere with Ca^{2+}-influx and/or CaM activation. To investigate this, we tested the effects of these peptides on FcεRI-degranulation, a mast cell parameter which has been shown to be dependent on Ca^{2+}-influx and CaM activation, which should be comparable to those on FcεRI-induced adhesion. Anti-DNP IgE sensitized BMMC were stimulated with DNP-HSA in the presence of various concentrations of CALP1 or CALP2 and the release of granular β-hexosaminidase, as a measure for degranulation, was determined. As is shown in figure 6A, CALP1 and more potently CALP2, parallel to adhesion, dose-dependently inhibited β-hexosaminidase release from BMMC (EC_{50}: 2 x 10^{-5}

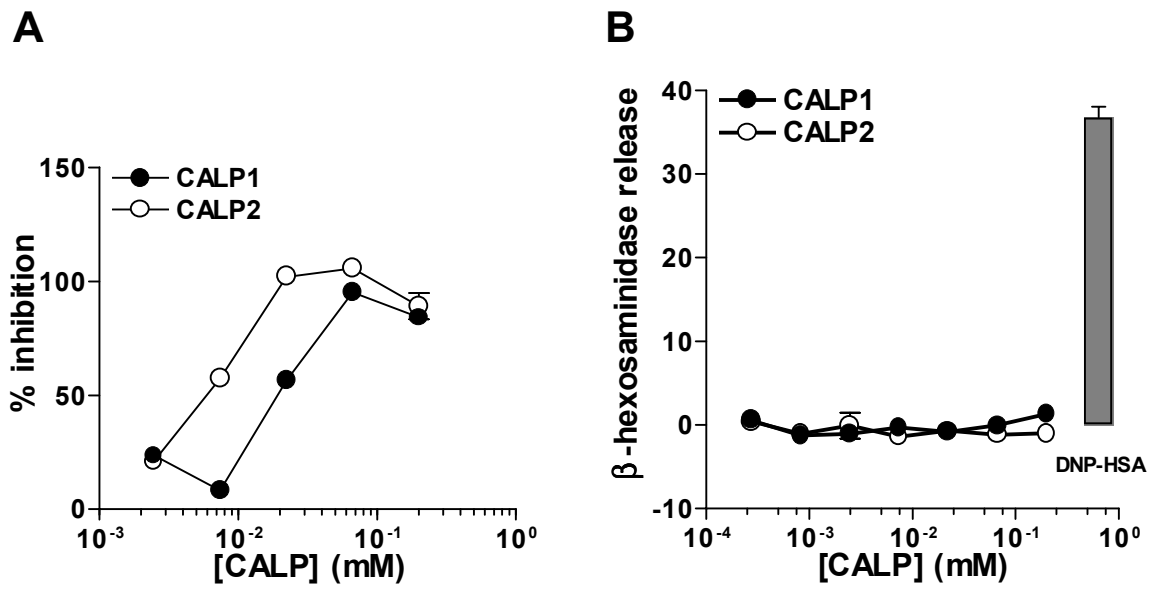


Figure 6. Effect of CALP1 and 2 on degranulation of BMMC. (A) *Anti-DNP IgE sensitized BMMC were incubated with various concentrations of CALP1 (closed circles) or CALP2 (open circles) for 30 minutes at 37°C. Additionally, cells were stimulated with 30 ng/ml DNP-HSA* ***(A)*** *or not* ***(B),*** *and after 30 minutes β-hexosaminidase release as a measure for degranulation was quantified. The bar in figure 6 B represents degranulation of these cells triggered with 30 ng/ml DNP-HSA in the absence of CALPs, as control for their function. (In several cases error bars are covered by symbols used)*

and. 6 x 10^{-6} M respectively). Both peptides lacked the intrinsic ability to induce mast cell degranulation, as is shown in figure 6B. From these data we conclude that CALP1 and 2 both attenuate FcεRI-induced degranulation by interfering with the same targets as those in FcεRI-induced adhesion, probably Ca^{2+}-influx and CaM activation.

Discussion

The observation that VLA-5 can modulate mast cell degranulation makes this receptor a possible target for future therapeutic strategies against allergy. Our data show that high-affinity of VLA-5 and subsequent adhesion of BMMC to fibronectin can be induced via two separate pathways, first by clustering of FcεRI, which is dependent on activation of CaM and extracellular Ca^2, and second by Mn^{2+}-stimulation which is independent of CaM and even antagonized by Ca^{2+}.

Because FcεRI-induced adhesion as well as degranulation are both dependent on extracellular Ca^{2+} and activation of CaM, it is logical to assume that both are mediated by the same signaling cascade and therefore affected similarly by CALP1 and 2. Inhibition by CALP2 seemed predictable as this peptide was shown to be an antagonist for CaM activation[165] and should therefore have the same affect as the CaM inhibitor, W7. Inhibition of adhesion and degranulation by CALP1 can not be explained by inhibition of CaM, since it was shown that this peptide mimics Ca^{2+} by activating CaM[38,165]. Importantly, it was shown

recently that CALP1 not only mimics Ca^{2+} in its binding and activating characteristics towards CaM, but is also able to directly, as well as indirectly, through CaM, block the N-methyl-D-aspartate type calcium channel of rat neuronal cells and the nonselective cation channel (NSCC) of Jurkatt cells which provide negative feedback for Ca^{2+} influx (Manion *et al. Submitted for publication*) Thes channels were shown to be present in mast cells and play a role in mast cell degranulation[72,73,129,130] and these receptors could serve as targets for CALP1 in our cells. The closure of cation channels also explains the lack of intrinsic ability of CALP1 to induce degranulation, which is characteristic of Ca^{2+} ionophores[15]. Of CALP1 and CALP2 the latter was the most potent inhibitor of both adhesion and degranulation which can be explained by the fact that this peptide has the higher affinity for the CaM EF-hand.

The EC_{50} of both peptides for inhibition of adhesion was approximately fivefold higher than that for inhibition of degranulation. In our experiments we find that the amount of allergen needed for optimal degranulation is approximately tenfold higher than that for optimal adhesion (unpublished observations), this suggest that a stronger FcεRI signal is needed for degranulation and is therefore more sensitive to the inhibiting effects of CALP1 and 2.

Mn^{2+}-induced adhesion is independent of CaM activation and an inhibiting effects of any CALP on adhesion is, therefore, mediated by different targets than those described for the FcεRI pathway. We find that CALP2 but not CALP1 is able to inhibit Mn^{2+}-induced adhesion of BMMC to fibronectin, which could be explained by the higher affinity of this peptide for EF-hands. The EC_{50} of CALP2 for inhibition of Mn^{2+}-induced adhesion is approximately threefold higher than that for inhibition of FcεRI-induced adhesion. This suggests either lower affinity of CALP2 for this EF-hand than for CaM, or a higher quantity of EF-hands that need to be bound for a comparable effect. We claim that the target for CALP2 in the inhibition of Mn^{2+}-induced adhesion is the EF-hand-like domain of VLA-5, and because this motif has a hydropathy profile identical to the CaM EF-hand, it should therefore bind CALP2 with the same affinity. Therefore we propose that the difference in target quantity is the explanation for this observation.

In our experiments using SA-complexed CALP2, a partial inhibition of FcεRI-induced adhesion can be observed. There are several explanations for this phenomenon. First, the presence of some unbound CALP-2-biotin could attenuate CaM activation, we show that an effect of CALP2 on IgE-mediated degranulation could already be observed at low concentrations. Second, there could be a direct effect of CALP2 on the EF-hand like domain of VLA-5 in FcεRI-induced adhesion. In the same experiment we also observed a partial attenuation of the inhibiting capacity of complexed CALP2 compared to free CALP2. This could be explained by the fact that the binding of CALP2 to the EF-hand like domain of VLA-5 is partially interfered spherically by the bulky SA-molecule to which it is bound.

Although we have no data showing direct interaction between VLA-5 and CALP2, several arguments support our conclusion that this integrin is targeted during inhibition of CaM independent adhesion. First, based on hydropathy and sequence homology to CaM EF-hands the EF-hand-like domain of VLA-5 forms a potential binding site for CALP2. Second, the affinity of CALP2 and Ca^{2+} for CaM was shown to be comparable[34,165]. Accordingly, we find that the EC_{50} of these agents for inhibition of Mn^{2+}-induced adhesion is also comparable. Third, although EF-hand motifs are present in numerous proteins that play a role in

various cellular processes[163], most of these are localized intracellularly. The observation that our target is located extracellularly, narrows the list of possible candidates drastically and mainly leaves us with the integrins. We and others have shown that in 4-6 weeks old BMMC, adhesion to fibronectin is entirely VLA-5-mediated[45,87], which strongly suggests this integrin as the most obvious candidate.
There are several mechanisms by which CALP2 could inhibit VLA-5 binding to fibronectin. Binding of the peptide to the VLA-5 EF-hand-like domain could induce a conformational change of the integrin to a low-affinity state, a mechanism which was also postulated for the antagonizing effect of Ca^{2+} in cation modulation of VLA-5 affinity[116,118]. Although it was shown that EF-hand-binding to troponin C of CALP2 induces a conformational change of this protein, this conformational change was different from the one induced by Ca^{2+} [165]. Another possible mechanism is that due to binding to the EF-hand-like domain, which is localized in the ligand-binding site of the integrin, binding of the ligand by the integrin is physically blocked and thereby adhesion is prevented.
Previous studies have shown that hydropathic patterning can be used to tailor make peptide ligands targeted to the EF-hand of CaM and that by increasing affinity the functional activity of the peptide can be modulated from agonistic to antagonistic[38,165]. In this report we show that these peptides can be applied to block cell adhesion by interfering with CaM and other targets, presumably the EF-hand-like domain of VLA-5. Their ability to block adhesion and thereby modulate the activity of mast cells indicates that these peptides could serve as possible lead compounds for the development of future therapies against allergy and other pathologies in which proteins with EF-hands are involved.

6

Cation-induced adhesion of bone marrow-derived mast cells to fibronectin induces degranulation *in vitro* and *in vivo*

(Submitted for publication)

René Houtman, Aletta D. Kraneveld, Andries S. Koster and Frans P. Nijkamp

Abstract

Previously, it was demonstrated that degranulation of RBL-2H3 mast cells is enhanced by $\alpha_5\beta_1$ integrin very late antigen-5 (VLA-5)-mediated adhesion to fibronectin. However, RBL-2H3 cells adhere nonspecifically to various substrates, and we therefore used mast cells cultured from mouse bone marrow (BMMC) to investigate the effect of adhesion to fibronectin on degranulation. It has been demonstrated previously that Mn^{2+} induces a conformational change of VLA-5 into high-affinity for fibronectin. In our experiments, Mn^{2+}-induced adhesion to fibronectin enhanced allergen-induced mast cell degranulation *in vitro*. Surprisingly, adhesion to fibronectin induced by Mn^{2+} but also by Zn^{2+}, Ni^{2+}, and Co^{2+}, metals that are known to induce contact hypersensitivity responses, directly triggered mast cell degranulation *in vitro* in absence of an antigenic stimulus. This effect could be blocked by soluble arginin-glycine-aspartic acid (RGD) confirming a role for VLA-5. In addition, local injection of BMMC stimulated *in vitro* with Mn^{2+}, into the skin of BALB/c mice resulted in spontaneous activation and enhanced allergen-induced activation of these cells *in vivo*. This response was also attenuated with soluble RGD peptide. In conclusion, our data show that VLA-5 can modulate mast cell sensitivity for antigenic stimulation and provide a plausible explanation for hypersensitivity responses to various metals in which cation-induced integrin-mediated adhesion directly triggers degranulation.

Introduction

Mast cells are localized in all vascularized tissues and play an important role in hypersensitivity type I responses[147]. Crosslinking of the high-affinity receptor for IgE (FcεRI)[i] on mast cells triggers a signal transduction cascade leading to the release of various inflammatory mediators, which cause clinical phenomena associated with allergy[12,14,59]. An increasing number of reports suggest that the function of the mast cell is affected by its microenvironment, consisting of neighboring cells, extracellular matrix and various humoral factors, probably by modulating the FcεRI signal transduction cascade[39,56,134,155]. Adhesion to the extracellular matrix component fibronectin has been shown to enhance FcεRI-induced degranulation of RBL-2H3 mast cells[62,64,131,155]. Although it was shown that this adhesion is mediated by the $\alpha_5\beta_1$-integrin very late antigen-5 (VLA-5) and the arginine-glycine-aspartic acid (RGD) sequence of fibronectin[150,177], the mechanism by which FcεRI signaling is influenced is not yet understood. Elucidation of the molecular basis of the role for VLA-5 in mast cell degranulation, which is potentially useful for the development of future therapy against allergy, is therefore topic of our research.

A model system was required in which adhesion and degranulation can be independently controlled. RBL-2H3 cells, a widely used model for mast cells, adhere to various surfaces without assembling specialized adhesion structures at the cell-substrate interface[8,124], and are therefore not suitable to study the effect of adhesion on degranulation. We used mast cells cultured from mouse bone marrow (BMMC)[84,133], because these cells do not show nonspecific adhesion. Although it has been shown that VLA-5 is expressed on BMMC[45,177], these cells do not adhere spontaneously to fibronectin. Importantly, it has been demonstrated that on resting cells integrins exist in a low-affinity conformation and that cell activation triggers the conversion of these receptors into a high-affinity state via a mechanism referred to as inside-out signaling[44,46,55,74,93]. Accordingly, it was shown that stimulation of BMMC by PMA induces VLA-5-mediated adhesion to fibronectin, via a phosphatidylinositol 3-kinase-dependent pathway[87].

The affinity of VLA-5 for fibronectin is also modulated directly by cations, as was demonstrated in K562 erythroleukemia cells[116,118]. The extracellular domain of the VLA-5 α-subunit contains several cation-binding motifs and binding of Mn^{2+} induces a conformational change of the integrin into high-affinity, a mechanism which is antagonized by Ca^{2+}. Additionally, integrin-ligand binding is stabilized by the cooperative coordination and binding of Mn^{2+} by negatively charged amino acid residues of the cation-binding domain localized in the ligand binding area of VLA-5, and the negatively charged aspartic acid of the RGD-sequence in fibronectin[7]. In this report we investigated the adhesion of BMMC to fibronectin induced by various cations. In addition, the effect of VLA-5-mediated adhesion on degranulation of BMMC were studied *in vitro* and *in vivo*.

[i] For abbreviations see page 101

Materials & Methods

Animals

Male BALB/c mice (6-8 week, Central Animal Laboratory (GDL), Utrecht University, Utrecht, The Netherlands) were used in all experiments. The experiments were approved by the Animal Care Committee, Utrecht University.

Cells

BMMC were obtained as described previously[84]. Briefly, bone marrow from femurs of BALB/c mice was flushed and cells were cultured at a density of 2 x 10^5/ml in complete RPMI (RPMI1640 medium, 4 mM L-glutamine, 5 x 10^{-5} M β-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.1 mM nonessential amino acids) supplemented with 20 %(vol/vol) culture supernatant from 7-day pokeweed mitogen (Sigma Aldrich NV/SA, Axel, The Netherlands)-stimulated BALB/c splenocytes. Medium was refreshed once per week. For all experiments 4-6 weeks old BMMC were used.

Immobilization of fibronectin

NUNC maxisorp 96 well plates were incubated for 3 hours at 37°C with 2.5 mg/ml human fibronectin (CLB, Amsterdam, The Netherlands) in Tris-buffered saline (TBS, 2.9 g/L Tris-Cl, 4 g/L NaCl, 0.2 g/L KCl, 0.4 g/L glucose, 0.1 % BSA), 200 μl/well, washed 3 times with 200 μl/well PBS, and used for cell adhesion experiments.
Polystyrene beads (mean diameter 11.9 μm, Sigma Aldrich NV/SA, Axel, The Netherlands) were incubated for 1 hour at room temperature with 2.5 mg/ml human fibronectin (CLB, Amsterdam, The Netherlands) or in TBS containing 1% BSA (Sigma Aldrich NV/SA, Axel, The Netherlands) as control for non-adhered cells, under constant rotation, followed by three times washing with an excess volume of TBS and used for mast cell degranulation experiments.

Preparation of the cells for degranulation and adhesion

Sensitization of BMMC was performed by incubation of the cells with culture supernatant of hybridoma 26.82 containing anti-dinitrophenyl (DNP)-specific IgE[98], optimal concentration was empirically determined, data not shown) for 1 hour at 37°C. Subsequently cells were washed once with PBS, once with 1 mM EDTA in PBS at 37°C, and once with TBS and resuspended in TBS for experimental use.

In vitro adhesion of BMMC

For adhesion to a fibronectin-coated 96 well plate, nonsensitized BMMC were prepared as described , and 5 x 10^4 cells per well were incubated with 0.2 mM Mn^{2+}, 3.0 mM Zn^{2+}, 3.0 mM Ni^{2+} or 2.0 mM Co^{2+} in TBS with or without 1 mg/ml soluble RGD (Sigma Aldrich NV/SA, Axel, The Netherlands) for 30 minutes on ice to synchronize adhesion. Subsequently, the 96 well plate was transferred to 37°C and after 30 minutes non-adhered cells were removed by washing of the plate.
For adhesion to beads, BMMC were prepared as described, stimulated with 0.2 mM Mn^{2+}, 3.0 mM Zn^{2+}, 3.0 mM Ni^{2+} or 2.0 mM Co^{2+} at a density of 7.5 x 10^6 cells/ml in TBS with or without 1 mg/ml soluble RGD, mixed with a pellet of fibronectin- or BSA coated beads (beads:cells ratio = 1:1) and centrifuged briefly to pellet the cell/bead mixture.

The cell/bead pellet was incubated at 37°C for 5 minutes, gently resuspended and incubated for an additional 25 minutes at 37°C.

Quantification of adhesion

Adhesion to fibronectin-coated 96 well plates, was quantified using Cyquant proliferation assay kit (Molecular Probes Europe B.V., Leiden, The Netherlands) according to the manufacturer's protocol. In short, samples were frozen overnight at -20°C, thawed and incubated for 60 minutes under continuous agitation at room temperature with a DNA-specific fluorescent dye and lysing reagent. Fluorescence was measured at $\lambda_{ex.}$: 485nm/$\lambda_{em.}$: 530nm using a Millipore Cytofluor 2350 microplate reader and adhesion was calculated as the residual fluorescence as percentage of input fluorescence.

Quantification of *in vitro* mast cell degranulation

As parameter for *in vitro* mast cell degranulation, the release of granular β-hexosaminidase was measured. Per volume of the BMMC/bead mixture, 14 volumes of Tyrode's buffer (0.1 M Tyrode salts: Life Technologies BV, Breda, The Netherlands; 100 mM HEPES/ 0.1 % $NaHCO_3$, pH 7.2; supplemented with 0.1 % BSA) were added to supplement extracellular Ca^{2+}. Per sample, 5 x 10^4 cells of the cell/bead mixture were stimulated with 0-100 ng/ml DNP-HSA (Sigma Aldrich NV/SA, Axel, The Netherlands). After 30 minutes at 37°C, 50 µl of supernatant of each sample was collected and samples of 5 x 10^4 unstimulated cells of each treatment-group were lyzed using 0.1 % NP-40 (Omnilabo International, Breda, The Netherlands) in order to quantify the total β-hexosaminidase activity present in these cells. Samples were analyzed for β-hexosaminidase activity as described previously[84]. In short, samples were incubated for 60 minutes with 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (4-MUG, Sigma Aldrich NV/SA, Axel, The Netherlands) and the reaction was stopped with 0.2 M glycin buffer (pH 10.7). 4-MUG hydrolysis was determined by fluorimetric measurement (λ_{ex}: 360nm/λ_{em}: 452nm) using a Millipore Cytofluor 2350 microplate reader. The percentage of β-hexosaminidase released, was calculated as follows:100% x $fluorescence_{supernatant}$/$fluorescence_{cell\ lysate}$ corrected for the β-hexosaminidase activity present in the supernatant of nonchallenged cells incubated with BSA-coated beads, representing nonadhered cells.

Quantification of *in vivo* mast cell degranulation

As parameter for *in vivo* mast cell degranulation we measured plasma leakage induced by a modified protocol for passive cutaneous anaphylaxis.
In vitro treatment of cells: BMMC were sensitized or not, prepared as described for *in vitro* adhesion and incubated at a density of 2.5 x 10^6 cells/ml in suspension with 0.2 mM Mn^{2+}, with or without 1 mg/ml soluble RGD, for 30 minutes at 37°C (see graph legends).
Passive cutaneous anaphylaxis: 15 minutes before the start of the experiment, mice were anaesthetized with sodium pentobarbitone (30 mg/kg, i.p.) and shaved dorsally. At t = 0 minutes, *in vitro* treated BMMC (5 x 10^4 cells, 20 µl per patch) and Evans blue dye (EB, 12.5 mg/ml, 50 µl, i.v.) were injected intradermally. The site of injection of each sample of cells was varied between mice to exclude site-dependent effects. After 30 minutes DNP-HSA (20 mg/ml, 50 µl, i.v.) was injected and after additional 30 minutes mice were sacrificed. Circular skin patches (9 mm in diameter) were excised and blood samples were taken. EB was extracted from skin samples by incubation in 0.5 ml formamid for 5 days at 50°C. The amount of EB in extracts and plasma samples

was quantified by measuring the OD at 595 nm using a Biorad Benchmark Microplate reader, corrected for its dilution in formamid, and extravasation was calculated resulting in μl extravasated plasma per skin patch.

