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(54) Title: THE USE OF PERITONEAL MAST CELLS AS A HEPARIN SOURCE

(57) Abstract: The present invention relates to peritoneal cell-derived mast cells that are able to respond to IgG stimuli. More specifically, the present invention relates to the capability of peritoneal cell-derived mast cells, expanded in vitro by culturing peri-
toneal cells with SCF, and to produce heparin. Accordingly, the present invention provides a new source of heparin. In an aspect of
the present invention, the heparin producing-cells (or heparin by itself) are used as a part of treatment regimens for treating cardiovas-
cular and/or hematological diseases. Further, the heparin producing-cells (or heparin by itself) may also be used in surgical-related
applications.



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THE USE OF PERITONEAL MAST CELLS AS A HEPARIN SOURCE

FIELD OF THE INVENTION

The present invention relates to peritoneal cell-derived mast cells that are able to respond to IgG antibodies. Further, the present invention relates to cell-models using the peritoneal cell-derived mast cells that respond to IgG stimuli. The cell-models of the present invention are applicable to dermatology, immunology, neurology and rheumatology. In a preferred embodiment, the cell-models of the present invention find utility as a model of multiple sclerosis and rheumatoid arthritis.

The present invention also relates to the capability of peritoneal cell-derived mast cells, obtained and cultured as described herein, to release molecules with proteolytic activity and to produce heparin. Accordingly, the present invention provides a new source of heparin. In an aspect of the present invention, the heparin producing-cells (or heparin by itself) are used as a part of treatment regimens for treating cardiovascular and/or hematological diseases. Further, the heparin producing-cells (or heparin by itself) may also be used in surgical-related applications.

BACKGROUND OF THE INVENTION

Mast cells

The incidence of both allergies and autoimmune diseases has dramatically increased over the last 3-4 decades (Bach 2002. N Engl J Med 347:911-920), calling for better understanding the cellular and molecular immunopathological processes underlying these diseases. A recent advance has been the discovery that antibody-dependent mast cell activation plays a pivotal role not only in IgE-dependent allergies (Nadler et al. 2000. Adv Immunol. 76:325-355), but also, as observed in murine models of arthritis (Lee et al. 2002. Science 297:1689-1692) and encephalitis (Robbie-Ryan et al. 2003. J Immunol 170:1630-1634), in IgG-dependent tissue-specific autoimmune diseases. These findings call for better understanding mast cell biology.

Mast cells, however, represent a minor population in tissues, from which they are not readily purified. Moreover, the biological properties of distinct mast cell populations that reside in different tissues are poorly known. Mast cells are not identical in different tissues, and different mast cells may not secrete the same mediators. Mast cells indeed differentiate and mature in peripheral tissues, into which mast cell-committed bone marrow progenitors migrate and where they receive tissue-specific signals (Gurish and Boyce. 2002. Clin Rev Allergy Immunol 22:107-118). Thus, mucosal-type mast cells develop in the mucosa of the gastro-intestinal tract and in the lamina propria of the respiratory tract

where their differentiation depends on T cell-derived cytokines among which IL-3 is critical (Ihle et al. 1983. J. Immunol. 131:282-287), while serosal-type mast cells develop in the skin, in the submucosa of the respiratory tract, in joint synovia and in the peritoneum where their differentiation primarily depends on fibroblast-derived Stem Cell Factor (SCF) (Galli et al. 1994. Adv. Immunology 55:1-96). Besides being dependent on different growth factors, mucosal- and serosal-type mast cells can be distinguished by their morphology, by their histamine content, and by a differential expression of mast cell-specific chymases and tryptases (Welle 1997. J Leukoc Biol 61:233-245).

Reliable models of defined types of murine mast cells, that can be obtained in high numbers and that can account for immunopathological processes, are therefore a requirement. Such models are not currently available. A good mast cell model should 1) be representative of mature differentiated tissue mast cells, and 2) respond not only to IgE, but also to IgG antibodies.

The only available source of significant numbers of homogenous non-transformed mouse mast cells is Bone Marrow-derived Mast Cells (BMMC). BMMC are often considered as an *in vitro* equivalent of mucosal-type mast cells. They, however, are immature cells whose physiological *in vivo* equivalent is not known. They can indeed reconstitute not only mucosal-type mast cells, but also serosal-type mast cells, when injected intravenously into mast cell-deficient mice (Wershil and Galli. 1994. Adv Exp Med Biol 347:39-54). BMMC may correspond to precursors of the mature tissue mast cells which initiate allergies and inflammatory diseases. BMMC express FcεRI, FcγRIIIA and FcγRIIB (Benhamou et al. 1990. J. Immunol. 144:3071-3077). They release mediators when sensitized by IgE and challenged with antigen, and they have been used extensively for studying FcεRI signaling. They, however, do not or hardly respond to IgG immune complexes. For the above two reasons, BMMC are a suitable model for studying either IgE-induced allergic reactions or IgG-induced mast cell-dependent inflammation. Bone Marrow-derived Mast Cells (BMMC) have been used extensively as a mast cell model. BMMC, however, are immature cells which have no known physiological equivalent in tissues. They do not respond to IgG immune complexes. They are therefore not appropriate for studying the physiopathology of IgE-induced allergies or IgG-induced tissue-specific inflammatory diseases.