Statistical analysis

All data are expressed as mean ± SEM. Statistically significant differences were determined using ANOVA followed by a Bonferroni post-hoc or Student's *t*-test considering $p<0.05$ to be significant. All analyses were performed using Systat for Windows (version 6.1, SPSS Inc., 1996).

Results

Effect of adhesion to fibronectin on degranulation of BMMC

To investigate the effect of Mn^{2+}-induced adhesion on degranulation, anti–DNP IgE-sensitized BMMC were prepared for adhesion by removal of extracellular Ca^{2+} with EDTA. Additionally cells were stimulated with 0.2 mM Mn^{2+} to convert VLA-5 to high-affinity and incubated with fibronectin- or BSA-coated beads. After 30 minutes, extracellular $Ca^{2+}$was supplemented, cells were antigenically challenged with various concentrations of DNP-HSA and β-hexosaminidase release was determined. As is shown in figure 1A, incubation of BMMC with

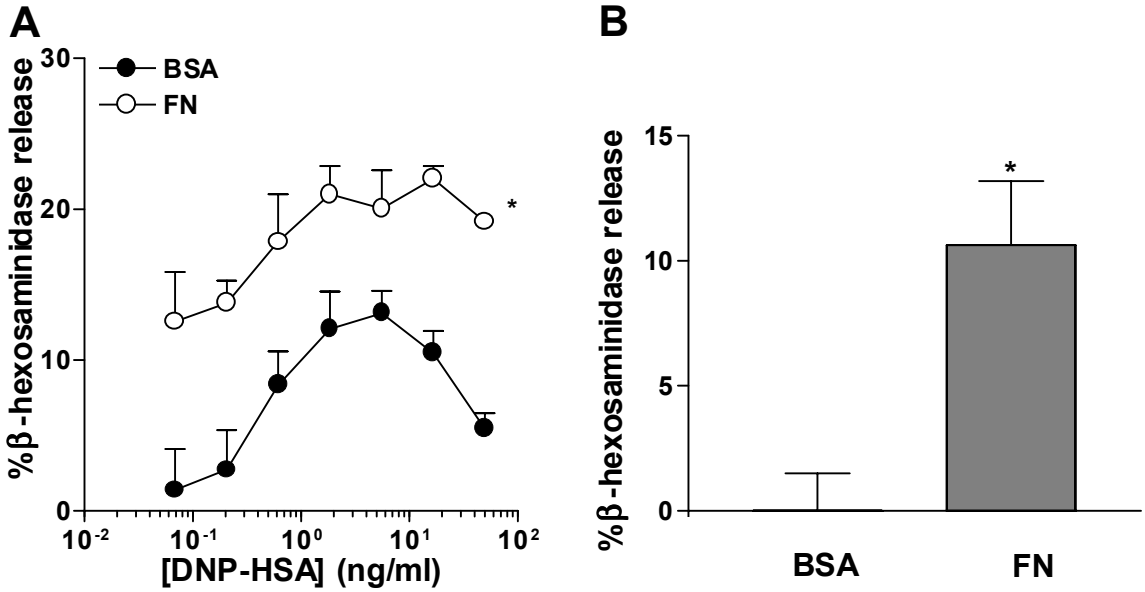


Figure 1. Effect of Mn^{2+}-induced adhesion to fibronectin on degranulation of BMMC. *Anti-DNP IgE-sensitized BMMC were prepared for adhesion, stimulated with 0.2 mM Mn^{2+}, incubated with fibronectin (FN)- or BSA-coated polystyrene beads and after 30 minutes extracellular Ca^{2+} was supplemented (final concentration: 1.8 mM). Cells were challenged **(A)** with various concentrations of DNP-HSA or **(B)** with buffer only. After 30 minutes β-hexosaminidase release was measured. β-hexosaminidase release was corrected for the β-hexosaminidase content in the supernatant of cation-stimulated nonchallenged BMMC incubated with BSA-coated beads. Means ± SEM, n=4 of a representative experiment are shown, * $p<0.05$, by (A) ANOVA followed by Bonferroni post hoc (B) Student's t-test, compared to cells incubated with BSA-coated beads.*

fibronectin-coated beads significantly enhances allergen-induced degranulation of these cells compared to cells incubated with BSA-coated beads. Figure 1B shows that adhesion of BMMC to fibronectin-coated beads itself significantly induces β-hexosaminidase release of these cells even when an antigenic stimulus is absent. From these data we conclude that adhesion of BMMC to fibronectin not only enhances allergen-induced degranulation but also directly triggers mast cell degranulation by an allergen-independent mechanism.

Effect of soluble RGD on adhesion-induced degranulation

VLA-5 plays a role in adhesion of BMMC to fibronectin and its function can be blocked by soluble RGD peptide[87]. To assess a role for VLA-5 in our model, we tested the effect of soluble RGD on adhesion-induced BMMC-activation. Nonsensitized BMMC were prepared for adhesion as described, stimulated with Mn^{2+} and incubated for 30 minutes with BSA-coated beads or with fibronectin-coated beads with or without 1 mg/ml soluble RGD and β-hexosaminidase release was determined. As is shown in figure 2, degranulation triggered by Mn^{2+}-induced adhesion to fibronectin can be completely blocked by soluble RGD. Soluble RGD itself did not affect degranulation (data not shown) From these data we conclude that degranulation triggered by adhesion to fibronectin is mediated by VLA-5.

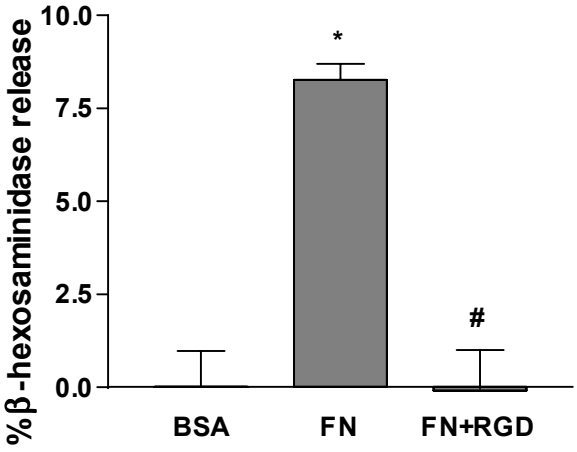


Figure 2. Effect of soluble RGD on adhesion-induced degranulation of BMMC. *BMMC were prepared for adhesion, stimulated with 0.2 mM Mn^{2+} and incubated for 30 minutes with BSA-coated polystyrene beads **(BSA)** or with fibronectin-coated polystyrene beads in the absence **(fibronectin)** or presence **(fibronectin+RGD)** of 1 mg/ml soluble RGD. Extracellular Ca^{2+} was supplemented and after 30 minutes β-hexosaminidase release was measured. Means ± SEM, n=4 of a representative experiment are shown. * p<0.05 by Student's t-test, compared to cells incubated with BSA-coated beads; # p<0.05 by Student's t-test, compared to cells incubated with fibronectin-coated beads in the absence of soluble RGD.*

Cation-induced adhesion and degranulation of BMMC

Our data show that Mn^{2+} is able to induce adhesion of mast cells to fibronectin and subsequently trigger degranulation. This raised the question if other cations from metals that are known for their ability to induce allergic contact dermatitis, such as zinc, nickel and cobalt have the potency to induce adhesion and thereby evoke degranulation. BMMC were prepared for adhesion and incubated with Mn^{2+} (0.2 mM) or Zn^{2+}, Ni^{2+} or Co^{2+} (0.1-10 mM) on a fibronectin-coated 96 well plate, which allows us to remove nonadhered cells by washing and to quantify adhesion. Figure 3 shows the adhesion of BMMC to fibronectin triggered by the optimal concentration of each cation. Mn^{2+} (0.2 mM), Zn^{2+} (3 mM), Ni^{2+} (3mM) and Co^{2+} (1 mM) are able to significantly induce adhesion of BMMC to fibronectin. Additionally, as shown in figure 3A, the adhesion induced

by each of these cations can be blocked by soluble RGD. This finding shows that, similar to Mn^{2+}, adhesion to fibronectin induced by these cations is mediated by VLA-5.
To test if adhesion of BMMC to fibronectin induced by Zn^{2+}, Ni^{2+} or Co^{2+} leads

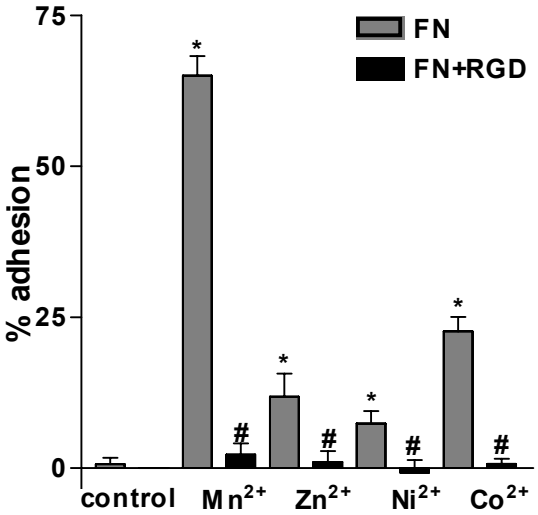


Figure 3. Adhesion of BMMC to fibronectin induced by divalent cations. *BMMC were prepared for adhesion, stimulated with 0.2 mM Mn^{2+}, 3 mM Zn^{2+}, 3 mM Ni^{2+} or 1 mM Co^{2+} or no cation (control) and incubated on a fibronectin (FN)-coated 96 well plate, with (FN+RGD) or without (FN) soluble RGD, 30 minutes on ice to synchronize adhesion, and 30 minutes at 37°C. Nonadhered cells were removed by washing and adhesion was quantified. Means ± SEM, n=4 of a representative experiment are shown. * p<0.05 by Student's t-test, compared to control.; # p<0.05 by Student's t-test, compared to cation-stimulated cells incubated in the absence of soluble RGD.*

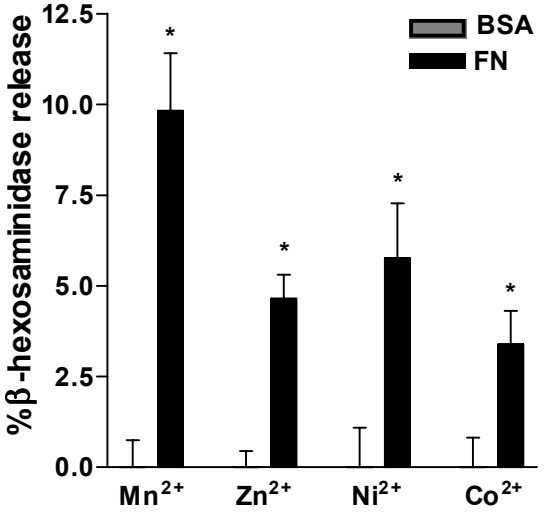


Figure 4. Degranulation of BMMC triggered by adhesion to fibronectin induced by divalent cations. *BMMC were prepared for adhesion, stimulated with 0.2 mM Mn^{2+}, 3 mM Zn^{2+}, 3 mM Ni^{2+} or 1 mM Co^{2+} and incubated for 30 minutes with fibronectin (FN)- or BSA-coated polystyrene beads. Extracellular Ca^{2+} was supplemented and after 30 minutes β-hexosaminidase release was measured. Means ± SEM, n=4 of a representative experiment are shown. * p<0.05 by Student's t-test, compared to cells incubated with BSA-coated beads.*

to mast cell degranulation, nonsensitized BMMC were prepared for adhesion, stimulated with optimal concentrations of Mn^{2+}, Zn^{2+}, Ni^{2+} or Co^{2+}, incubated with BSA- or fibronectin-coated polystyrene beads and β-hexosaminidase release was determined. Figure 4 shows that, in addition to Mn^{2+}, adhesion of BMMC to fibronectin induced by Ni^{2+}, Co^{2+} or Zn^{2+} triggers significant mast cell degranulation compared to cells incubated with BSA-coated beads. From these data we conclude that in addition to Mn^{2+}, Ni^{2+}, Co^{2+} and Zn^{2+} induce VLA-5 mediated adhesion of BMMC to fibronectin and subsequently induce degranulation of these cells.

Effect of Mn^{2+}-stimulation on mast cell function *in vivo*

Our data show induction of adhesion by cations and subsequent triggering of degranulation of BMMC in an *in vitro* setup, where cells are incubated in an artificial microenvironment. To assess the relevance of our findings for mast cell physiology we extrapolated our model to an *in vivo* setup. BMMC of BALB/c

origin were used in a modified protocol for passive cutaneous anaphylaxis, in which *in vitro* treated cells are locally injected in the skin of BALB/c mice providing a physiological microenvironment for these cells. Because Mn^{2+} has the highest potency to induce adhesion of BMMC to fibronectin and subsequent degranulation *in vitro* according to our data, this cation was used for our *in vivo* investigations.

Four groups of BMMC were treated *in vitro* (see also legend figure 5): two groups were sensitized with anti-DNP IgE, all four groups were treated with EDTA to remove extracellular Ca^{2+} and finally one nonsensitized and one sensitized group were stimulated for 30 minutes with Mn^{2+}. Mice were injected intradermally at different sites with 5 x 10^4 cells of each group and simultaneously injected intravenously with Evans Blue dye (EB). Subsequently, after 30 minutes mice were challenged by intravenous injection with 1 mg DNP-HSA to trigger sensitized BMMC and analyzed for EB extravasation (figure 5B) after another subsequent 30 minutes. As is shown in figure 5A, at the site of sensitized BMMC significant plasma leakage occurred (Mn^{2+}/IgE: -/+) compared to untreated cells (Mn^{2+}/IgE: -/-), which indicates that this method evokes an identical response as the classical passive cutaneous anaphylaxis protocol. Significant plasma leakage was also observed at the site of Mn^{2+}-stimulated nonsensitized BMMC (Mn^{2+}/IgE: +/-), although somewhat lower than the leakage induced by allergen. This response was already visible macroscopically before the mice were challenged with antigen (data not shown), and indicates IgE-independent activation of these cells. In all our *in vivo* experiments we observed a trend, where stimulation with Mn^{2+} enhanced

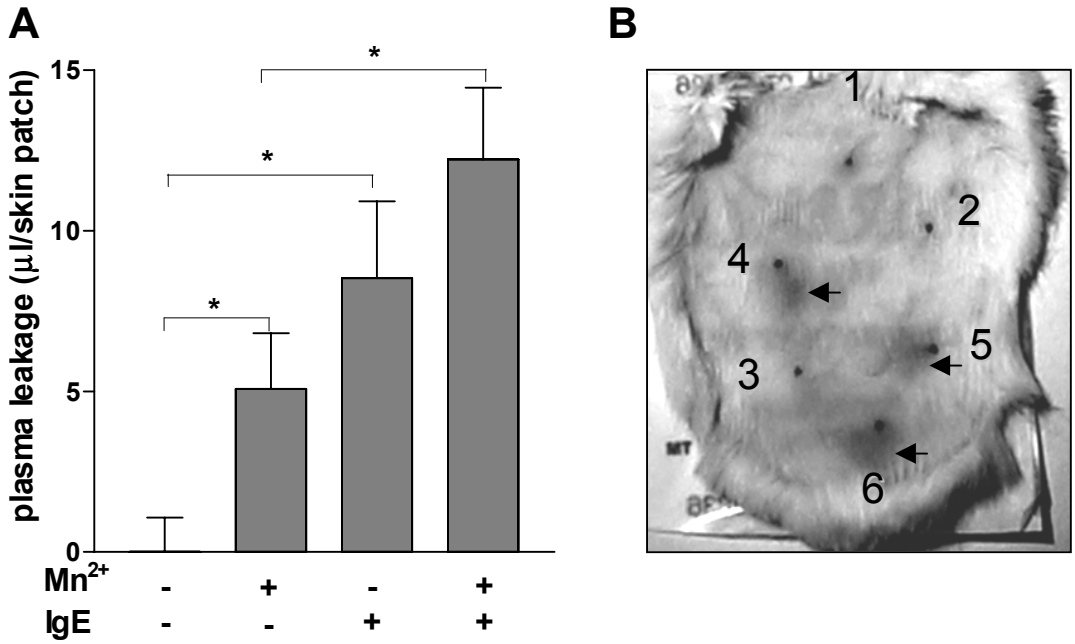


Figure 5. In vivo mast cell activation induced by Mn^{2+}-stimulation and antigenic challenge. (A) *Naive BALB/c mice were injected intradermally with in vitro-treated BMMC (see legend, 0.2 mM Mn^{2+}, anti-DNP IgE), and simultaneously injected intravenously with Evans blue dye (EB). After 30 minutes, mice were antigenically challenged intravenously with 1 mg DNP-HSA and after additional 30 minutes mice were sacrificed and EB extravasation at the sites of injected cells was quantified. Blood samples were analyzed for EB content and used for the calculation of µl extravasated plasma per skin patch. Mean ± SEM, n=6 of a representative experiment are shown. * p<0.05 by Student's t-test.* ***(B)*** *Photograph of a representative example of a stripped dorsal mouse skin, arrows indicate EB extravasation. Injected samples: 1. no injection; 2. TBS; 3. IgE/Mn^{2+} -/-; 4. IgE/Mn^{2+} -/+; 5. IgE/Mn^{2+} +/-; 6. IgE/Mn^{2+} +/+.*

allergen-induced mast cell degranulation (Mn^{2+}/IgE: +/+) compared to cells stimulated with allergen alone (Mn^{2+}/IgE:-/+), however this effect was never significant.
From these data we conclude that, parallel to our *in vitro* results, Mn^{2+}-induced adhesion evokes mast cell degranulation and tends to enhance allergen-induced degranulation.

Effect of soluble RGD on the function of Mn^{2+}-stimulated cells *in vivo*
To show that the observed *in vivo* effects are indeed the result of adhesion to fibronectin through VLA-5 and exclude a direct effect of Mn^{2+} on skin reactivity, we tested the effect of soluble RGD in our *in vivo* setup. Five groups of cells received *in vitro* treatment (see also legend of figure 6): two groups were sensitized with anti-DNP IgE, all five groups were treated with EDTA to remove extracellular Ca^{2+} and finally a sensitized and an nonsensitized group were stimulated for 30 minutes with Mn^{2+} alone or with Mn^{2+} and soluble RGD. Subsequently, the function of these cells was tested *in vivo* as described before. As is shown in figure 6, *in vivo* mast cell activation by Mn^{2+}-stimulation (Mn^{2+}/IgE/RGD: +/-/-) is almost abolished by soluble RGD (Mn^{2+}/IgE/RGD: +/-/+). Soluble RGD also largely attenuated leakage induced by BMMC that were stimulated with Mn^{2+} and challenged with allergen, shown by (Mn^{2+}/IgE/RGD: +/+/+) compared to (Mn^{2+}/IgE/RGD: +/+/-). This suggests that inhibition of VLA-5 function also affects allergen-induced degranulation. These data show that Mn^{2+} stimulation of BMMC induces degranulation of mast cells *in vivo* by a VLA-5-dependent mechanism, and suggest that the inhibition of VLA-5 function also affects FcεRI-induced degranulation.

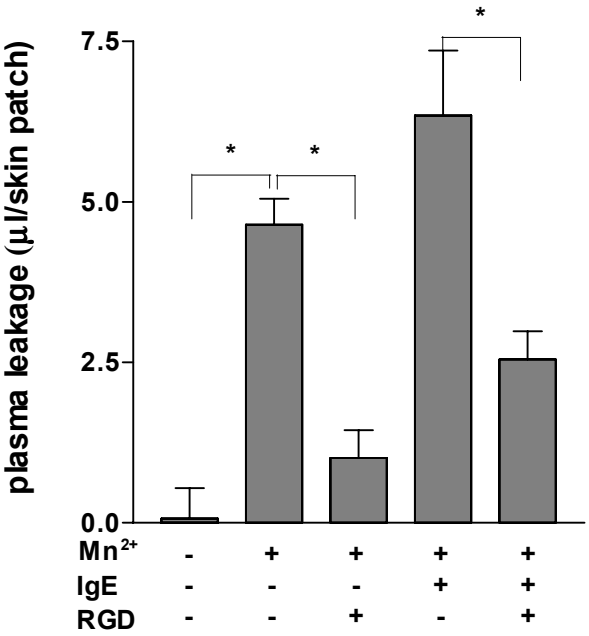


Figure 6. Effect of soluble RGD on in vivo mast cell activation induced by Mn^{2+}-stimulation and antigenic challenge. *Naive BALB/c mice were injected intradermally with in vitro-treated BMMC (see legend), and simultaneously injected intravenously with EB. After 30 minutes, mice were antigenically challenged intravenously and after additional 30 minutes mice were sacrificed and EB extravasation at the sites of injected cells was quantified. Blood samples were analyzed for EB content and used for the calculation of µl extravasated plasma per skin patch. Mean ± SEM, n=6 of a representative experiment are shown. * p<0.05 by Student's t-test.*

Discussion

Understanding of VLA-5-mediated modulation of mast cell function is potentially useful for the development of future therapy against mast cell-mediated diseases and therefore topic of our investigations. In this report we show that adhesion of BMMC to fibronectin enhanced FcεRI-induced degranulation *in vitro*, which is according to previous data obtained with RBL-2H3 cells[62,64,131,155]. Surprisingly, we observed that in absence of an antigenic stimulus, adhesion of BMMC to fibronectin triggered the degranulation of these cells. Our data show that adhesion-induced degranulation as well as adhesion-enhanced FcεRI-mediated degranulation *in vitro* is mediated by VLA-5, the integrin that was previously identified as key receptor for mast cell adhesion to fibronectin[45,87]. The use of BMMC derived from Balb/c origin enables us to study the function of these cells after *in vitro* treatment in their physiological microenvironment, thereby excluding, for example, possible intrinsic effects of fibronectin density[170], coated to beads in our *in vitro* setup. The data obtained from our *in vivo* experiments confirm the conclusions drawn from our *in vitro* experiments stating that cation-induced adhesion of BMMC through VLA-5 triggers degranulation of these cells. The effect of Mn^{2+}-induced adhesion on FcεRI-induced degranulation did not reach statistical significance when compared to cells stimulated with allergen alone in our *in vivo* experiments, although a trend for enhancement was observed. This may be explained by a limitation of plasma leakage per area of skin and therefore nonlinearity with mast cell degranulation at sites of strong activation.

Activation of integrins leading to adhesion and migration of various cells can be induced by a range of mediators such as interleukins[79,141], chemokines[99,171] and growth factors such as SCF[13,36,167]. Our findings show that VLA-5-mediated adhesion can augment FcεRI-induced degranulation. This could be the result of a lowering of the activation threshold of FcεRI. This implies that by local induction of adhesion, for example at sites of tissue inflammation, mast cells can be primed for antigenic stimulation by their microenvironment. Our data suggest that *in vivo* blocking of VLA-5 attenuates allergen-induced degranulation, which implies that by prevention of mast cell adhesion these cells become less sensitive for antigenic stimulation. This conclusion is supported by previous data showing that passive cutaneous anaphylaxis is blocked by injection of anti-VLA-5 antibody[177]. In *in vitro* experiments this phenomenon will not be observed, because the availability of allergen for a sensitized mast cell is virtually unlimited, therefore the activation threshold can always be exceeded. However, *in vivo* allergen concentrations reaching the local sensitized mast cells is limited and therefore the mast cell will no longer be optimally activated because the priming effect of VLA-5 is lost. On resting mast cells located in uninflamed tissues, VLA-5 will be present in a low-affinity state and therefore these cells, although sensitized, will not be primed for an antigenic challenge. Vice versa it has been demonstrated that FcεRI-stimulation induces VLA-5 mediated adhesion to fibronectin[87]. In our experiments we find that the optimal allergen concentration needed for mast cell adhesion to fibronectin is, at least tenfold, lower than for degranulation (unpublished observations). VLA-5 on nonprimed mast cells could therefore be initially converted to high-affinity by low concentrations of allergen, subsequently

inducing adhesion to fibronectin and priming of the cells for antigenic challenge, via this 'positive-feedback' mechanism degranulation and adhesion can be mutually enhanced.
In case of optimal stimulation of VLA-5, which in our experiments is induced by Mn^{2+}-stimulation the FcεRI activation threshold appears to be exceeded spontaneously and degranulation is induced without the presence of an antigenic stimulus. As discussed earlier, integrin-mediated adhesion can be induced by various inflammatory mediators and therefore optimal stimulation of VLA-5 i*n vivo* may easily occur in chronically inflamed tissues or at sites of severe anaphylaxis where inflammatory mediators are abundantly present[2,90,101,137].
In our experiments, we find that various divalent cations are able to induce mast cell adhesion and subsequent degranulation in absence allergen. In literature there are numerous examples of contact hypersensitivity responses towards manganese, zinc, nickel and cobalt[9,57,92,119,148], however the underlying mechanisms are poorly understood. Our data suggest a plausible explanation for hypersensitivity responses to various metals. These responses could very well be the result of cation-induced high-affinity of VLA-5 on local mast cells; thereby inducing adhesion to fibronectin and subsequent triggering of allergic mediator release from mast cells.
In summary, we have shown that VLA-5-mediated adhesion of mast cells to fibronectin primes these cells for antigenic stimulation and that prevention of adhesion reduces allergen-induced degranulation, which indicates possible future targets for therapy against allergy. Furthermore, our data show that cations can induce VLA-5-mediated adhesion and subsequent activation of mast cells, providing a possible mechanism for allergic reactions to metals *in vivo.*

VLA-5-mediated adhesion of bone marrow-derived mast cells to fibronectin triggers FcεRI signaling and subsequent degranulation

(Submitted for publication)

René Houtman, Jeanette H. W. Leusen†, Janine Schuurman, Frank A. Redegeld, Andries S. Koster and Frans P. Nijkamp

†Immunotherapy Laboratory, Department of Immunology, University Medical Center Utrecht, The Netherlands

Abstract

Adhesion to fibronectin through very late antigen-5 (VLA-5) enhances allergen-induced mast cell degranulation in RBL-2H3 cells. In the present study, we use mast cells derived from murine bone marrow (BMMC) to study this effect in more detail. Adhesion of BMMC was induced through Mn^{2+}-stimulation, which converts VLA-5 into a high-affinity conformation for fibronectin. Our data show that adhesion not only enhances allergen-induced degranulation but also directly triggers degranulation in absence of allergen. Absence of FcεRI-γ did not affect adhesion but abrogated adhesion-induced degranulation. Furthermore, degranulation and confocal microscopy indicated membrane colocalization of VLA-5 and FcεRI on resting BMMC. Adhesion of BMMC to fibronectin-coated beads induced accumulation of FcεRI-γ and Syk with the cell fraction associated with these beads, suggesting association with focal adhesion complexes. Moreover adhesion and allergen stimulation induced an identical protein tyrosine phosphorylation pattern.This indicates that both stimuli trigger the same signal transduction cascade. Disruption of rafts by cholesterol depletion, inhibited adhesion as well as adhesion-induced degranulation, while ionomycin-induced degranulation was not affected. This indicates a role for these membrane microdomains in VLA-5 function. In summary, our data suggest that adhesion of BMMC to fibronectin induces clustering of VLA-5 and physically associated FcεRI at the cell-substrate interface. This aggregation of FcεRI and possible association with rafts could induces the initiation of the FcεRI signaling cascade and subsequent degranulation. Regulation of the degree of adhesion and subsequent (pre-)clustering of FcεRI could thereby serve as a modulating mechanism for allergen-induced mast cell activation.

Introduction

Mast cells are localized in all vascularized tissues and play an important role in hypersensitivity type I responses[147]. Antigenic stimulation of these cells via the high affinity receptor for IgE (FcεRI)[i] triggers a signal transduction cascade leading to the release of various inflammatory mediators, which cause clinical phenomena associated with allergy[12,14,59].

Adhesion of mast cells to extracellular matrix component fibronectin enhances allergen-induced degranulation[62,64,131,155], and it has been established that interaction of mast cells with fibronectin is mediated by the $\alpha_5\beta_1$-integrin very late antigen-5 (VLA-5)[150,177]. Interestingly, we recently found that adhesion of murine bone marrow-derived mast cells (BMMC) to fibronectin, induced by conversion of VLA-5 to high affinity with Mn^{2+} [7,116,118], triggers degranulation in absence of an antigenic stimulus. The mechanism by which adhesion itself induces degranulation and modulates allergen-induced degranulation is not yet understood and is topic of this report.

Aggregation of FcεRI by binding of multivalent allergen to FcεRI-bound IgE causes this receptor to associate rapidly with rafts, which are specialized cholesterol-enriched plasma membrane regions[47-49,152]. Subsequently, active protein tyrosine kinase (PTK) Lyn, enriched within rafts, phosphorylates the tyrosine residues of the immunoreceptor tyrosine-based activation motifs (ITAM) located in the cytoplasmic tails of the FcεRI β- and γ-subunits[161]. Phosphorylation of the FcεRI-γ ITAMs leads to the recruitment, tyrosine phosphorylation and activation of cytosolic Syk, which has been shown to be critical for the induction of degranulation[32,132,161]. Disruption of rafts by cholesterol depletion impairs the interaction of aggregated FcεRI and Lyn and attenuates initiation of the FcεRI signal transduction cascade in RBL-2H3 mast cells[143].

Clustering of integrins by binding to extracellular matrix components, induces the formation of focal adhesion complexes (at the locus of cell-matrix attachment[22,33,100]. Integrin-mediated modulation of signaling of the growth factor receptor family has been well documented[27,113,135] and several reports suggest that this modulation takes place within the focal adhesion complexes where these receptors are physically associated with integrins[126,153]. Considering the similarities between FcεRI and growth factor receptors[63,136,161], we investigated whether modulation of degranulation by VLA-5 may also take place in the focal adhesion complexes by physical interaction between this integrin and FcεRI.

In this report we have investigated if FcεRI plays a role in adhesion-induced degranulation of BMMC. Furthermore we studied the distribution of FcεRI and VLA-5 on these cells, analyzed the contents of the insoluble fraction of adhered and nonadhered cells for the presence of FcεRI signaling components, and propose a possible mechanism for VLA-mediated modulation of FcεRI signaling.

[i] For abbreviations see page 101

Materials & Methods

Animals

6-8 Weeks old male BALB/c mice were obtained from Central Animal Laboratory (GDL), Utrecht University, Utrecht, The Netherlands. FcεRI-γ knock out mice[164] were kindly provided by Prof. T. Saito, Div. Mol. Genet., Chiba Univ. Grad. Sch. Med., Chiba, Japan. The experiments were approved by the Animal Care Committee of the Utrecht University.

Cells

BMMC were obtained as described previously[84]. Briefly, bone marrow from mouse femurs, was flushed and cells were cultured at a density of 2 x 10^5/ml in complete RPMI (RPMI 1640 medium, 4 mM L-glutamine, 5 x 10^{-5} M β-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.1 mM nonessential amino acids) supplemented with 20 %(vol/vol) supernatant from BALB/c splenocytes stimulated for 7 days with pokeweed mitogen (Sigma Aldrich NV/SA, Axel, The Netherlands). Medium was refreshed once per week. In all experiments 4-6 weeks old BMMC were used.

Immobilization of fibronectin

NUNC maxisorp 96-well plates were incubated for 3 hours at 37°C with 2.5 mg/ml human fibronectin (C.L.B., Amsterdam, The Netherlands) in Tris-buffered saline (TBS, 2.9 g/L Tris-Cl, 4 g/L NaCl, 0.2 g/L KCl, 0.4 g/L glucose, 0.1 % BSA), 200 μl/well, washed 3 times with 200 μl/well PBS, and used for cell adhesion experiments.
Polystyrene beads (average diameter 11.9 μm, Sigma Aldrich NV/SA, Axel, The Netherlands) were incubated for 1 hour at room temperature with 2.5 mg/ml human fibronectin (CLB, Amsterdam, The Netherlands) or 1% BSA (Sigma Aldrich NV/SA, Axel, The Netherlands) in TBS as control for non-adhered cells, under constant rotation, followed by three times washing with an excess volume of TBS and used for mast cell degranulation experiments.

Cholesterol depletion of cells

BMMC were washed twice with complete RPMI without FCS, and incubated at a density of 2.5 x 10^6 cells/ml for 30 minutes at 37°C with 10 mM methyl-β-cyclodextrin (Sigma Aldrich NV/SA, Axel, The Netherlands) in complete RPMI. Subsequently, cells were prepared for adhesion.

Preparation of cells for degranulation and adhesion

Sensitization of BMMC was performed by incubation of the cells with culture supernatant of hybridoma 26.82 containing dinitrophenyl(DNP)-specific IgE[98], for 1 hour at 37°C. For adhesion cells were washed once with PBS, once with 1 mM EDTA in PBS at 37°C, once with TBS and resuspended in TBS for experimental use.

Adhesion of BMMC to fibronectin

For adhesion on a fibronectin-coated 96-well plate, BMMC were prepared as described, 5 x 10^4 cells per well were incubated with 0.2 mM Mn^{2+} (the concentration that induces maximal adhesion, empirically determined) for 30 minutes on ice to

synchronize adhesion of the cells. Subsequently, the 96-well plate was transferred to 37°C and after 30 minutes non-adhered cells were removed by washing of the plate (three times, 200 μl/well).
For adhesion to beads, BMMC were prepared as described, incubated with 0.2 mM Mn^{2+}, mixed with a pellet of fibronectin- or BSA coated beads (beads:cells ratio = 1:1) and centrifuged briefly to pellet the cells and beads. The pellet was then incubated at 37°C for 5 minutes, gently resuspended and incubated for an additional 25 minutes at 37°C.

Quantification of adhesion

Adhesion to fibronectin-coated 96-well plates was quantified using Cyquant proliferation assay kit (Molecular Probes Europe BV, Leiden, The Netherlands) according to the manufacturer's protocol. In short, samples were frozen overnight at -20°C, thawed and incubated for 60 minutes under continuous agitation at room temperature with a DNA-specific fluorescent dye and lysing reagent. Fluorescence was measured at $\lambda_{ex.}$: 485nm/$\lambda_{em.}$: 530nm using a Millipore Cytofluor 2350 microplate reader and adhesion was calculated as the residual fluorescence as percentage of input fluorescence.

Measurement of degranulation

Per volume of the BMMC/bead mixture, 14 volumes of Tyrode's buffer (0.1 M Tyrode salts: Life Technologies BV, Breda, The Netherlands; 100 mM HEPES/ 0.1 % $NaHCO_3$, pH 7.2; supplemented with 0.1 % BSA) were added to supplement extracellular Ca^{2+}. Per sample, 5 x 10^4 cells of the cell/bead mixture were incubated with 10 ng/ml human serum albumin (HSA)-conjugated DNP (DNP-HSA, Sigma Aldrich NV/SA, Axel, The Netherlands) or with Tyrode's buffer alone, and after 30 minutes at 37°C 50 μl supernatant of each sample was collected. Samples of 5 x 10^4 unstimulated cells of each treatment-group were lyzed using 0.1 % NP-40 (Omnilabo International, Breda, The Netherlands) in order to quantify the total granular β-hexosaminidase activity present in these cells. Supernatants and lysates were analyzed for β-hexosaminidase activity as described previously[84]. In short, samples were incubated for 60 minutes at 37°C with 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (4-MUG, Sigma Aldrich NV/SA, Axel, The Netherlands) and the reaction was stopped with glycine buffer (0.2 M, pH 10.7). Hydrolysis of 4-MUG was determined by fluorimetric measurement (λ_{ex}: 360nm/λ_{em}: 452nm) using a Millipore Cytofluor 2350 microplate reader. The percentage of degranulation was calculated as follows:100% x $fluorescence_{supernatant}$/$fluorescence_{cell\ lysate}$. β-Hexosaminidase release was corrected for β-hexosaminidase activity found in the supernatant of nonchallenged BMMC incubated with BSA-coated beads. All graphs show mean values ± SEM of a representative of three experiment in which each condition was performed in quadruplicate.

Membrane expression of VLA-5

Cells were incubated for 30 minutes at 4°C with Fluorescein Isothiocyanate (FITC)-conjugated[103] anti-VLA-5 antibody, BMA5 (kindly provided by Dr B.M.C. Chan, Transplantation and Immunobiology group, Univ. Western Ontario, Canada)[45], in FACS buffer (0.1 % BSA, 0.1 % sodium azide in PBS), washed and resuspended in FACS buffer. Fluorescence was analyzed by flow cytometry using FACScan, and PC-lysis software.

Membrane distribution of FcεRI and VLA-5 on BMMC

BMMC were incubated in complete RPMI in suspension for 60 minutes at 37°C with FITC-conjugated anti-VLA-5 antibody, BMA5, and Indocabocyanine Cy3 (Cy3)-conjugated IgE (purified from 26.82 culture supernatant). Subsequently cells were washed twice with TBS, incubated for 30 minutes at 37°C on poly-L-lysine coated object slides, fixed for 20 minutes at room temperature with 4% paraformaldehyde and washed twice with PBS. Samples were mounted in Mowiol containing 2.5 % DABCO and examined with a 63x planapo objective on a Leitz DMIRB fluorescence microscope (Leica, Voorburg, The Netherlands) interfaced with a Leica TCS4D confocal laser microscope (Leica, Heidelberg, Germany) using the following filters: FITC, λ_{ex} = 488 nm and λ_{em} = 520 nm band pass; and Cy3, λ_{ex} = 568 nm and λ_{em} = 590 nm long pass. Images were imported and processed in Adobe Photoshop 4.0 (Adobe Systems Inc, San Jose, CA, USA).

Western blot analysis of bead-associated proteins

Mn^{2+}-stimulated BMMC were incubated for indicated time intervals with fibronectin- or BSA-coated beads as described above and, when indicated, antigenically challenged for 1 minute with 300 ng/ml DNP-HSA. Per sample, 5 x 10^6 cells were briefly centrifuged to pellet cells and beads, supernatant was discarded and cells were lysed with icecold lysis buffer (0.15 M NaCl, 50 mM, Tris, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM tetrasodium pyrophosphate, 0.5 mM sodiumfluoride, 1 mM PMSF, 1 % NP-40, pH 7.4, and Complete Mini™ protease inhibitors (Boehringer Mannheim BV, The Netherlands)) After 30 minutes incubation on ice, samples were centrifuged at 4°C 15.000 g.for 30 minutes and supernatants were discarded. Pellets were resuspended in 20 µl sample buffer (3 % SDS, 7.5 %(vol/vol) glycerol, 0.05 M Tris and 0.5 mg/ml bromophenol blue and 10 mM DTT) boiled for 5 minutes, separated on 12.5 % bis-/acrylamide gel (Bio-Rad Laboratories BV, Veenendaal, The Netherlands) and blotted on PVDF membrane (Costar Europe Ltd., Badhoevedorp, The Netherlands). Tyrosine phosphorylation was detected with horseradish peroxidase (HRP)-conjugated PY99 (Santa Cruz Biotechnology, Inc). FcεRI-γ was detected with a rabbit polyclonal antibody (a generous gift from Dr. M. -H. Jouvin, Beth. Israel Deaconess Med. Ctr., Boston, USA)[123] and Syk was detected with a rabbit polyclonal antibody (kindly donated by Dr. U Blank, Inst. Pasteur, Paris, France)[110] and HRP-conjugated horse anti-rabbit polyclonal (C.L.B., Amsterdam, The Netherlands) and visualized with Supersignal® Substrate (Pierce, Rockford IL, U.S.A.) and exposure to KODAK X-OMAT-AR film. Films were scanned and analyzed on a GS710 Calibrated Imaging Densitometer and Quantity One v. 4.0.3 software (both Bio-Rad Laboratories BV, Veenendaal, The Netherlands).