Peritoneal mast cells are mature serosal-type mast cells that respond vigorously to IgG immune complexes. Resident peritoneal mast cells are a minor population of differentiated cells that cannot be readily purified. They represent less than 5% of cells recovered in peritoneal washings from normal mice. They vigorously degranulate not only when sensitized with IgE and challenged with antigen, but also when challenged with

preformed soluble IgG immune complexes (Vaz and Prouvost-Danon. 1969. *Progr. Allergy* 13:111-173). They can be separated from other peritoneal cells by techniques based on centrifugation through high-density medium (Sterk and Ishizaka. 1982. *J Immunol* 128:838-843), but in very low numbers only ($\leq 1 \times 10^5$ /mouse), and with a variable purity. Isolating the cells from a large number of mice is not a solution either, since this needs an extensive amount of work, and leads to decreased quality of the cells.

Yamada et al. (2003. *J Invest Dermatol.* 121(6):1425-32) suggests that mature mast cells could be generated by culturing murine fetal skin cells with IL-3 and SCF, and that these cells retained most features of skin mast cells. However, only low numbers of mast cells were obtained using this protocol as well. In addition, these mast cells apparently differentiated in cultures from mast cell precursors.

Preliminary works by Malbec et al. (2004. *J. Immunol.* 173:5086-5094) suggested that cultured peritoneal mast cells might be a model of serosal-type mast cells. In these works, however, Malbec et al. (2004) did not characterize the cultured peritoneal mast cells. Although Malbec et al. (2004) taught that the cultured peritoneal mast cells exhibit at least some of the features of resident peritoneal mast cells, such as activation by IgG antibodies, they did not teach whether other essential features such as the number and the content of the granules are retained. Since the biological properties of primary cells are usually modified upon culture, it was unpredictable whether the peritoneal mast cells disclosed in Malbec et al. (2004) would retain the morphological, phenotypic and functional features of resident peritoneal mast cells upon culture. In addition, Malbec et al. (2004) neither taught whether such cells could be expanded and cultured over a longer period of time, nor whether a number of cells sufficient for using them as a model could be obtained.

There is therefore a need for a mast cell model that is representative of mature differentiated tissue mast cells, and that responds not only to IgE, but also to IgG antibodies.

Heparin

Heparin is a mucopolysaccharide with an average molecular weight ranging from 5,000 to 40,000 Da. The polymeric chain is composed of repeating disaccharide units of D-glucosamine and uronic acid linked by 1→4 interglycosidic bond. The uronic acid residue could be either D-glucuronic acid or L-iduronic acid. Few hydroxyl groups on each of these monosaccharide residues may be sulfated giving rise to a polymer with that is highly negatively charged. The average negative charge of individual saccharide

residues is about 2.3. At least one 3-D structure of heparin exists corresponding to protein data bank code 1HPN.

This highly sulfated glycosaminoglycan finds significant utility in treatment regimens for cardiovascular and/or hematological diseases, as well as in surgical applications. Heparin is widely used as an injectable anticoagulant and has the highest negative charge density of any known biological molecule. It also may be used to form an inner anticoagulant surface on various experimental and medical devices such as test tubes and renal dialysis machines.

Currently, most of the heparin used is isolated from pig intestinal mucosa, from where it is extracted by proteolysis, followed by purification on anion exchange resin (for a review on the various methods for preparing heparin, cf. Duclos, "L'héparine: fabrication, structure, propriétés, analyse", Ed. Masson, Paris, 1984). However, the heparin content in the mucosa-containing aqueous medium is very low and consequently large amounts of mucosa tissue have to be processed.

WO 03/035886 describes an alternative method for producing heparin, wherein heparin is obtained from mast cells derived from porcine foetal liver. However, such mast cells are immature. Immature mast cells comprise fewer granules, and therefore less heparin, than mature mast cells.

There is therefore a need for mature mast cells that can be used for producing heparin.

DETAILED DESCRIPTION OF THE INVENTION

It has been found that peritoneal mast cells can be expanded in culture to generate large numbers of homogenous cells. At least 1×10^8 mast cells could be recovered by culturing the peritoneal cells harvested from two mice for one month.