Results

Effect of Mn^{2+}-induced adhesion of BMMC to fibronectin on degranulation

To show that Mn^{2+}-induced adhesion of BMMC to fibronectin triggers degranulation and enhances FcεRI-induced degranulation, BMMC were sensitized with anti–DNP IgE, prepared for adhesion by removal of extracellular Ca^{2+} with EDTA, and stimulated with 0.2 mM Mn^{2+} to convert VLA-5 to its high-affinity state. Additionally, cells were incubated for 30 minutes at 37°C with fibronectin-coated beads or with BSA-coated beads as control for nonadhered

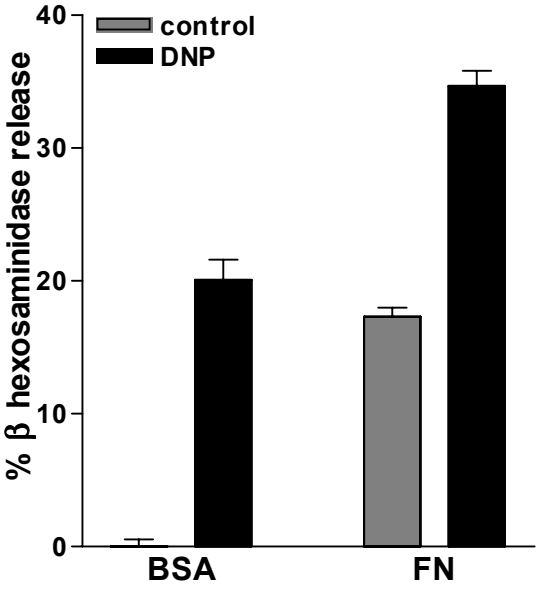


Figure 1. Effect of adhesion to fibronectin on β-hexosaminidase release of BMMC. *Anti-DNP IgE-sensitized BMMC were prepared for adhesion, stimulated with 0.2 mM Mn^{2+} and incubated for 30 minutes with fibronectin (**FN**)- or **BSA**-coated polystyrene beads. Subsequently, extracellular Ca^{2+} was supplemented (final concentration: 1.8 mM) and cells were antigenically challenged **(DNP)** or not **(control)** with 10 ng/ml DNP-HSA. After 30 minutes β-hexosaminidase release was quantified.*

cells. Extracellular Ca^{2+}, critical for degranulation[11], was supplemented, cells were antigenically challenged with DNP-HSA and β-hexosaminidase release as measure for mast cell degranulation was quantified. As is shown in figure 1, incubation of BMMC with fibronectin- but not BSA-coated beads induces release of β-hexosaminidase in the absence of allergen. Furthermore, adhesion to fibronectin enhances allergen-induced degranulation compared to cells incubate with BSA-coated beads. These results suggest that adhesion to fibronectin only generates sufficient signals to induce granule exocytosis from mast cells.

Adhesion of BMMC derived from FcεRI-γ knockout mice

To be able to test for a possible role for FcεRI, in adhesion-induced degranulation, we generated BMMC from mice lacking the γ-subunit of FcεRI due to targeted gene disruption, and characterized them. As is shown in figure 2A, the average VLA-5 expression on wild type and FcεRI-γ knock out BMMC, determined by FACS analysis, was found to be comparable. Additionally, we tested if an equivalent level of VLA-5 expression correlates with comparable adhesion of wild type and knock out BMMC to fibronectin. Cells were prepared for adhesion, stimulated with Mn^{2+} and incubated on a fibronectin-coated 96-well plate, a setup which allows us to remove nonadhered cells by washing and quantify adhesion. As is shown in figure 2B, Mn^{2+}-induced adhesion of BMMC to fibronectin is comparable between cells with and without FcεRI-γ. From these data we conclude that the γ-subunit of FcεRI is not required for VLA-5 mediated adhesion of BMMC to fibronectin.

Adhesion-induced degranulation of BMMC lacking FcεRI-γ

To investigate if the γ-subunit of FcεRI plays a role in degranulation triggered by adhesion of BMMC to fibronectin, wild type and knock out cells were prepared for adhesion, stimulated with Mn^{2+}, incubated with fibronectin- or BSA-coated beads and analyzed for β-hexosaminidase release. As is shown in figure 3, incubation of wild type BMMC with fibronectin- but not BSA-coated beads

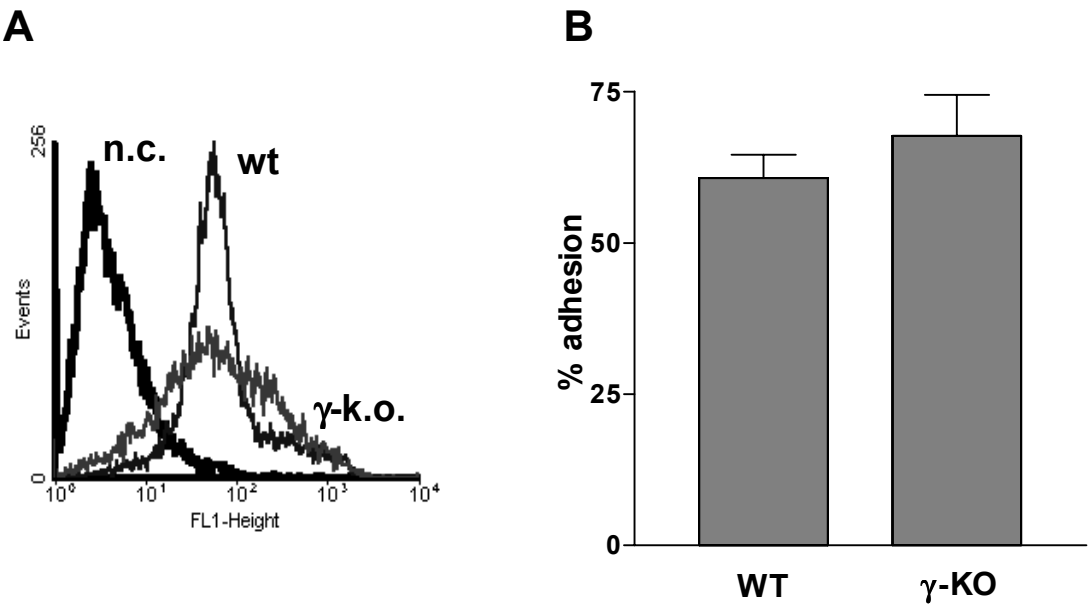


Figure 2. Characterization of BMMC derived from FcεRI-γ knock out mice. (A) *Histogram of VLA-5 expression of BMMC derived from wild type* ***(WT)*** *and FcεRI-γ knock out* ***(γ-KO)*** *mice, as determined by staining with FITC-conjugated anti-VLA-5 antibody BMA-5 and FACS analysis. Nonstained cells serve as negative control* ***(n.c.)****. **(B)** BMMC derived from wild type* ***(WT)*** *and FcεRI-γ knock out* ***(γ-KO)*** *mice were prepared for adhesion, stimulated with 0.2 mM Mn^{2+}, and incubated on a fibronectin-coated 96-well plate, 30 minutes on ice to synchronize adhesion, and 30 minutes at 37°C. Nonadhered cells were removed by washing and adhesion was quantified.*

triggers release of β-hexosaminidase, however in BMMC lacking the γ-subunit of FcεRI this phenomenon was not observed. Ionomycin-induced degranulation of wild type and knock out cells, bypassing the initial steps of the FcεRI-signaling cascade, was found to be comparable indicating that knock out BMMC are fully capable of degranulation (data not shown). From these data we conclude that FcεRI-γ plays a critical role in degranulation of BMMC induced by adhesion of these cells to fibronectin.

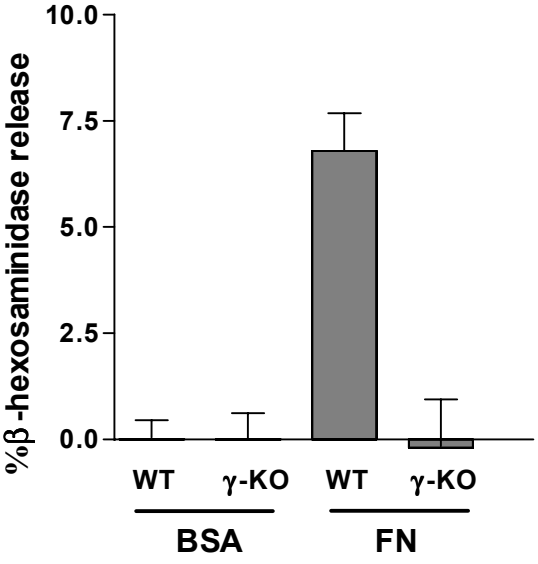


Figure 3. A role for FcεRI-γ in adhesion-induced β-hexosaminidase release of BMMC. *BMMC derived from wildtype* ***(WT)*** *and FcεRI-γ knock out* ***(γ-KO)*** *mice, were prepared for adhesion, stimulated with 0.2 mM Mn^{2+} and incubated for 30 minutes with fibronectin (**FN**)- or **BSA**-coated polystyrene beads. Subsequently, extracellular Ca^{2+} was supplemented (final concentration: 1.8 mM) and after 30 minutes β-hexosaminidase release was determined.*

Membrane distribution of FcεRI and VLA-5 on BMMC

One mechanism by which FcεRI-γ could play a role in adhesion-induced degranulation is through physical interaction between these proteins. We therefore investigated whether VLA-5 and FcεRI are colocalized on resting BMMC. These receptors were visualized by incubation of BMMC with TRITC-labeled IgE, to detect FcεRI, and FITC-labeled anti-VLA-5 antibody BMA5. Cells were fixed on poly-L-lysine-coated object slides and examined by confocal microscopy. As is shown in figure 4, the distribution of VLA-5, shown in green (left panel), largely corresponds with that of FcεRI, shown in red (middle panel), on BMMC. This is confirmed by overlaying the two pictures as shown in the right panel of figure 4, of which the yellow staining is indicative for codistribution on the plasma membrane. From these data we conclude that, within the spatial resolution of the confocal microscope (0.3 μm), VLA-5 and FcεRI are, at least partially, colocalized on the surface of BMMC.

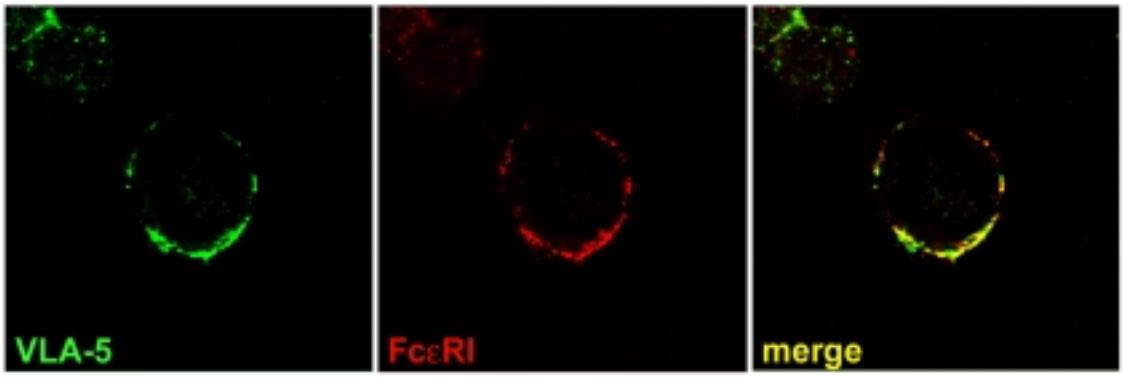


Figure 4. Distribution of FcεRI and VLA-5 on BMMC. *BMMC were stained in suspension with TRITC-conjugated IgE* ***(left panel)*** *and FITC-conjugated anti-VLA-5 antibody BMA5* ***(middle panel)****, fixed on object slides and analyzed by confocal microscopy. The obtained pictures were merged and resulting yellow signal indicated colocalization of the FITC- and TRITC-signal* ***(right panel)****.*

Effect of adhesion to fibronectin on the distribution of FcεRI signal transduction components

The observation that FcεRI colocalizes with VLA-5 raised the question if adhesion of these cells to fibronectin-coated beads induces a redistribution of FcεRI signal transduction components to the sites where VLA-5 is clustered. IgE-sensitized BMMC were prepared for adhesion, stimulated with Mn^{2+} and incubated with BSA- or fibronectin-coated beads. Next, cells were incubated in the absence or presence of DNP-HSA and subsequently lyzed, leaving a mix of detergent-soluble and -insoluble components. The latter fraction that contains cytoskeletal proteins and beads with -associated cell components, was isolated by centrifugation and boiled with SDS to disrupt this association. Subsequently, these samples were analyzed by Western blotting. As shown in figure 5A, incubation of cells with fibronectin-coated beads increases the amount of FcεRI-γ in the insoluble fraction of cells compared to cells incubated with BSA-coated beads. This suggests that FcεRI-γ associates with beads upon adhesion, probably within the focal adhesion complexes where interaction with fibronectin takes place. Antigenic stimulation of the cells increased the amount of FcεRI-γ

takes place. Antigenic stimulation of the cells increased the amount of FcεRI-γ in the insoluble fraction only in cells incubated with fibronectin-coated beads. This suggests that antigenic stimulation induces aggregation of additional, detergent-soluble or 'free', FcεRI with FcεRI that is already immobilized in focal adhesion complexes due to adhesion.
To investigate if adhesion of BMMC induces association of Syk with beads in adhered cells, we analyzed the insoluble fraction of adhered cells for Syk content by western blotting. As shown in figure 5B, adhesion to fibronectin also triggers a time-dependent redistribution of Syk to the insoluble fraction. This probably reflects the recruitment of Syk from the cytoplasm to phosphorylated focal adhesion complex-associated FcεRI-γ.
Additional analysis of the insoluble fractions of adhered and non-adhered cells shows that adhesion triggers the accumulation of various tyrosine phosphorylated proteins within the fraction of adhered cells (figure 5C). Interestingly, the pattern of phosphorylation induced by adhesion is similar to that found in antigenically challenged non-adhered cells. Furthermore, the highest degree of tyrosine phosphorylation is achieved in cells that are stimulated, both by adhesion and allergen. These data suggest that clustering of FcεRI in focal adhesion complexes, due to adhesion of BMMC to fibronectin, and clustering of this receptor by multivalent allergen can lead to the initiation of the same signal transduction cascade.

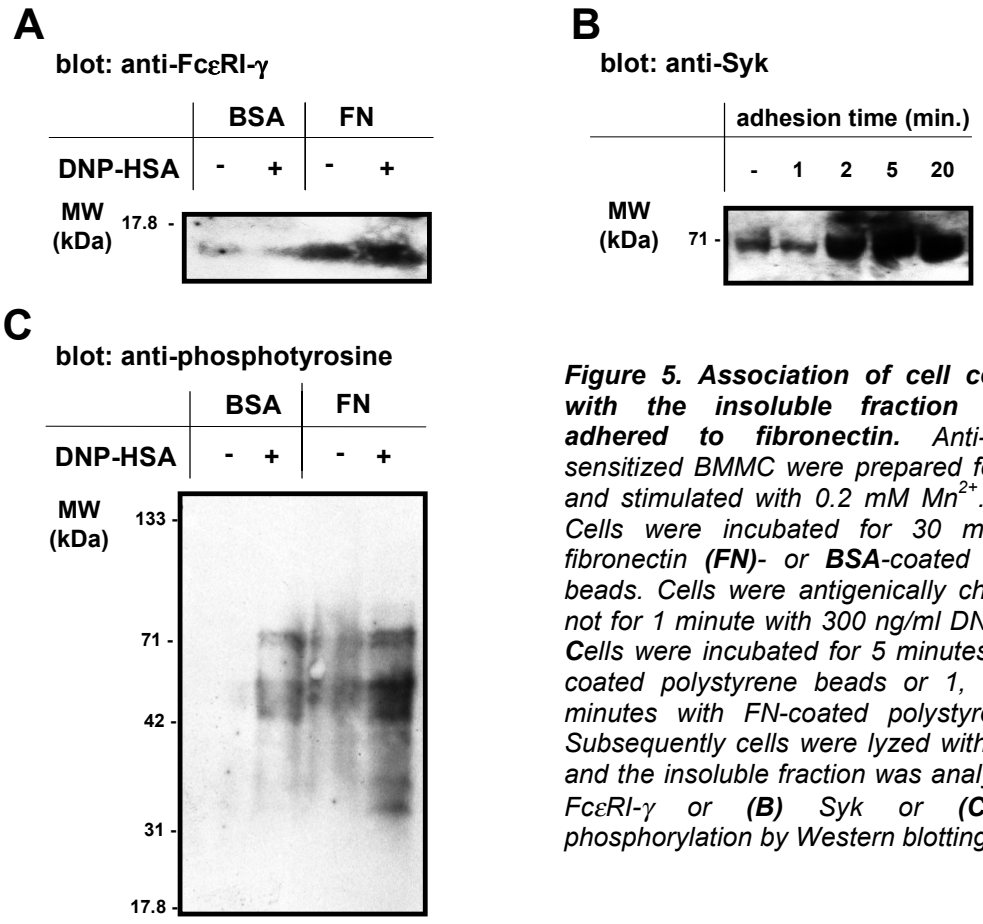


Figure 5. Association of cell components with the insoluble fraction of BMMC adhered to fibronectin. *Anti-DNP IgE-sensitized BMMC were prepared for adhesion and stimulated with 0.2 mM Mn^{2+}. **(A** and **C)** Cells were incubated for 30 minutes with fibronectin **(FN)**- or **BSA**-coated polystyrene beads. Cells were antigenically challenged or not for 1 minute with 300 ng/ml DNP-HSA. **(B)** **C**ells were incubated for 5 minutes with **BSA**-coated polystyrene beads or 1, 2, 5 or 15 minutes with FN-coated polystyrene beads. Subsequently cells were lyzed with 1 % NP40 and the insoluble fraction was analyzed for **(A)** FcεRI-γ or **(B)** Syk or **(C)** tyrosine phosphorylation by Western blotting.*

Effect of cholesterol depletion on adhesion and adhesion-induced degranulation

To investigate a possible role for rafts in adhesion-induced degranulation, BMMC were treated with methyl-β-cyclodextrin (β-CD) to deplete membrane cholesterol to disrupt raft architecture and function[78]. This treatment did not affect membrane integrity as determined by measurement of propidium iodide cell entrance (data not shown). Treated and nontreated BMMC were prepared for adhesion, stimulated with Mn^{2+} and incubated on a fibronectin-coated 96-well plate. Nonadhered cells were removed by washing and adhesion was quantified. As is shown in figure 6A, β-CD treatment of BMMC severely disturbs adhesion to fibronectin.

Attenuated adhesion should also result in abrogation of adhesion-induced degranulation. To confirm this, β-CD treated and nontreated sensitized BMMC were incubated with BSA- or fibronectin coated beads, and β-hexosaminidase release was determined. As is shown in figure 6B, β-hexosaminidase release induced by incubation with fibronectin-coated beads is completely inhibited after β-CD treatment. From these data we conclude that rafts are important for VLA-5 function and adhesion of BMMC to fibronectin and thereby play a role in adhesion-induced degranulation of these cells.

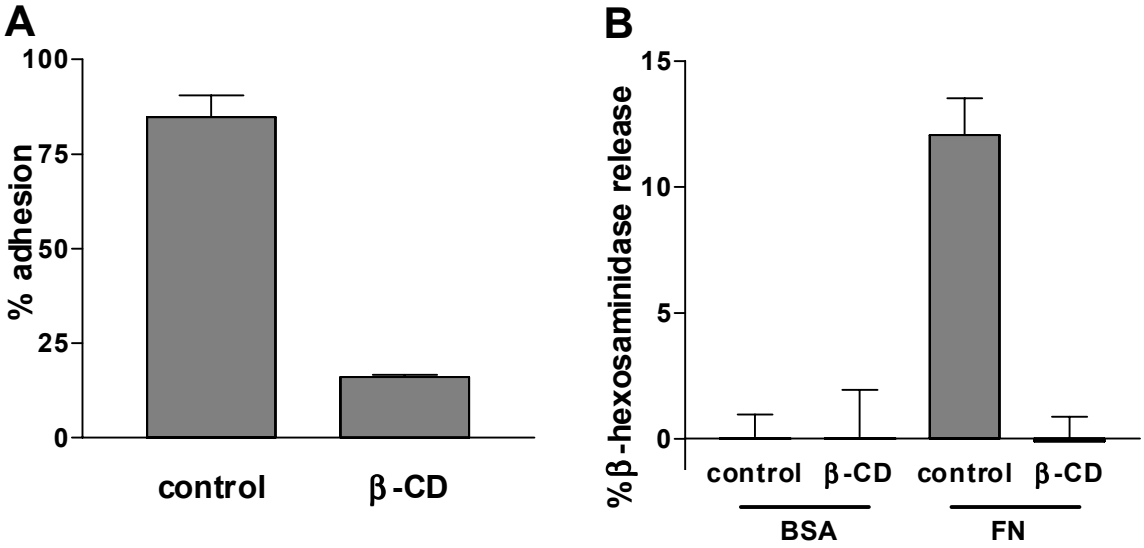


Figure 6. Effect of cholesterol-depletion on adhesion and adhesion-induced degranulation. *BMMC were treated or not with methyl-β-cyclodextrin **(β-CD)** or not **(control)**, prepared for adhesion and stimulated with 0.2 mM Mn^{2+}. **(A)** Cells were incubated on a fibronectin-coated 96-well plate, 30 minutes on ice to synchronize adhesion, and 30 minutes at 37°C. Nonadhered cells were removed by washing and adhesion was quantified. **(B)** Cells were incubated for 30 minutes with fibronectin (**FN**)- or **BSA**-coated polystyrene beads, extracellular Ca^{2+} was supplemented (final concentration: 1.8 mM) and after 30 minutes β-hexosaminidase release was determined.*

Discussion

In this report we show that adhesion of BMMC to fibronectin not only enhances allergen-induced degranulation, but also directly induces degranulation.

Adhesion of BMMC to fibronectin has been shown to be mediated by VLA-5[45,87] and we previously showed that adhesion-induced degranulation can be blocked by soluble arginin-glycine-aspartic acid peptide.
Our data show that presence of FcεRI-γ, although not needed for adhesion itself, is required for the induction of degranulation by adhesion and this strongly suggests physical interaction between VLA-5 and FcεRI. Within the spatial resolution of the confocal microscope, VLA-5 and FcεRI appear to be colocalized on resting BMMC. In our experiments the α-subunit of FcεRI was detected by fluorescently stained IgE. Because FcεRI-α can not exist independently of β- or γ-subunits on the cell membrane, we consider the observed fluorescence as indicative for the presence of the complete FcεRI complex. Previous transfectant studies have shown physical association between $\alpha_m\beta_2$ integrin CR3 and Fcγ receptors[127,174], which suggests that physical association between VLA-5 and FcεRI may be possible. Moreover, adhesion of BMMC to fibronectin-coated beads and subsequent clustering of VLA-5 results in association of FcεRI-γ with these beads which suggests that FcεRI comigrates with VLA-5, possibly due to physical association, into focal adhesion complexes.
Our study shows that cholesterol depletion attenuates adhesion of BMMC to fibronectin and this indicates that membrane rafts play a role in VLA-5 function, as was previously shown to be the case for $\alpha_L\beta_2$ integrin LFA-1 on T-lymphocytes[89]. Rafts are enriched with Src-kinases[20], which play an important role in focal adhesion complex-formation[22,33]. Association of clustered VLA-5 with rafts would also lead to redistribution of co-associated FcεRI to these microdomains, where the FcεRI signaling is initiated by phosphorylation of the receptor subunits by Lyn. Interestingly, attachment of bronchial epithelial cells to fibronectin through β_1 integrins, prevents these cells from dying when deprived from growth factors[5], and integrin-mediated adhesion of fibroblasts to fibronectin induces platelet-derived growth factor receptor phosphorylation in absence of growth factors[153]. In these two studies the growth factor receptors were shown to be localized in the focal adhesion complexes and this suggests that fibronectin serves as “pseudo-growth factor” because integrin aggregation induces clustering and activation of physically associated growth factor receptors. Analogous to this mechanism, fibronectin could function as “pseudo-allergen”, clustering FcεRI that is physically associated with aggregated VLA-5.
Syk activation by integrins has been demonstrated in various reports[53,60,97,109,176]. and the presence of the γ-subunit normally used by Fc receptors, was found to be obligatory for integrin-induced Syk activation[128]. Accordingly, we show that adhesion-induced degranulation is FcεRI-γ-dependent and that adhesion induces the recruitment of Syk to the insoluble fraction, probably to the phosphorylated γ-subunit within the focal adhesion complexes. Activation of Syk, through binding to FcεRI-γ ITAM, is critical as well as sufficient for degranulation[32,132]. In our experiments, adhesion and FcεRI-stimulation induce a similar pattern of tyrosine phosphorylation associated with the insoluble fraction of the cells. This suggests that adhesion induces Syk recruitment and initiation of the signal transduction cascade leading to degranulation, which is also triggered by clustering of FcεRI by multivalent allergen.
In our experiments we use cells with optimally stimulated VLA-5, as assessed empirically (data not shown), and our data suggest that adhesion of these cells

leads to clustering of a sufficient amount of FcεRI to exceed the activation threshold of this receptor in absence of allergen. Suboptimal VLA-5 stimulation, could induce clustering of a small amount of FcεRI. In this case the activation threshold is not exceeded but these cells are primed for FcεRI-stimulation due to the preclustered state of this receptor. According to 'kinetic proofreading', stabilization of FcεRI aggregates at focal adhesion complexes, thereby enhancing their lifetime in the aggregate, may also enhance degranulation. The concept of kinetic proofreading postulates that the biological potency of an individual receptor is determined by its lifetime within a signaling aggregate[71,104] and was shown to apply for FcεRI signaling[159].

Activation of integrins can be induced by a range of mediators such as interleukins[79,141], chemokines[99,171] and growth factors such as SCF[13,36,167]. Subtle variations in conditions of the mast cell microenvironment, for example at sites of inflammation where integrin-activating mediators are abundantly present, could thereby regulate mast cell sensitivity for antigenic stimulation.

In summary, our data suggest that VLA-5 clustering through adhesion to fibronectin induces aggregation of FcεRI in focal adhesion complexes and thereby triggers the FcεRI signal transduction cascade leading to mast cell degranulation.

8

General discussion

Although the underlying mechanisms remain elusive, the incidence of allergy has increased rapidly during the last century and has reached epidemic proportions[69]. Various lines of evidence indicate that the function of mast cells, the major effector cell type in allergy, is modulated by microenvironmental factors. In all tissues, mast cells are surrounded by the extracellular matrix-component fibronectin. A key receptor involved in mast cell-fibronectin interaction is very late antigen-5 (VLA-5)[i], and this integrin has been shown to be a potent enhancer of allergen-induced mast cell degranulation *in vitro* and *in vivo*[35,64,91,131,155,177]. Understanding of the mechanism by which VLA-5 modulates mast cell function is of potential interest for the development of future therapy against allergy and was therefore chosen to be the subject of this thesis. The main findings of the thesis are summarized in this general discussion.

Choice of experimental setup

Enhancing effects on degranulation induced by VLA-5-mediated adhesion to fibronectin, were initially reported in RBL-2H3 mast cells[35,64,155]. These cells have been shown invaluable for the investigation of signaling through the high-affinity for IgE (FcεRI), and are still widely used as *in vitro* model for mast cells. For detailed study of adhesion-mediated modulation of mast cell degranulation, an *in vitro* model system is required in which these cell functions can be controlled independently. A typical feature of RBL-2H3, however, is adhesion to various surfaces without the assembly of specialized adhesion structures at the cell-substrate interface[8,124]. This major discrepancy with *in vivo* mast cells makes RBL-2H3 cells unsuitable for our investigations.

The development of techniques to culture mast cells from murine bone-marrow (BMMC) has opened a new field of possibilities for *in vitro* mast cell research[84,133]. Through variations in culture conditions, maturation into different BMMC subtypes can be induced. The phenotypes and responses to various stimuli, such as allergen, of these cells are comparable to the different mast cell populations found *in vivo*. Most importantly BMMC cells do not adhere spontaneously or nonspecifically to fibronectin. In our opinion, BMMC are the most accurate *in vitro* representation of mast cells currently available, and we therefore use these cells for our investigations.

For adhesion of BMMC to fibronectin, a conformational change of VLA-5 into a high-affinity state is required. In chapter 4, we present a protocol using Mn^{2+} as a stimulus to induce a high-affinity state of VLA-5. A major advantage of Mn^{2+} compared to other known adhesion–inducing stimuli, is that this cation does not affect FcεRI signaling directly.

Characterization of Mn^{2+}-induced adhesion indicates two phases. During the first phase, a conformational change of the integrin enables ligand binding and during the second stage, increased intracellular kinase and phosphatase activity leads to a stabilized cell-substrate bond. These features, referred to as inside-out and outside-in signaling respectively, are characteristic for integrin physiology[58,74] and make this model suitable for our purposes.

[i] For abbreviations see page 101

VLA-5: enhancer and initiator of degranulation

The effects of VLA-5-mediated adhesion on degranulation of BMMC are presented in chapter 6. We demonstrate that adhesion enhances allergen-induced degranulation. This confirms a regulatory role in degranulation for VLA-5 in BMMC, cells that are a proper reflection of mast cells *in vivo*. Besides enhancing the response to allergen, we demonstrated that VLA-5-mediated adhesion to fibronectin directly triggers degranulation in the absence of an allergic stimulus, both *in vitro* and *in vivo*.

Overall, our data suggest that the degree of adhesion, determined by the affinity-state of VLA-5, correlates with allergen sensitivity and magnitude of degranulation of mast cells. Further analysis, as discussed below, suggests that both initiation and enhancement of degranulation by adhesion may be the result of the same mechanism through which VLA-5 and FcεRI interact.

A model for VLA-5 mediated modulation of mast cell degranulation

In chapter 7, the characteristics of adhered and nonadhered BMMC were studied and described in more detail. We found that on the membrane of BMMC VLA-5 and FcεRI are colocalized and that the FcεRI γ-subunit is required for, allergen-independent, adhesion-induced degranulation. Analogous to integrin CR3 and FcγRIIA, as demonstrated by Worth *et al.*[174], VLA-5 and FcεRI may be physically associated on the mast cell membrane. Additionally, we demonstrate that in adhered BMMC FcεRI-γ and subsequently Syk are accumulated in the fibronectin-associated cell fraction. Furthermore, adhesion to fibronectin and FcεRI-stimulation trigger tyrosine phosphorylation of an identical set of proteins, which is additive when both stimuli are applied. Similar data have been demonstrated for β_1 integrins in relation to members of the growth factor-receptor (GF-R) family by Aoshiba *et al.* and Sundberg *et al.*. In these studies, adhesion to fibronectin-induced coclustering of β_1 integrins and GF-R in focal adhesion complexes, induced growth factor-independent phosphorylation of the GF-R and prevented apoptosis due to growth factor deprivation[5,153]. There appears to be a parallel between integrin-GF-R and integrin-FcεRI interaction. In both cases fibronectin serves as a “pseudo-ligand” and the integrin as a “pseudo-ligand binding receptor subunit” of the physically associated receptor (GF-R or FcεRI). Hence, adhesion triggers the signal transduction cascade of the latter. Finally, our data show that rafts play a role in VLA-5 mediated adhesion, possibly due to their enrichment with Src-kinases that are important for focal adhesion complex formation[58]. Since these membrane microdomains have also been shown to be important for FcεRI-signaling[144], this suggests a role for rafts in adhesion-induced degranulation.

Based on these data we propose the following model (figure 1). On the mast cell membrane VLA-5 and FcεRI are colocalized, and may be physically associated in small assemblies. On resting mast cells (A), due to a low affinity conformation of VLA-5, adhesion is absent and these assemblies are randomly distributed on the mast cell membrane. Lipid rafts are distributed on the membrane in an identical “patchy” manner and there is no association between the raft and receptor pools. Conversion of VLA-5 into a high-affinity conformation by an external stimulus induces binding to fibronectin and leads to an accumulation of the integrin and FcεRI at the cell-fibronectin interface. (B)

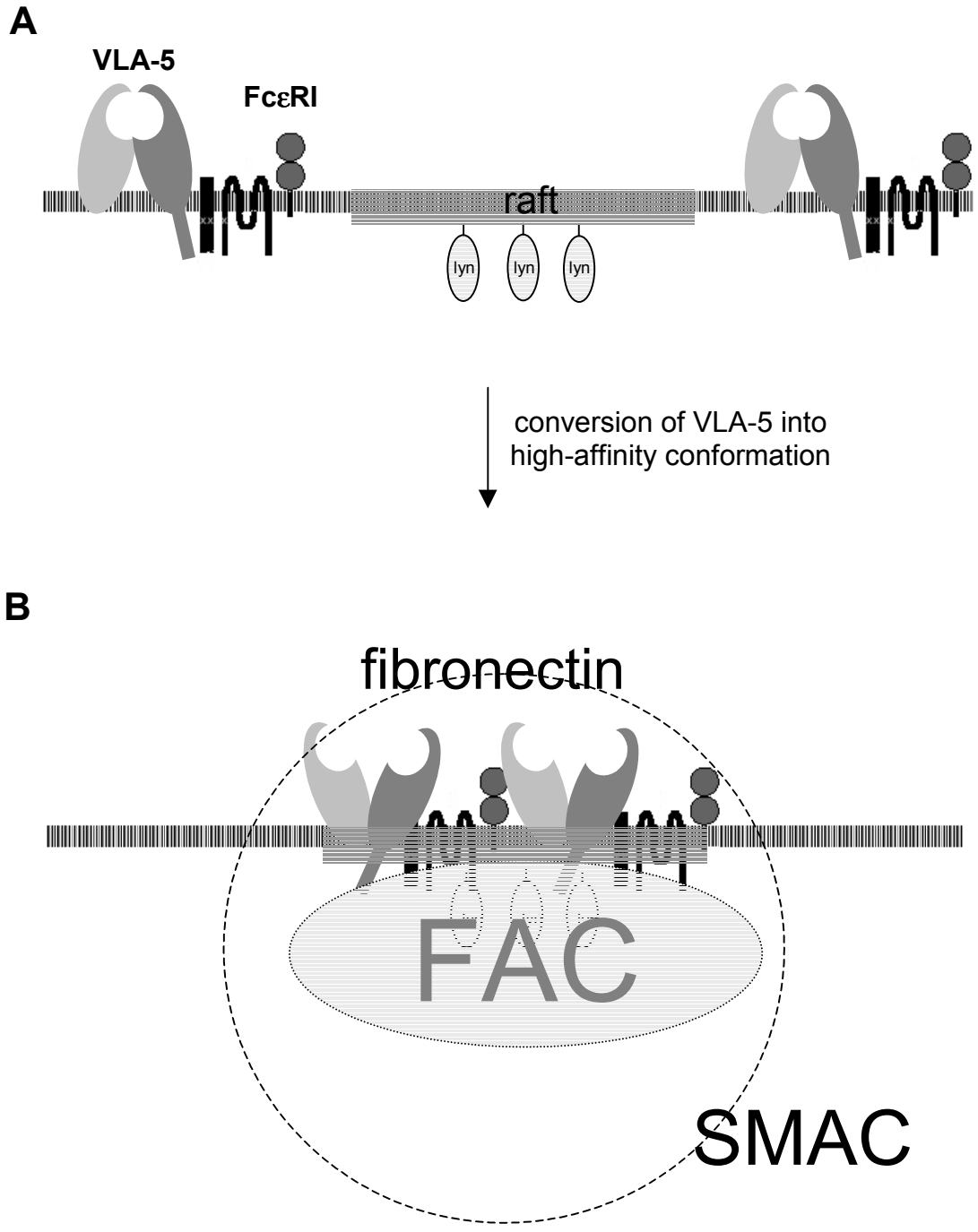


Figure 1. Adhesion to fibronectin induces the formation of supramolecular activation clusters in mast cells. A. *In resting mast cells, small low-affinity VLA-5-FcεRI assemblies and rafts are not associated but randomly distributed in a patchy manner on the plasma membrane.* ***B.*** *Conversion of VLA-5 to a high-affintity conformation induces the accumulation of VLA-5 and FcεRI at the cell-fibronectin interface. Subsequent association with rafts and focal adhesion complex (FAC) formation leads to the assembly of supramolecular activation clusters (SMACs).*

Association with rafts and formed focal adhesion complexes leads to the assembly of supramolecular activation clusters (SMACs)[3,68,114]. Interaction between FcεRI and components of focal adhesion complexes have been demonstrated previously by others[65,86,122] .
The formation of SMACs could account for both adhesion-enhanced and adhesion-induced degranulation, and both phenomena are discussed below. First, stabilization of FcεRI aggregates in SMACs could enhance FcεRI signaling according to the concept of 'kinetic proofreading', which states that a cascade of intracellular events unfold as long as the initiating event is maintained[71,104,159]. Secondly, they could affect the activation threshold of FcεRI (the minimal amount of allergen required for sufficient FcεRI and raft aggregation in order to trigger degranulation). Adhesion-induced aggregation, or 'preclustering', of FcεRI and rafts into SMACs, could lower the activation threshold and thus increase the sensitivity for allergen stimulation. Overall, we imagine that degranulation of mast cells depends on the degree of SMAC assembly (figure 2), and that several processes may contribute to this process either alone or in combination. In the case of resting IgE-sensitized mast cells, bearing only VLA-5 in a low-affinity conformation, no SMACs are formed (situation A). In these cells degranulation can only be triggered by allergen and these cells therefore have the lowest sensitivity (a high activation threshold). Increase in the number of high-affinity VLA-5 molecules (situation B) and subsequent SMAC assembly lowers the FcεRI activation threshold and adhesion-enhanced degranulation is observed. At a certain degree of SMAC formation (situation C) the FcεRI activation threshold is reduced to zero. At this point allergen is no longer required, because by adhesion a sufficient amount of FcεRI is clustered to trigger degranulation by itself. The latter situation is probably the case in our experimental setup and explanatory for adhesion-induced degranulation of BMMC.

Microenvironmental regulation of mast cell function

Our data suggest that the affinity status of VLA-5 determines the sensitivity of mast cells for allergen. A range of mediators that can be produced by various cell types can trigger inside-out signaling of integrins. Local variations in the mast cell microenvironment could thereby regulate mast cell sensitivity and prime these cells for allergen stimulation. The observation that skin atopy does not always correlate with allergic disease could be explained by low sensitivity of mast cells in the body as described in situation 2.A. Organ restriction of allergy could be due to priming of local mast cells by VLA-5-activating mediators produced by the mast cell surrounding tissue, as in situation 2.B. At locations of severe tissue inflammation the concentration of inflammatory, and integrin-activating, mediators reaches high levels and situation 2.C could occur. In chapter 6 we show that in addition to Mn^{2+}, Zn^{2+}, Ni^{2+} and Co^{2+} are able to induce VLA-5 mediated adhesion of BMMC to fibronectin and subsequently trigger adhesion-induced degranulation. These metals are known for their ability to induce clinical phenomena of allergy, but the mechanism of action is not completely understood. Our data suggest that divalent cations might induce a conformational change of VLA-5 on mast cells and induce degranulation due to situation 2.C and thereby provide a plausible explanation for metal-induced hypersensitivity responses.

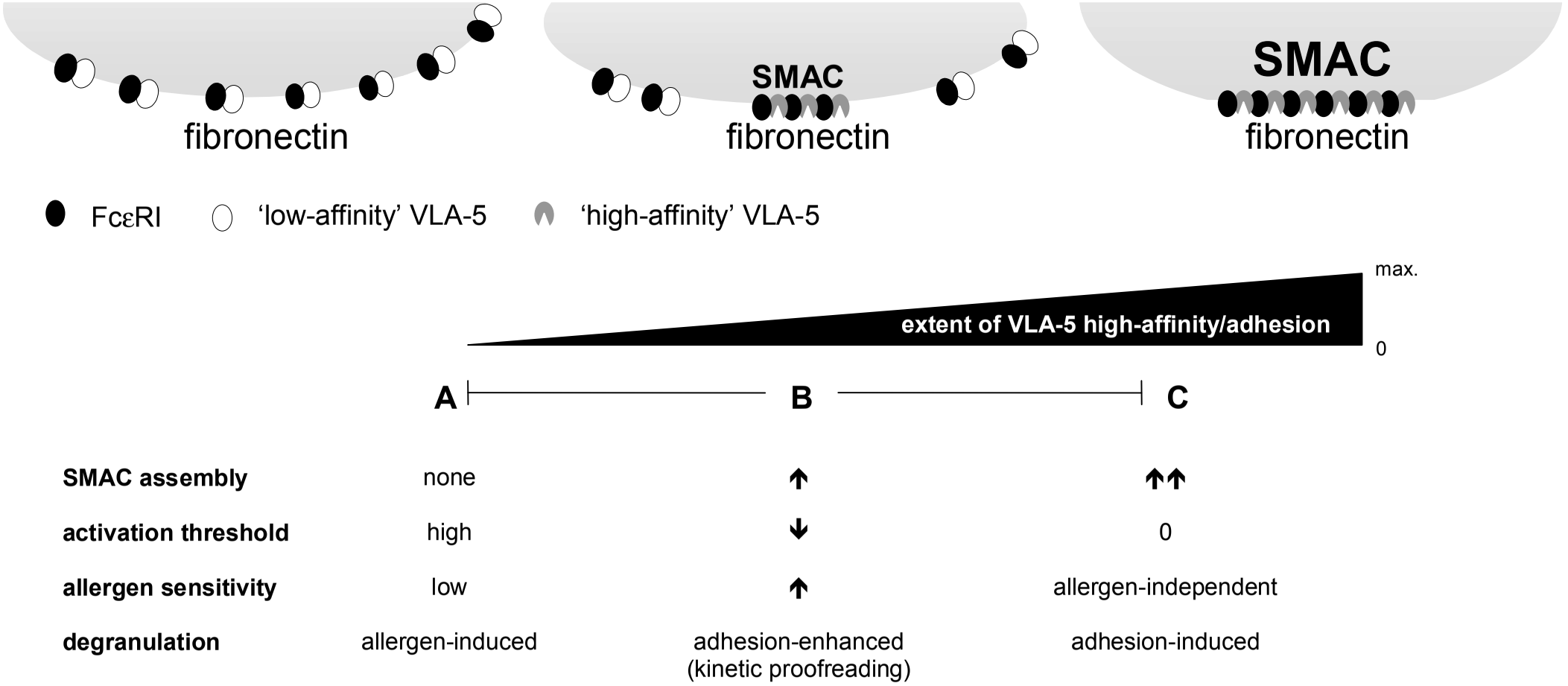


Figure 2 Regulation of mast cell function by VLA-5. *The extent of VLA-5 in high-affinity conformation correlates with supramolecular activation cluster (SMAC) assembly in mast cells. Through SMACs sensitivity towards allergen and magnitude of degranulation of mast cells are regulated.*

An additional modulating factor from the mast cell microenvironment is suggested by data presented in chapter 3. Our data suggest that limited availability of cholesterol in the mast cell microenvironment could impair the maintenance of rafts in the mast cell membrane. Subsequently affected raft integrity could disturb SMAC assembly and thereby affect mast cell sensitivity for allergen.