Surprisingly, these Peritoneal Cell-derived Mast Cells (PCMC) are mature serosal-type mast cells which retain most morphological, phenotypic and functional features of resident peritoneal mast cells. These PCMCs can therefore be used as a mast cell model that is representative of mature differentiated tissue mast cells. As shown in the examples, the PCMCs respond both to IgE and to IgG antibodies and this property is due to mild SHIP1-dependent negative regulation. It has been found that the PCMCs contain and release massive amounts of preformed vasoactive granular mediators and proteases, but secrete no or small amounts of newly formed pro-inflammatory molecules, including eicosanoids, chemokines and cytokines.

In addition, it has surprisingly been found that the granules of cultured PCMC contain massive amounts of heparin. These PCMCs can therefore advantageously be used for producing heparin.

It has further been found that peritoneal mast cells can be expanded *in vitro* by culturing cells in the presence SCF. While BMMCs are obtained by culturing bone-marrow mast cells in the presence of IL-3, it has been unexpectedly been found that peritoneal mast cells can be cultured in the absence of IL-3. On the other hand, peritoneal mast cells can be cultured in the presence of SCF only, which is not the case for bone-marrow mast cells.

More specifically, the experimental data shown in the examples demonstrates that like peritoneal mast cells, PCMC respond to IgG antibodies. IgG immune complex-induced responses depended on FcγRIIIA and were negatively regulated by FcγRIIB. It has been found that a moderate FcγRIIB-dependent negative regulation, due not to a higher FcγRIIIA / FcγRIIB ratio but to a relatively inefficient use of the lipid phosphatase SHIP1, determines this property of PCMC.

PCMC also respond to IgE antibodies. IgE-induced PCMC responses, however, differed quantitatively and qualitatively from BMMC responses. PCMC secreted no or much lower amounts of newly formed lipid mediators, chemokines and cytokines, but they contain and, upon stimulation, they release much higher amounts of preformed granular mediators. PCMC, but not BMMC, also contained and released upon degranulation molecules with a potent proteolytic activity.

Taken together, these properties make PCMC a useful new *in vitro* model for understanding the physiopathology of mast cells in IgE- and IgG-dependent tissue inflammation.

Cells Producing Heparin

The present invention provides peritoneal mast cells, obtained and cultured by the method described herein and in particular in the examples. As shown in Example 2, these cells contain large amounts of heparin. Such cells thus represent a new source of heparin. The produced cells find use both as mast cell models and in production of heparin.

For culture production, peritoneal cells are seeded and cultured in SCF-containing medium. In one embodiment, seeding is at 1×10^6 /ml in complete Opti-MEM supplemented with 4% supernatant of CHO transfectants secreting murine SCF. After twenty four hours (could range from 12-36 hours), non-adherent cells are removed and fresh culture medium added to adherent cells. Three days later (could be 2-4 days), non-adherent cells and adherent cells recovered with trypsin-EDTA, harvested, pelleted and resuspended in

fresh culture medium at a concentration of 3×10^5 /ml. The same procedure is repeated twice a week. The length of the culture and the number of repeats of the foregoing process can be determined by monitoring the amount of heparin produced. In a preferred embodiment, culturing is conducted for at least two weeks. In a more preferred embodiment, culturing is conducted for at least one month.

One month-old cultures of peritoneal cells consisted of $Fc\epsilon RI^+$, Kit^+ , $CD19^-$, $GR1^-$, $Mac1^-$ homogenous cells. This phenotype is characteristic of mast cells. About 1×10^8 mast cells could be recovered, after one month, from cultures seeded with the peritoneal cells from two mice. Large numbers of pure mast cells can therefore be generated by culturing mouse peritoneal cells with SCF. These cells contained granules stained with alcian blue/safranin. PCMC granules were much more intensely colored in red than BMMC granules (Fig. 1A), indicating a higher heparin content.

Of course, heparin may be recovered from the PCMCs by standard isolation and/or purification techniques that have been heretofore utilized for recovering heparin from other sources.

Therefore, the invention is directed to a method of producing a cell producing heparin comprising culturing peritoneal mast cells in the presence of Stem Cell Factor (SCF), in particular fibroblast-derived SCF. Such a culture is preferably performed for a time and under conditions suitable for producing heparin. Such conditions are further detailed below, and may be modified and/or adapted by the skilled in the art.

The term "fibroblast-derived Stem Cell Factor" is used interchangeably with the terms "Stem Cell Factor" and "SCF" within this specification. These terms refer to the natural kit ligand (see e.g. Galli et al. 1994. Adv. Immunology 55:1-96). SCF may be of any origin, e.g. of mouse, human or porcine origin. The term "fibroblast-derived" may refer in particular to a SCF isolated from fibroblasts. However, SCF could be isolated from any other cell type since the sequence of the SCF protein is the same within a given mammal (e.g. human, porcine or mouse) irrespective of the cell expressing SCF. The sequence of SCF is well-known in the art (see e.g. Swiss-Prot entries P05532, P10721 and Q2HWD6 for SCF of mouse, human and porcine origin respectively). Preferably, SCF is derived from the same organism as the peritoneal mast cells that are cultured in vitro.