Future directions in anti-allergic therapy

Ra *et al.* and Yasuda *et al.* have shown that blocking of VLA-5 with specific antibodies inhibits passive cutaneous anaphylaxis responses in mice[131,177]. Blocking of VLA-5 mimics situation 2.A of our model because SMAC assembly in mast cells is prevented. Decreased mast cell sensitivity and limited allergen concentrations that reach the location of these mast cells could thereby be explanatory for abrogation of activation *in vivo*.

Instead of using antibodies we suggest a different approach to block VLA-5 adhesion based on the experiments described in chapter 5. We show that calcium-like peptides are able to block adhesion of BMMC to fibronectin. These peptides probably bind to the cation-binding domains of the VLA-5 α-subunit that are homologous to calmodulin EF-hands, and thereby interfere with VLA-5 function. Making mast cells less responsive to allergen by blocking VLA-5 could be a strategy for therapy in allergic responses. The observation that CALPs can block VLA-5 function suggests that these peptide calcium-mimetics may be possible lead compounds for anti-allergic drugs.

1. **Abraham, S. N., and Malaviya, R.** 1997. Mast cells in infection and immunity. *Infect. Immun. 65:3501*.
2. **Adler, K. B., Fischer, B. M., Wright, D. T., Cohn, L. A., and Becker, S.** 1994. Interactions between respiratory epithelial cells and cytokines: relationships to lung inflammation. *Ann. N. Y. Acad. Sci. 725:128*.
3. **Alberts, B.** 1998. The cell as a collection of protein machines: preparing the next generation of molecular biologists. *Cell 92:291*.
4. **Aldenborg, F., and Enerback, L.** 1985. Thymus dependence of connective tissue mast cells: a quantitative cytofluorometric study of the growth of peritoneal mast cells in normal and athymic rats. *Int. Arch. Allergy Appl. Immunol. 78:277*.
5. **Aoshiba, K., Rennard, S. I., and Spurzem, J. R.** 1997. Fibronectin supports bronchial epithelial cell adhesion and survival in the absence of growth factors. *Am. J. Physiol. 273:L684*.
6. **Artuc, M., Hermes, B., Steckelings, U. M., Grutzkau, A., and Henz, B. M.** 1999. Mast cells and their mediators in cutaneous wound healing -active participants or innocent bystanders? *Exp. Dermatol. 8:1*.
7. **Baneres, J. L., Roquet, F., Green, M., LeCalvez, H., and Parello, J.** 1998. The cation-binding domain from the asubunit of integrin $\alpha_5\beta_1$ is a minimal domain for fibronectin recognition. *J. Biol. Chem. 273:24744*.
8. **Barsumian, E. L., Isersky, C., Petrino, M. G., and Siraganian, R. P.** 1981. IgE-induced histamine release from rat basophilic leukemia cell lines: isolation of releasing and nonreleasing clones. *Eur. J. Immunol. 11:317*.
9. **Basketter, D. A., Briatico-Vangosa, G., Kaestner, W., Lally, C., and Bontinck, W. J.** 1993. Nickel, cobalt and chromium in consumer products: a role in allergic contact dermatitis? *Contact Dermatitis 28:15*.
10. **Beasley, R.** 1998. Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. *Lancet 351:1225*.
11. **Beaven, M. A., and Cunha-Melo, J. R.** 1988. Membrane phosphoinositide-activated signals in mast cells and basophils. *Prog. Allergy 42:123*.
12. **Beaven, M. A., and Kassessinoff, T.** 1997. Role of phospholipases, protein kinases and calcium in FcεRI-induced secretion. In *IgE receptor (FcεRI) function in mast cells and basophils*. M. M. Hamawy, ed. Springer-Verlag, Heidelberg, p. 55.
13. **Bendall, L. J., Makrynikola, V., Hutchinson, A., Bianchi, A. C., Bradstock, K. F., and Gottlieb, D. J.** 1998. Stem cell factor enhances the adhesion of AML cells to fibronectin and augments fibronectin-mediated anti-apoptotic and proliferative signals. *Leukemia 12:1375*.
14. **Benhamou, M. 1997**. FcεRI-induced protein tyrosine phosphorylation. In *IgE receptor (FcεRI) function in mast cells and basophils*. M. M. Hamawy, ed. Springer-Verlag, Heidelberg, p. 33.
15. **Bennett, J. P., Cockcroft, S., and Gomperts, B. D.** 1979. Ionomycin stimulates mast cell histamine secretion by forming a lipid- soluble calcium complex. *Nature 282:851*.
16. **Bernard, M., Voisin, P., and Klein, D. C.** 1996. Hydroxyindole-O-methyltransferase in Y-79 cells: regulation by serum. *Brain. Res. 727:118*.
17. **Bienenstock, J., and Befus, A. D.** 1982. Mast cell heterogeneity: basic questions and clinical implications. In *Mast cell differentiation and heterogeneity*. A. D. Befus, J. Bienestock, and J. A. Denburg, eds. Raven, New York, p. 391.
18. **Bienenstock, J., MacQueen, G., Sestini, P., Marshall, J. S., Stead, R. H., and Perdue, M. H.** 1991. Mast cell/nerve interactions in vitro and in vivo. *Am. Rev. Respir. Dis. 143:S55*.
19. **Blalock, J. E.** 1995. Genetic origins of protein shape and interaction rules. *Nature Med. 1:876*.
20. **Brown, D. A., and London, E.** 1998. Functions of lipid rafts in biological membranes. *Annu. Rev. Cell Dev. Biol. 14:111*.
21. **Bungy, G. A., Mossawi, J., Nojoumi, S. A., and Brostoff, J.** 1996. Razi's report about seasonal allergic rhinitis (hay fever) from the 10th century AD. *Int. Arch. Allergy Immunol. 110:219*.
22. **Burridge, K., Chrzanowska-Wodnicka, M., and Zhong, C.** 1997. Focal adhesion assembly. *Tr. Cell Biol. 7:342*.

23. **Cansell, M., Gouygou, J. P., Jozefonvicz, J., and Letourneur, D.** 1997. Lipid composition of cultured endothelial cells in relation to their growth. *Lipids. 32:39*.
24. **Caviedes, R., Caviedes, P., Liberona, J. L., and Jaimovich, E.** 1994. Ion channels in a skeletal muscle cell line from a Duchenne muscular dystrophy patient. *Muscle Nerve 17:1021*.
25. **Chakravarty, N., and Nielsen, E. H.** 1985. Calmodulin in mast cells and its role in histamine secretion. *Agents Actions 16:122*.
26. **Chen, M. S., Almeida, E. A., Huovila, A., Takahashi, Y., Shaw, L. M., Mercurio, A. M., and White, J. M.** 1999. Evidence that distinct states of the integrin $\alpha_6\beta_1$ interact with laminin and an ADAM. *J. Cell Biol. 144:549*.
27. **Chen, Q. M., Lin, T. H., Der, C. J., and Juliano, R. L.** 1996. Integrin-mediated activation of mitogen activated protein (MAP) or extracellular signal-related kinase kinase (MEK) and kinase is independent of Ras. *J. Biol. Chem. 271:18122*.
28. **Choquet, D., Felsenfeld, D. P., and Sheetz, M. P.** 1997. Extracellular matrix rigidity causes strengthening of integrin-cytoskeleton linkages. *Cell 88:39*.
29. **Columbo, M., Horowitz, E. M., Botana, L. M., MacGlashan, D. W., Jr., Bochner, B. S., Gillis, S., Zsebo, K. M., Galli, S. J., and Lichtenstein, L. M.** 1992. The human recombinant c-kit receptor ligand, rhSCF, induces mediator release from human cutaneous mast cells and enhances IgE-dependent mediator release from both skin mast cells and peripheral blood basophils. *J. Immunol. 149:599*.
30. **Cookson, W.** 1999. The alliance of genes and environment in asthma and allergy. *Nature 402:B5*.
31. **Corry, D. B., and Kheradmand, F.** 1999. Induction and regulation of the IgE response. *Nature 402:B18*.
32. **Costello, P. S., Turner, M., Walters, A. E., Cunningham, C. N., Bauer, P. H., Downward, J., and Tybulewicz, V. L. J.** 1996. Critical role for the tyrosine kinase Syk in signalling through the high affinity IgE receptor of mast cells. *Oncogene 13:2595*.
33. **Craig, S. W., and Johnson, R. P.** 1996. Assembly of focal adhesions: progress, paradigms, and portents. *Curr. Opin. Cell Biol. 8:74*.
34. **Crivici, A., and Ikura, M.** 1995. Molecular and structural basis of target recognition by calmodulin. *Annu. Rev. Biophys. Biomol. Struct. 24:85*.
35. **Dastych, J., Costa, J. J., Thompson, H. L., and Metcalfe, D. D.** 1991. Mast cell adhesion to fibronectin. *Immunology 73:478*.
36. **Dastych, J., and Metcalfe, D. D.** 1994. Stem cell factor induces mast cell adhesion to fibronectin. *J. Immunol. 152:213*.
37. **Deanin, G. G., Cutts, J. L., Pfeiffer, J. R., and Oliver, J. M.** 1991. Role of isoprenoid metabolism in IgE receptor-mediated signal transduction. *J. Immunol. 146:3528*.
38. **Dillon, J., Woods, W. T., Guarcello, V., LeBoeuf, R. D., and Blalock, J. E.** 1991. A peptide mimetic of calcium. *Proc. Natl. Acad. Sci. U. S. A. 88:9726*.
39. **Dines, K. C., and Powell, H. C.** 1997. Mast cell interactions with the nervous system: relationship to mechanisms of disease. *J. Neuropathol. Exp. Neurol. 56:627*.
40. **Ebisawa, M., Saito, H., Reason, D. C., Sakaguchi, N., Katsunuma, T., and Iikura, Y.** 1991. Changes in filament actin accompanying IgE-dependent and -independent histamine release from IL-3-dependent cultured human basophils. *Int. Arch. Allergy Appl. Immunol. 94:71*.
41. **Egesten, A., Gullberg, U., Olsson, I., and Richter, J.** 1993. Phorbol ester-induced degranulation in adherent human eosinophil granulocytes is dependent on CD11/CD18 leukocyte integrins. *J. Leukoc. Biol. 53:287*.
42. **Ehrlich, P.** 1877. Beitrage zur kenntniss der quilinfarbunger und ihrer verivendigung in der mikroskopischen technik. *Alch. Mikros. Anat. 13:263*.
43. **Fassina, G., Roller, P. P., Olson, A. D., Thorgeirsson, S. S., and Omichinski, J. G.** 1989. Recognition properties of peptides hydropathically complementary to residues 356-375 of the c-raf protein. *J. Biol. Chem. 264:11252*.
44. **Faull, R. J., and Ginsberg, M. H.** 1996. Inside-out signaling through integrins. *J. Am. Soc. Nephrol. 7:1091*.
45. **Fehlner-Gardiner, C. C., Uniyal, S., Vonballestrem, C. G., and Chan, B. M. C.** 1996. Differential utilization of VLA-4 ($\alpha_4\beta_1$) and -5 ($\alpha_5\beta_1$) integrins during the development of mouse bone marrow-derived mast cells. *Differentiation 60:317*.

46. **Fernandez, C., Clark, K., Burrows, L., Schofield, N. R., and Humphries, M. J.** 1998. Regulation of the extracellular ligand binding activity of integrins. *Front. Biosc. 3:D684*.
47. **Field, K. A., Holowka, D., and Baird, B.** 1995. FcεRI-mediated recruitment of p53/56lyn to detergent-resistant membrane domains accompanies cellular signaling. *Proc. Natl. Acad. Sci. U. S. A. 92:9201*.
48. **Field, K. A., Holowka, D., and Baird, B.** 1997. Compartmentalized activation of the high affinity immunoglobulin E receptor within membrane domains. *J. Biol. Chem. 272:4276*.
49. **Field, K. A., Holowka, D., and Baird, B.** 1999. Structural aspects of the association of FcεRI with detergent-resistant membranes. *J. Biol. Chem. 274:1753*.
50. **Galli, S. J.** 1990. New insights into "the riddle of the mast cells": microenvironmental regulation of mast cell development and phenotypic heterogeneity. *Lab. Invest. 62:5*.
51. **Galli, S. J.** 2000. Mast cells and basophils. *Curr. Opin. Hematol. 7:32*.
52. **Galli, S. J., and Wershil, B. K.** 1996. The two faces of the mast cell. *Nature 381:21*.
53. **Gao, J., Zoller, K. E., Ginsberg, M. H., Brugge, J. S., and Shattil, S. J.** 1997. Regulation of the pp72syk protein tyrosine kinase by platelet integrin $\alpha_{IIb}\beta_3$. *EMBO. J. 16:6414*.
54. **Garcia, A. J., Takagi, J., and Boettiger, D. 1998.** Two-stage activation for $\alpha_5\beta_1$ integrin binding to surface- adsorbed fibronectin. *J. Biol. Chem. 273:34710*.
55. **Garratt, A. N., and Humphries, M. J.** 1995. Recent insights into ligand binding, activation and signalling by integrin adhesion receptors. *Acta Anat. 154:34*.
56. **Gaston, B., Drazen, J. M., Loscalzo, J., and Stamler, J. S.** 1994. The biology of nitrogen oxides in the airways. *Am. J. Respir. Crit. Care Med. 149:538*.
57. **Gawkrodger, D. J.** 1996. Nickel dermatitis: how much nickel is safe? *Contact Dermatitis 35:267*.
58. **Giancotti, F. G., and Ruoslahti, E.** 1999. Integrin signaling. *Science 285:1028*.
59. **Gordon, J. R., Burd, P. R., and Galli, S. J.** 1990. Mast cells as a source of multifunctional cytokines. *Immunol. Today 11:458*.
60. **Gotoh, A., Takahira, H., Geahlen, R. L., and Broxmeyer, H. E.** 1997. Cross-linking of integrins induces tyrosine phosphorylation of the proto-oncogene product Vav and the protein tyrosine kinase Syk in human factor-dependent myeloid cells. *Cell Growth Differ. 8:721*.
61. **Grakoui, A., Bromley, S. K., Sumen, C., Davis, M. M., Shaw, A. S., Allen, P. M., and Dustin, M. L.** 1999. The immunological synapse: a molecular machine controlling T cell activation. *Science 285:221*.
62. **Hamawy, M. M., Mergenhagen, S. E., and Siraganian, R. P.** 1994. Adhesion molecules as regulators of mast-cell and basophil function. *Immunol. Today 15:62*.
63. **Hamawy, M. M., Mergenhagen, S. E., and Siraganian, R. P.** 1995. Protein tyrosine phosphorylation as a mechanism of signalling in mast cells and basophils. *Cell. Sign. 7(6):535*.
64. **Hamawy, M. M., Oliver, C., Mergenhagen, S. E., and Siraganian, R. P.** 1992. Adherence of rat basophilic leukemia (RBL-2H3) cells to fibronectin-coated surfaces enhances secretion. *J. Immunol. 149:615*.
65. **Hamawy, M. M., Swieter, M., Mergenhagen, S. E., and Siraganian, R. P.** 1997. Reconstitution of high affinity IgE receptor-mediated secretion by transfecting protein tyrosine kinase pp125FAK. *J. Biol. Chem. 272:30498*.
66. **Harder, T., and Simons, K.** 1997. Caveolae, DIGs, and the dynamics of sphingolipid-cholesterol microdomains. *Curr. Opin. Cell Biol. 9:534*.
67. **Hartfield, P. J., Greaves, M. W., and Camp, R. D.** 1993. β_1 Integrin-mediated T cell adhesion is regulated by calcium ionophores and endoplasmic reticulum Ca^{2+}-ATPase inhibitors. *Biochem. Biophys. Res. Commun. 196:1183*.
68. **Hartwell, L. H., Hopfield, J. J., Leibler, S., and Murray, A. W.** 1999. From molecular to modular cell biology. *Nature 402:C47*.
69. **Holgate, S. T.** 1999. The epidemic of allergy and asthma. *Nature 402:B2*.
70. **Holt, P. G., Macaubas, C., Stumbles, P. A., and Sly, P. D.** 1999. The role of allergy in the development of asthma. *Nature 402:B12*.
71. **Hopfield, J. J.** 1974. Kinetic proofreading: a new mechanism for reducing errors in biosynthetic processes requiring high specificity. *Proc. Natl. Acad. Sci. U.S.A. 71:4135*.

72. **Hoth, M., Fasolato, C., and Penner, R.** 1993. Ion channels and calcium signaling in mast cells. *Ann. N.Y. Acad. Sci. 707:198*.
73. **Hoth, M., and Penner, R.** 1993. Calcium release-activated calcium current in rat mast cells. *J. Physiol. 465:359*.
74. **Hughes, P. E., and Pfaff, M.** 1998. Integrin affinity modulation. *Tr. Cell Biol. 8:359*.
75. **Hunter, T.** 1995. Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell 80:225*.
76. **Hynes, R. O.** 1992. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell 69:11*.
77. **Ilangumaran, S., He, H.-T., and Hoessli,** D. C. 2000. Microdomains in lymphocyte signalling: beyond GPI-anchored proteins. *Immunol. Today 21:2*.
78. **Ilangumaran, S., and Hoessli, D. C.** 1998. Effects of cholesterol depletion by cyclodextrin on the sphingolipid microdomains of the plasma membrane. *Biochem. J. 335:433*.
79. **Issekutz, A. C.** 1998. Adhesion molecules mediating neutrophil migration to arthritis in vivo and across endothelium and connective tissue barriers in vitro. *Inflamm. Res. 47 Suppl 3:S123*.
80. **Izumi, Y., Hirai, S., Tamai, Y., Fujise-Matsuoka, A., Nishimura, Y., and Ohno, S.** 1997. A protein kinase C_{δ}-binding protein SRBC whose expression is induced by serum starvation. *J. Biol. Chem. 272:7381*.
81. **Jacobson, K., and Dietrich, C.** 1999. Looking at lipid rafts? *Tr. Cell Biol. 9:87*.
82. **Janeway, J. A., and Travers, P.** 1997. The humoral immune response. In *Immunobiology: the immune system in health and disease*. J. A. Janeway, and P. Travers, eds. Current Biology Inc./Garland Publishing Inc., New York.
83. **Jones, C. A., Kilburn, S. A., Warner, J. A., and Warner, J. O.** 1998. Intrauterine environment and fetal allergic sensitization. *Clin. Exp. Allergy 28:655*.
84. **Karimi, K., Redegeld, F. A., Heijdra, B., and Nijkamp, F. P.** 1999. Stem cell factor and interleukin-4 induce murine bone marrow cells to develop into mast cells with connective tissue type characteristics in vitro. *Exp. Hematol. 27:654*.
85. **Kawasaki, H., Inagaki, N., Kimata, M., Nakai, N., and Nagai, H.** 1995. Selective potentiation of IgE-dependent histamine release from rat peritoneal mast cells by stem cell factor. *Life. Sci. 57:2377*.
86. **Kawasugi, K., French, P. W., Penny, R., and Ludowyke, R. I.** 1995. Focal adhesion formation is associated with secretion of allergic mediators. *Cell Motil. Cytoskeleton. 31:215*.
87. **Kinashi, T., Asaoka, T., Setoguchi, R., and Takatsu, K.** 1999. Affinity modulation of very late antigen-5 through phosphatidylinositol 3-kinase in mast cells. *J. Immunol. 162:2850*.
88. **Kinet, J. P., Jouvin, M. H., Paolini, R., Numerof, R., and Scharenberg, A.** 1996. IgE receptor (FcεRI) and signal transduction. *Eur. Resp. J. 9:S116*.
89. **Krauss, K., and Altevogt, P.** 1999. Integrin leukocyte function-associated antigen-1-mediated cell binding can be activated by clustering of membrane rafts. *J. Biol. Chem. 274:36921*.
90. **Krishnaswamy, G., Kelley, J., Yerra, L., Smith, J. K., and Chi, D. S.** 1999. Human endothelium as a source of multifunctional cytokines: molecular regulation and possible role in human disease. *J. Interferon Cytokine Res. 19:91*.
91. **Kruger-Krasagakes, S., Grutzkau, A., Krasagakis, K., Hoffmann, S., and Henz, B. M.** 1999. Adhesion of human mast cells to extracellular matrix provides a co- stimulatory signal for cytokine production. *Immunology 98:253*.
92. **Lansdown, A. B.** 1995. Physiological and toxicological changes in the skin resulting from the action and interaction of metal ions. *Crit. Rev. Toxicol. 25:397*.
93. **Lasky, L. A.** 1997. Cell adhesion - How integrins are activated. *Nature 390:15*.
94. **Lavens, S. E., Goldring, K., Thomas, L. H., and Warner, J. A.** 1996. Effects of integrin clustering on human lung mast cells and basophils. *Am. J. Respir. Cell. Mol. Biol. 14:95*.
95. **Levenberg, S., Katz, B. Z., Yamada, K. M., and Geiger, B.** 1998. Long-range and selective autoregulation of cell-cell or cell-matrix adhesions by cadherin or integrin ligands. *J. Cell Sci. 111:347*.
96. **Lichtenfels, R., Biddison, W. E., Schulz, H., Vogt, A. B., and Martin, R.** 1994. CARE-LASS (calcein-release-assay), an improved fluorescence-based test system to measure cytotoxic T lymphocyte activity. *J. Immunol. Methods. 172:227*.

97. **Lin, T. H., Rosales, C., Mondal, K., Bolen, J. B., Haskill, S., and Juliano, R. L.** 1995. Integrin-mediated tyrosine phosphorylation and cytokine message induction in monocytic cells. A possible signaling role for the Syk tyrosine kinase. *J. Biol. Chem. 270:16189*.
98. **Liu, F.-T., Bohn, J. W., Ferrie, E. L., Yamamoto, H., Molinaro, C. A., Sherman, L. A., Klinman, N. R., and Katz, D. H.** 1980. Monoclonal dinitrophenyl-specific murine IgE antibody: preparation, isolation, and characterisation. *J. Immunol. 124:2728*.
99. **Lloyd, A. R., Oppenheim, J. J., Kelvin, D. J., and Taub, D. D.** 1996. Chemokines regulate T cell adherence to recombinant adhesion molecules and extracellular matrix proteins. *J. Immunol. 156:932*.
100. **Longhurst, C. M., and Jennings, L. K.** 1998. Integrin-mediated signal transduction. *Cell. Mol. Life Sci. 54:514*.
101. **MacDermott, R. P., Sanderson, I. R., and Reinecker, H. C.** 1998. The central role of chemokines (chemotactic cytokines) in the immunopathogenesis of ulcerative colitis and Crohn's disease. *Inflamm. Bowel Dis. 4:54*.
102. **Maier, C. C., Moseley, H. N., Zhou, S. R., Whitaker, J. N., and Blalock, J. E.** 1994. Identification of interactive determinants on idiotypic-anti-idiotypic antibodies through comparison of their hydropathic profiles. *Immunomethods 5:107*.
103. **Mason, D. W., Penhale, W. J., and Sedgwick, J. D.** 1987. Preparation of lymphocyte subpopulations. In *Lymphocytes, a practical approach*. G.G. B. Klaus, ed. IRL Press Ltd., Oxford, p. 35.
104. **McKeithan, T. W.** 1995. Kinetic proofreading in T-cell receptor signal transduction. *Proc. Natl. Acad. Sci. U.S.A. 92:5042*.
105. **Metcalfe, D. D., Baram, D., and Mekori, Y. A.** 1997. Mast cells. *Physiol. Rev. 77:1033*.
106. **Metzger, H.** 1999. It's spring, and thoughts turn to...allergies. *Cell 97:287*.
107. **Metzger, H., Chen, H., Goldstein, B., Haleem-Smith, H., Inman, J. K., Peirce, M., Torigoe, C., Vonakis, B., and Wofsy, C.** 1999. A quantitative approach to signal transduction. *Immunol. Lett. 68:53*.
108. **Miller, H. R. P., Huntley, J. F., Newlands, G. F. J., MacKellar, A., Irvine, J., Haig, D. M., MacDonald, A., Lammas, A. D., Wakelin, D., and Woodbury, R. G.** 1989. Mast cell granular proteases in mouse and rat: a guide to mast cell heterogeneity and activation in the gastrointestinal tract. In *Mast cell and basophil differentiation and function in health and disease*. S. J. Galli, and K. F. Austen, eds. Raven, New York, p. 81.
109. **Miller, L. A., Hong, J. J., Kinch, M. S., Harrison, M. L., and Geahlen, R. L.** 1999. The engagement of β_1 integrins on promonocytic cells promotes phosphorylation of Syk and formation of a protein complex containing Lyn and β_1 integrin. *Eur. J. Immunol. 29:1426*.
110. **Minoguchi, K., Benhamou, M., Swaim, W. D., Kawakami, Y., Kawakami, T., and Siraganian, R. P.** 1994. Activation of protein tyrosine kinase p72syk by FcεRI aggregation in rat basophilic leukemia cells. p72syk is a minor component but the major protein tyrosine kinase of pp72. *J. Biol. Chem. 269:16902*.
111. **Miyamoto, S., Akiyama, S. K., and Yamada, K. M.** 1995. Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. *Science 267:883*.
112. **Miyamoto, S., Teramoto, H., Coso, O. A., Gutkind, J. S., Burbelo, P. D., Akiyama, S. K., and Yamada, K. M.** 1995. Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. *J. Cell Biol. 131:791*.
113. **Miyamoto, S., Teramoto, H., Gutkind, J. S., and Yamada, K. M.** 1996. Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. *J. Cell Biol. 135:1633*.
114. **Monks, C. R., Freiberg, B. A., Kupfer, H., Sciaky, N., and Kupfer, A.** 1998. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature 395:82*.
115. **Mosmann, T.** 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicicity assays. *J. Immunol. Methods. 65:55*.

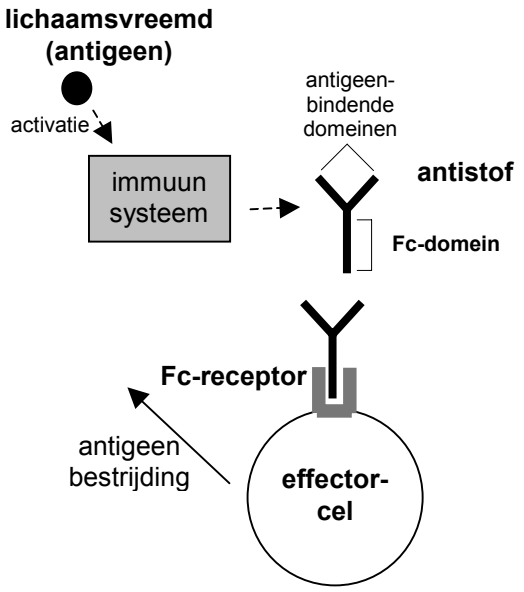
116. **Mould, A. P., Akiyama, S. K., and Humphries, M. J.** 1995. Regulation of integrin $\alpha_5\beta_1$-fibronectin interactions by divalent cations. Evidence for distinct classes of binding sites for Mn^{2+}, Mg^{2+}, and Ca^{2+}. *J. Biol. Chem. 270:26270*.
117. **Mould, A. P., Garratt, A. N., Askari, J. A., Akiyama, S. K., and Humphries, M. J.** 1995. Regulation of integrin $\alpha_5\beta_1$ function by anti-integrin antibodies and divalent cations. *Biochem. Soc. Trans. 23:395S*.
118. **Mould, A. P., Garratt, A. N., Puzon-McLaughlin, W., Takada, Y., and Humphries, M. J.** 1998. Regulation of integrin function: evidence that bivalent-cation-induced conformational changes lead to the unmasking of ligand-binding sites within integrin $\alpha_5\beta_1$. *Biochem. J. 331:821*.
119. **Nemery, B.** 1990. Metal toxicity and the respiratory tract. *Eur. Respir. J. 3:202*.
120. **Ninio, J.** 1975. Kinetic amplification of enzyme discrimination. *Biochimie 57:587*.
121. **Ogasawara, T., Murakami, M., Suzukinishimura, T., Uchida, M. K., and Kudo, I.** 1997. Mouse bone marrow-derived mast cells undergo exocytosis, prostanoid generation, and cytokine expression in response to G protein-activating polybasic compounds after coculture with fibroblasts in the presence of c-kit ligand. *J. Immunol. 158:393*.
122. **Okazaki, H., Zhang, J., Hamawy, M. M., and Siraganian, R. P.** 1997. Activation of protein-tyrosine kinasePyk2 is downstream of Syk in FcεRI signaling. *J. Biol. Chem. 272:32443*.
123. **Paolini, R., Jouvin, M. H., and Kinet, J. P.** 1991. Phosphorylation and dephosphorylation of the high-affinity receptor for immunoglobulin E immediately after receptor engagement and disengagement. *Nature. 353:855*.
124. **Pfeiffer, J. R., and Oliver, J. M.** 1994. Tyrosine kinase-dependent assembly of actin plaques linking FcεRI cross-linking to increased cell substrate adhesion in RBL-2H3 tumor mast cells. *J. Immunol. 152:270*.
125. **Pierini, L., Holowka, D., and Baird, B.** 1996. FcεRI-mediated association of 6-μm beads with RBL-2H3 mast cells results in exclusion of signaling proteins from the forming phagosome and abrogation of normal downstream signaling. *J. Cell Biol. 134:1427*.
126. **Plopper, G. E., McNamee, H. P., Dike, L. E., Bojanowski, K., and Ingber, D. E.** 1995. Convergence of integrin and growth factor receptor signalling pathways within the focal adhesion complex. *Mol. Biol. Cell 6:1349*.
127. **Poo, H., Krauss, J. C., Mayo-Bond, L., Todd, R. F., 3rd, and Petty, H. R.** 1995. Interaction of Fcγreceptor type IIIB with complement receptor type 3 in fibroblast transfectants: evidence from lateral diffusion and resonance energy transfer studies. *J. Mol. Biol. 247:597*.
128. **Poole, A., Gibbins, J. M., Turner, M., van Vugt, M. J., van de Winkel, J. G., Saito, T., Tybulewicz, V. L., and Watson, S. P.** 1997. The Fc receptor γ-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. *EMBO J. 16:2333*.
129. **Purcell, W. M., Doyle, K. M., Bagga, L., and Derks, M.** 1994. Histamine release from mast cells by polyamines: an NMDA receptor- mediated event? *Biochem. Soc. Trans. 22:398S*.
130. **Purcell, W. M., Doyle, K. M., Westgate, C., and Atterwill, C. K.** 1996. Characterisation of a functional polyamine site on rat mast cells: Association with a NMDA receptor macrocomplex. *J. Neuroimmunol. 65:49*.
131. **Ra, C., Yasuda, M., Yagita, H., and Okumura, K.** 1994. Fibronectin receptor integrins are involved in mast cell activation. *J. All. Clin. Immunol. 94:625*.
132. **Rivera, V. M., and Brugge, J. S.** 1995. Clustering of Syk is sufficient to induce tyrosine phosphorylation and release of allergic mediators from rat basophilic leukemia cells. *Mol. Cell. Biol. 15:1582*.
133. **Rodewald, H. R., Dessing, M., Dvorak, A. M., and Galli, S. J.** 1996. Identification of a committed precursor for the mast cell lineage. *Science 271:818*.
134. **Rubinchik, E., and Levi-Schaffer, F.** 1994. Mast cells and fibroblasts: two interacting cells. *Int. J .Clin. Lab. Res. 24:139*.
135. **Schlaepfer, D. D., and Hunter, T.** 1998. Integrin signalling and tyrosine phosphorylation: just the FAKs? *Tr. Cell Biol. 8:151*.
136. **Schlessinger, J., and Ullrich, A.** 1992. Growth factor signaling by receptor tyrosine kinases. *Neuron 9:383*.
137. **Schroder, J. M., and Mochizuki, M.** 1999. The role of chemokines in cutaneous allergic inflammation. *Biol. Chem. 380:889*.

β-CD	methyl-β-cyclodextrin
BMMC	bone marrow-derived mast cells
CALP	calcium-like peptide
CaM	calmodulin
CFDA	4-(and-5)-carboxyfluorescin diacetate
CTMC	connective tissue-type mast cells
DNP	dinitrophenyl
FcεRI	high-affinity receptor for IgE
FCS	fetal calf serum
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
MIRR	multichain immune recognition receptor
MMC	mucosal mast cells
MWCO	molecular weight cut-off
PAO	phenylarsene oxide
PTK	protein tyrosine kinase
RGD	arginine-glycine-aspartic acid
SCF	stem cell factor
SDS	sodiumdodecyl sulfate
SH2	Src-homology-2
TBS	tris-buffered saline
VLA-5	very late antigen-5

Nederlandse samenvatting

(voor iedereen dus…)

Iedere dag staan we bloot aan ontelbare schadelijke micro-organismen en stoffen. Om te overleven is het *immuunsysteem* constant in actie om deze factoren in ons lichaam te bestrijden. Als voorbeeld nemen we een bacterie. Na het lichaam te zijn binnengedrongen, via bijvoorbeeld een huidwond, wordt de bacterie gesignaleerd door het immuunsysteem wat leidt tot de productie van *antistoffen* (figuur 1). Binding van de geproduceerde antistoffen aan de bacterie zorgt ervoor dat deze herkend kan worden door cellen van het immuunsysteem die gemoeid zijn met de opruiming van lichaamsvreemde substanties, de *effectorcellen*. Op grond van een bepaald gedeelte van antistoffen, het *Fc-domein*, worden deze onderverdeeld in verschillende klassen. De klasse van de geproduceerde antistof is bepalend voor het verdere verloop van de afweerreactie. Voor iedere Fc-domeinklasse bestaat namelijk een specifieke *Fc-receptor*, IgG antistoffen bijvoorbeeld worden specifiek gebonden door Fcγ-receptoren. Doordat verschillende Fc-receptoren op verschillende effectorcel-types voorkomen, bepaald de klasse van het antilichaam welke effectorceltype aangewend wordt ter bestrijding van de indringer.

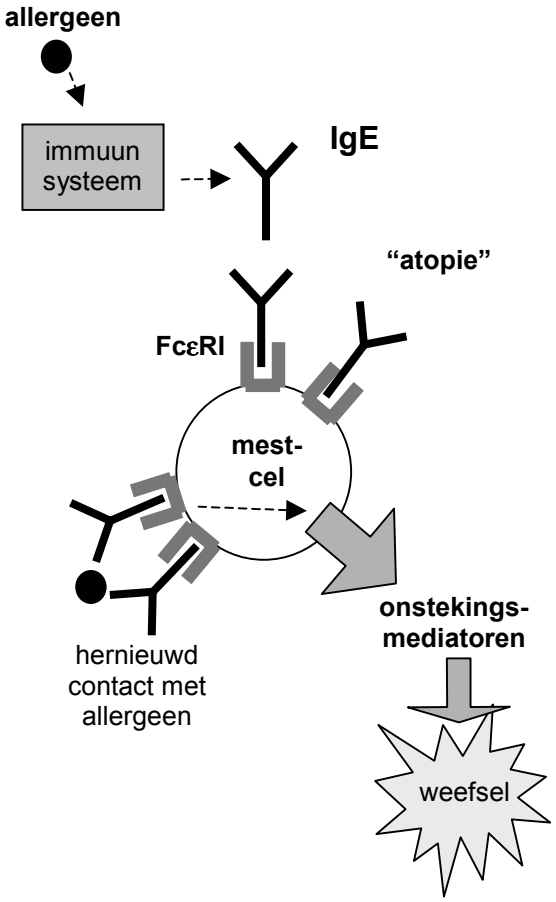


Figuur 1. *Een afweerreactie tegen een lichaamsvreemde substantie*

Sommige lichaamsvreemde stoffen zoals bijvoorbeeld huisstofmijt, zogenaamde *allergenen,* bevatten tot op heden onopgehelderde eigenschappen die leiden tot de productie van *IgE* antistoffen. Het geproduceerde IgE bindt aan de Fc-receptor voor IgE, *FcεRI,* die zich bevindt op *mestcellen* (figuur 2). IgE-productie en –binding aan de mestcel, leidt niet tot verdere reacties maar bewapent deze cel wel voor een volgend contact met hetzelfde allergeen, het betreffende individu is nu *allergisch*[1]. Bij hernieuwd contact wordt het allergeen gebonden door het IgE op de mestcel wat leidt tot activatie van deze cel. De naam “mestcel” werd afgeleid van het Duitse “*Mastzelle*”, wat “weldoorvoede cel” betekent. Hun “volgevreten” uiterlijk danken mestcellen aan het feit dat ze dat ze vol zitten met blaasjes, *granules*, gevuld met ontstekingsmediatoren zoals histamine en serotonine (zie ook hoofdstuk 1 figuur 2). Mestcelactivatie leidt tot het legen van de granules, degranulatie, waarna de vrijgekomen ontstekingsmediatoren in het omliggende weefsel de kenmerken van een allergische reactie, zoals jeuk en zwelling, veroorzaken.
In de westerse wereld is het aantal gevallen van allergische aandoeningen, zoals bijvoorbeeld *astma*, zo sterk toegenomen gedurende de afgelopen eeuw,

[1] De termen atopie en allergie zijn tegenwoordig bijna synoniem en worden willekeurig doorelkaar gebruikt. Kenmerken van atopie zijn: verhoogde hoeveelheden allergeen-specifiek IgE in het bloed en een allergische reactie in een huidtest.

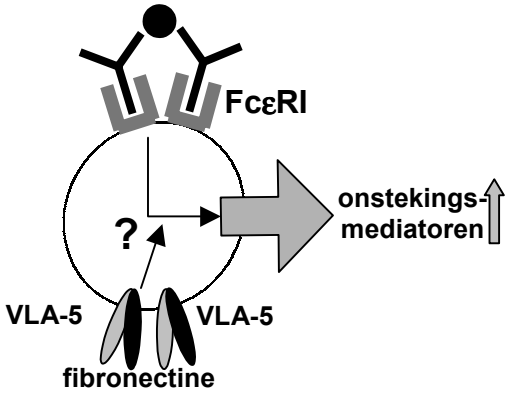
dat vandaag de dag iedereen er wel direct of indirect mee geconfronteerd wordt. Onderzoek heeft aangetoond dat zowel genetische als omgevingsfactoren verantwoordelijk zijn voor het ontstaan van atopie. Deze onderzoeksresultaten geven echter geen verklaring voor de observatie dat bij twee personen die beiden atopisch zijn voor huisstofmijt, dit allergeen bij de één astmatische klachten veroorzaakt en bij de ander niet. Verder kan een allergeen in de ene persoon leiden tot een aandoening in de bovenste, bij een ander in de onderste luchtwegen en bij een derde persoon aanleiding geven tot klachten in meerdere organen. In al deze voorbeelden zijn de personen atopisch, dat wil zeggen, in het lichaam bevinden zich met "IgE-bewapende" mestcellen, maar dit hoeft dus niet per se te leiden tot een allergische reactie. Deze observatie impliceert dat factoren uit de naaste omgeving van de mestcel de activatie van deze cel kunnen moduleren.



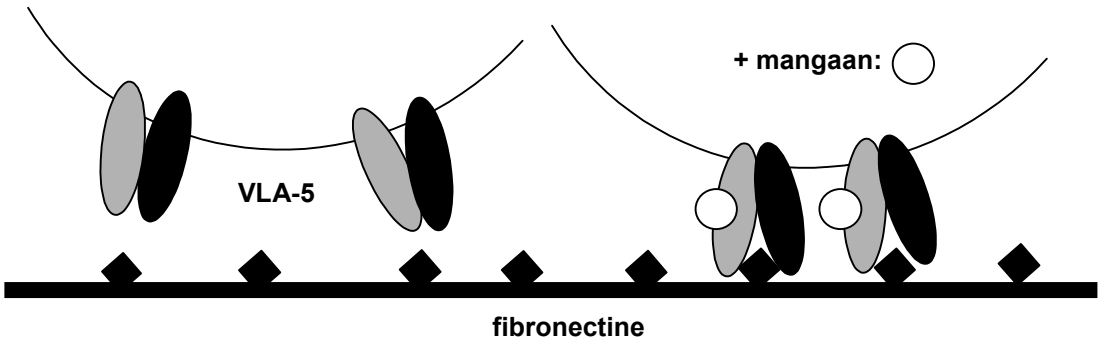
Figuur 2. *Een allergische reactie*

Mestcellen bevinden zich in alle weefsels en organen van het lichaam en zijn daar omgeven door diverse factoren waaronder de *extracellulaire matrix*, een netwerk van eiwitten dat in organen het cement tussen de cellen vormt. *Adhesie,* hechting, van mestcellen aan *fibronectine*, een bestanddeel van de extracellulaire matrix, vindt plaats via oppervlaktemolecuul *VLA-5*. Onderzoek heeft aangetoond dat adhesie van mestcellen aan fibronectine leidt tot verhoogde degranulatie na allergeen-stimulatie.

De manier waarop adhesie aan fibronectine ervoor zorgt dat de uitstoot van mestcelmediatoren verhoogd wordt, is onduidelijk. Opheldering hiervan kan potentiële aangrijpingspunten opleveren voor de ontwikkeling van toekomstige therapie tegen allergie en is daarom onderwerp van dit proefschrift (figuur 3).

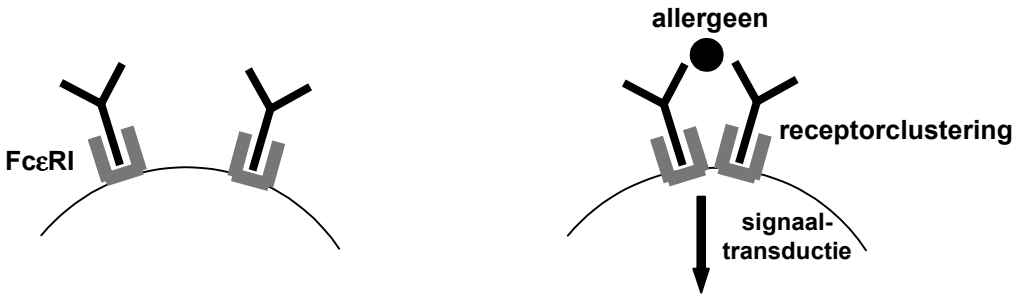


Figuur 3. Vraagstelling van dit proefschrift
"Op welke manier moduleert adhesie aan fibronectine de degranulatie van mestcellen?" (Ofwel: hoe beinvloedt VLA-5 de functie van FcεRI?)



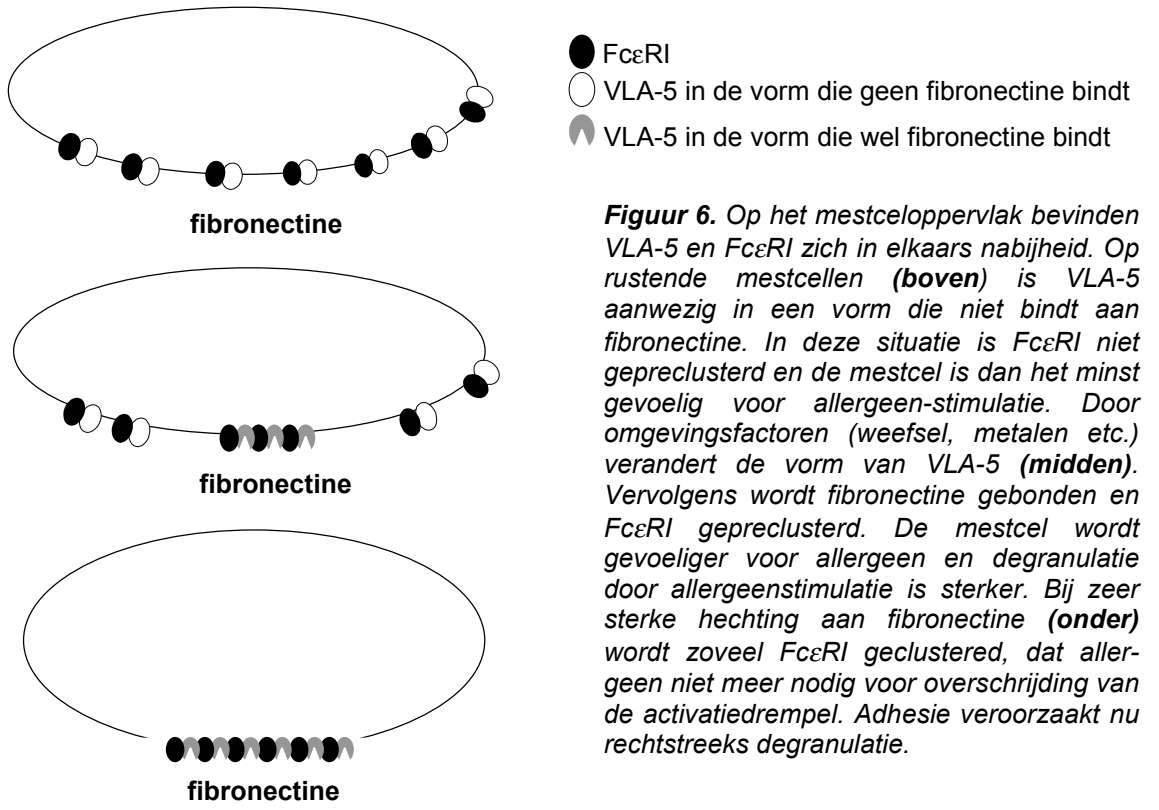
Figuur 4. *In de linker situatie bevindt VLA-5 zich in een vorm die niet bindt aan fibronectine. Toegevoegd mangaan (rechts) bindt aan VLA-5 zodat deze verandert van vorm en adhesie aan fibronectine mogelijk wordt.*

Voor ons onderzoek maakten wij gebruik van mestcellen die gekweekt worden uit het beenmerg van muizen. Op deze cellen bevindt VLA-5 zich in een vorm die niet hecht aan fibronectine. Om adhesie te stimuleren werd mangaan aan deze cellen toegevoegd. Dit metaal bindt aan VLA-5 en zorgt voor een vormverandering die binding aan fibronectine mogelijk maakt (figuur 4).
Onze resultaten laten twee belangrijke fenomenen zien. Ten eerste verhoogt adhesie aan fibronectine mestceldegranulatie als gevolg van allergeen-stimulatie. Ten tweede blijkt dat adhesie aan fibronectine zelf al voldoende om uitstoot van ontstekingsmediatoren op te wekken, zonder dat daar allergeenstimulatie voor nodig is.
FcεRI en VLA-5 zijn beide *receptoren,* eiwitten op het celoppervlak met als functie een signaal van buiten de cel naar binnen door te geven. Deze *signaaltransductie* leidt vervolgens tot een reactie van de cel. Aanwezigheid van allergeen buiten de cel wordt door FcεRI doorgegeven en leidt tot mestceldegranulatie. FcεRI-signaaltransductie komt op gang na clustering van FcεRI door binding aan verschillende bindingsplaatsen op hetzelfde allergeenpartikel (figuur 5). De sterkte van degranulatie wordt bepaald door een aantal factoren. Ten eerste is een minimale hoeveelheid allergeen nodig om voldoende receptoren te clusteren voor mestcelactivatie, dit wordt de *activatiedrempel* van de receptor genoemd. Ten tweede leidt een hogere mate van receptorclustering, bepaald door de hoeveelheid aanwezig allergeen, tot een sterkere activatie. Ten derde geldt dat de kracht van een individuele receptor bepaald wordt door de lengte van zijn verblijftijd in het receptorcluster, ofwel: hoe stabieler een receptorcluster des te sterker de reactie.



Figuur 5. *In afwezigheid van allergeen is FcεRI random verspreid over het mestceloppervlak. IgE-binding aan allergeen veroorzaakt clustering van FcεRI. Deze clustering geldt als signaal voor de mestcel om over te gaan tot degranulatie.*

Onze resultaten laten zien dat VLA-5 en FcεRI zich op het mestceloppervlak in elkaars nabijheid bevinden, en misschien zelfs wel verbonden zijn. Dat houdt in dat wanneer VLA-5 geclusterd wordt door adhesie aan fibronectine, FcεRI meeverhuist naar het VLA-5 cluster en daardoor ook geclusterd wordt (figuur 6). Door deze "pre"-clustering wordt de activatiedrempel van FcεRI verlaagd en wordt de mestcel gevoeliger voor allergeen. Een aan fibronectine gehechte mestcel zal sterker reageren op een bepaalde hoeveelheid allergeen dan een niet gehechte mestcel doordat FcεRI-clustering veroorzaakt door allergeen en die door adhesie optelbaar zijn.
Bij eenzelfde hoeveelheid allergeen, leidt in gehechte mestcellen een grotere hoeveelheid geclusterd FcεRI tot verhoogde degranulatie. Verder zorgt de pre-clustering van FcεRI in het VLA-5 cluster voor verhoogde stabiliteit en daardoor sterkere reactie. Bij een bepaalde mate van VLA-5-adhesie is de preclustering van FcεRI dermate dat de activatiedrempel van deze receptor spontaan overschreden wordt en er degranulatie optreedt zonder dat daar allergeen voor nodig is. De observatie dat mestcellen ontstekingsmediatoren uitscheiden door clustering van VLA-5, zou verklarend kunnen zijn voor het ontstaan van



Figuur 6. *Op het mestceloppervlak bevinden VLA-5 en FcεRI zich in elkaars nabijheid. Op rustende mestcellen* ***(boven****) is VLA-5 aanwezig in een vorm die niet bindt aan fibronectine. In deze situatie is FcεRI niet gepreclusterd en de mestcel is dan het minst gevoelig voor allergeen-stimulatie. Door omgevingsfactoren (weefsel, metalen etc.) verandert de vorm van VLA-5* ***(midden)****. Vervolgens wordt fibronectine gebonden en FcεRI gepreclusterd. De mestcel wordt gevoeliger voor allergeen en degranulatie door allergeenstimulatie is sterker. Bij zeer sterke hechting aan fibronectine* ***(onder)*** *wordt zoveel FcεRI geclustered, dat allergeen niet meer nodig voor overschrijding van de activatiedrempel. Adhesie veroorzaakt nu rechtstreeks degranulatie.*

metaalallergieën, waarvan de manier van ontstaan grotendeels onduidelijk is. Van mangaan, kobalt, nikkel en zink is bekend dat ze een allergische reaktie teweeg kunnen brengen. In onze experimenten vonden wij al deze metalen in staat zijn mestcel adhesie aan fibronectine te veroorzaken. Deze adhesie leidde vervolgens tot degranulatie.
Zoals hierboven reeds beschreven, bevindt VLA-5 zich op rustende mestcellen in een vorm die niet bindt aan fibronectine. In deze cellen is FcεRI dus niet gepreclusterd en deze cellen zijn dus relatief ongevoelig voor allergeenstimulatie. Verandering van VLA-5 naar een vorm die fibro-

nectinebinding mogelijk maakt kan, behalve door de hierboven beschreven metalen, worden geïnduceerd door verscheidene stoffen die plaatselijk in het weefsel aangemaakt worden. De gevoeligheid van de mestcel, correlerend met de vorm van VLA-5 en hechting aan fibronectine, kan dus per locatie in het lichaam gemoduleerd worden door gereguleerde aanmaak van deze stoffen.
Samenvattend kan geconcludeerd worden dat het al dan niet optreden van een allergische respons in een weefsel in een atopische situatie afhangt van de gevoeligheid van de mestcellen ter plaatse. Het uitblijven van een allergische reactie zou in dit geval verklaard kunnen worden door te zeggen dat de hoeveelheid allergeen ter plaatse te laag is om de activatiedrempel van FcεRI te overschrijden.
Door adhesie aan fibronectine te blokkeren, zou een mestcel dus in een relatief minder gevoelige staat gehouden kunnen worden, waardoor een allergische reactie minder snel optreedt. Blokkering van VLA-5 functie biedt volgens het hierboven beschreven model mogelijkheden als toekomstige therapie tegen allergische aandoeningen en verdient daarom nadere bestudering.

Bedankt !!!!

Hè, Hè……. Ja, ja eindelijk is het dan zover!!! Klaar met (ruim) vier jaar werk. De tijd is wel voorbij gevlogen !! En dat komt niet in de laatste plaats door een heleboel mensen uit mijn omgeving waarvan ik er hier een aantal ga bedanken…
Allereerst: de "gabs' **Robbemans** en **Jeroen** ('leuke filmpjes') van het GRT, **Ritsjurt** (limbo) en **Richard** (voetbal), (amu)**Khalil**, **Gerard** (verkeerde club), **Daphne** (auw auw !!), **Betty** (ècht niet gedronken??), **Joossie** (Hollandsche hits uit den ouden doos en improvisaties in de bus), **Annick** (cosmopoliet), **Joris Debby** (Húúúh !!), **Joris K.** (Ameirika-liefhebber), **Hannie** (na twaalven komt 't tot leven..), **Sjef** (my dutch twin from Harrogate), **Gert** ('dezelfde interesses'), **Aletta** (*in vivo…* publicatie?), **Henk** (dat liedje van Rowwen Hèze, dat was volgens mij echt over jou geschreven!!), **Corien** (Fries zonnestraaltje van 120 decibel… bier maakte jou altijd héél gezellig!!), **Bianca** (toch ook wel een beetje gevoelig…), **Cornelie** (lekker effe kankeren op de analistenkamer of tijdens de BMMC kweek … en mijn heerlijkste verkleedpartij ooit !!), **John Henneveld** (datdoejeniegoedjochie), **Andrys** (Friese 'queen of the night'), **Frank** (practische begeleiding en manuscriptcorrecties), **Paul** (manuscriptcorrecties, 'king of typos'), **Nanne** (verhalen van 'vroeger', tegengas in de koffiehoek…. en natuurlijk dat onvergetelijke zeemansduet !!), Robert (W041A rules !!), **Janine** (onze Sjaan, voor de gezelligheid op de kamer en op de fiets…. en af en toe kritisch, wanneer ik dat nodig had!!!), **Arwin** (dubbele stembanden?)en **Lovina** (Hand in Hand) van de superelf, en last but not least: natuurlijk **Edwin** de rode draad door mijn 'carrière'**,** altijd even naar de zesde verdieping voor de dagelijkse 'kijk op de wereld volgens professor doctor ingenieur E. van den Worm'.

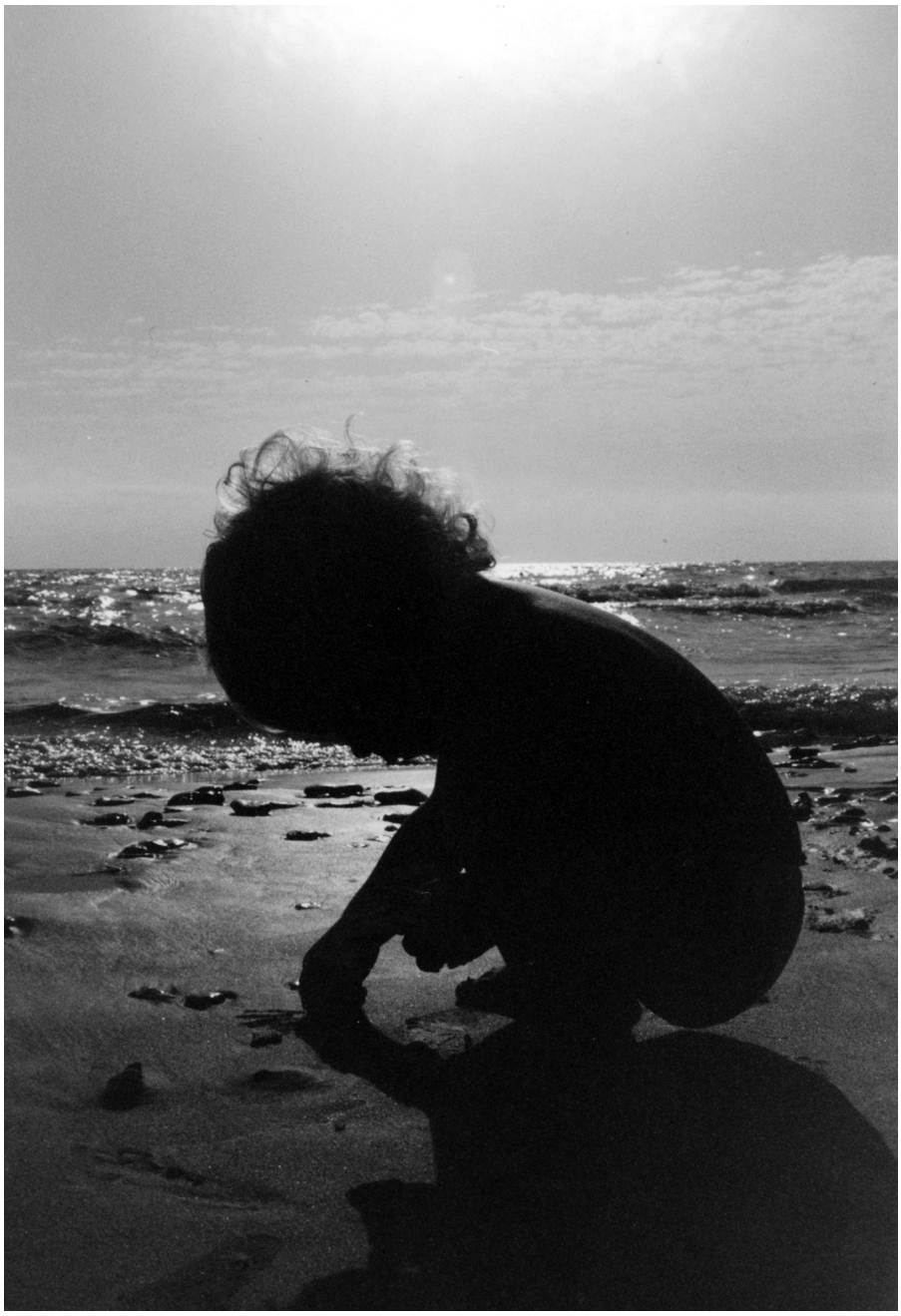
Frans, bedankt voor de mogelijkheid en vrijheid die je me hebt gegeven om me binnen je afdeling te ontplooien, en voor de extra tijd aan het eind, toen het allemaal niet zo afliep zoals we hadden gehoopt…

Andries, bedankt voor het vertrouwen. Jouw kritische kijk op dingen was de benodigde handrem wanneer ik soms wel eens wat kort door de bocht ging met mijn conclusies…. Soms had ik toch wel wat twijfel, maar ik heb jou nooit kunnen betrappen op een spoortje van paniek, dus het zal wel goed gezeten hebben…

Verder iedereen van de afdeling Farmacologie die ik hier nier genoemd heb. Bedankt voor de talloze borrels, feestjes en gezelligheid in de koffiehoek….. dáár zouden ze nou eens een lied over moeten schrijven…

Bep en **Leon**, mijn ouders, ik hoop dat ik jullie een beetje trots gemaakt heb…

En dan natuurlijk m'n meiden: **Marleen** en **Mayke.**
Lieve Marleen, de laatste maanden was ik waarschijnlijk nog chaotischer dan normaal….. bedankt voor je geduld, zonder jou was deze periode veel zwaarder geweest!!
Lieve Mayke, je bent in ieder geval het leukste experiment van de afgelopen periode !!! Omdat ik me een leven zonder jou niet meer kan voorstellen krijg je een blijvend plekje in dit boekje.



Mayke, Terenc Playa, Mallorca, september ’99

Curriculum Vitae

De auteur van dit proefschrift werd op 28 mei 1970 geboren te Vlaardingen. Zijn V.W.O. diploma behaalde hij in mei 1988 aan het Koningin Wilhelmina College in Culemborg. Vervolgens begon hij in 1988 aan een opleiding aan het H.L.O van de Hogeschool Utrecht. Tijdens deze opleiding verrichte hij een stage van 9 maanden bij de afdeling Klinische Immunologie van de Faculteit Geneeskunde aan de Universiteit Utrecht, onder begeleiding van Ing. G. Spierenburg en Dr. M Tilanus. Deze opleiding werd beëindigd met het behalen van het diploma in januari 1992. Vervolgens begon hij in januari 1992 een studie Biologie aan de Universiteit Utrecht. Tijdens deze studie werd een stage verricht van 10 maanden bij de afdeling Experimentele Immunologie van de Faculteit Geneeskunde van de Universiteit Utrecht, onder begeleiding van Dr. S.J. Verbeek en Dr. J.G.J. van de Winkel. Het doctoraal examen werd vervolgens behaald in september 1995. In de periode van augustus 1995 tot en met december 1999 was hij werkzaam als assistant in opleiding bij de afdeling Farmacologie & Pathofysiologie van de Faculteit Farmacie aan de Universiteit Utrecht. Tijdens deze periode werd hij bij zijn werkzaamheden begeleid door Dr. A.S. Koster en Prof. dr. F.P. Nijkamp, en werd het project gefinancierd door GlaxoWellcome Nederland.