The SCF to be added to the cell culture may be produced by any method known in the art. It may for example be obtained from the supernatant of recombinant cells producing SCF such as CHO-KL cells (Plo et al. 2001 Oncogene. 20(46):6752-63), or from a supplier such as R&D (Abingdon, UK). In a preferred embodiment, SCF is produced by CHO transfectants secreting murine, porcine or human SCF. In other terms, the SCF

may be added to the culture medium by addition of the supernatant of SCF-secreting recombinant cells (e.g. CHO-KL).

The peritoneal mast cells may be cultured e.g. in the presence of about 10-1000, 20-800, 30-500, 40-400, 50-300, 60-200, 70-100 or 75-90 ng/ml of SCF. In one embodiment, CHO-KL cells are seeded at 1×10^5 /ml in RPMI medium supplemented with 10% FCS + penicillin/streptomycin and cultured for 4 days. Cell-free supernatant is harvested and added to peritoneal cell cultures. As assessed by ELISA, the conditioned medium thus obtained contained about 80 ng/ml of SCF.

The peritoneal mast cells may be of any origin. Preferably, the peritoneal mast cells are murine, porcine or human peritoneal mast cells. The peritoneal mast cells are most preferably obtained from a mouse.

As used herein, the terms "PCMC" and "peritoneal cell-derived mast cell" refer to a cell expanded and/or cultured in accordance with the invention. The term "peritoneal mast cell" refers to a cell that is isolated from an organism, but that has not been cultured.

The cells may be cultured for instance for at least 2 weeks, at least one month or at least two months.

Preferred culture conditions suitable for producing heparin are detailed in the examples.

In one preferred embodiment, the method of culturing comprises the steps of:

- a) providing peritoneal cells;
- b) seeding said peritoneal cells in a liquid culture medium supplemented with a supernatant obtained from a culture of CHO transfectants secreting murine SCF;
- c) twelve to thirty-six hours later, removing non-adherent cells and adding fresh liquid culture medium to adherent cells;
- d) two to four days later, recovering non-adherent and adherent cells, and harvesting, pelleting and resuspending said non-adherent and adherent cells in fresh liquid culture medium; and
- e) repeating step (d) twice a week.

Step (a) may be carried out e.g. by harvesting peritoneal cells by peritoneal washing. Step (b) may be carried out by seeding e.g. 1×10^6 cells/ml in a complete liquid culture medium such as Opti-MEM. The liquid culture medium may be supplemented with 1-10%, 3-6% or preferably 4% supernatant of CHO transfectants secreting SCF, in particular murine, porcine or human SCF. The final concentration of SCF in the culture medium may be e.g. of 10-1000, 20-800, 30-500, 40-400, 50-300, 60-200, 70-100 or 75-90 ng/ml of SCF. At step (d), the cells may be recovered e.g. with trypsin-EDTA, and the

cells may then be seeded in the fresh medium at a concentration of 3×10^5 cells/ml. The steps are preferably carried out at 37°C. SCF is preferably added to the culture medium used at each of steps (b), (c) and (d). The term "fresh" liquid culture medium refers to a liquid culture medium in which cells have not yet been cultivated.

Such conditions are suitable for recovering about 1×10^8 mast cells after one month of culture. However, it is within the knowledge of the skilled in the art to slightly modify these conditions without impairing the number of cells recovered and/or the amount of heparin produced by the cells.

In one embodiment, the heparin-producing cell is further immortalized in order to facilitate its cultivation. Such immortalized cells do not constitute a suitable mast cell model, but may be useful in the frame of industrial heparin production. The cell may be immortalized using any method well-known in the art such as viral transformation using Epstein-Barr virus (EBV), Simian virus 40 (SV40) T antigen, adenovirus E1A and E1B, or human papillomavirus (HPV) E6 and E7, or through expression of the telomerase reverse transcriptase protein (TERT). While such an immortalized cell may lose some of the properties of the primary cell, the skilled in the art can easily verify that it retains the capacity to produce massive amounts of heparin using e.g. staining with alcian blue/safranin.

Use of the heparin-producing cells as a model for mast cells

The present invention provides non-transformed peripheral mast cells that are able to respond to IgG stimuli. These cells find application as cell-models in dermatology, immunology, neurology and rheumatology, mainly in the field of autoimmune diseases such as multiple sclerosis and rheumatoid arthritis.

As shown in the examples, Peritoneal Cell-derived Mast Cells (PCMC) are mature serosal-type mouse mast cells which retain most morphological, phenotypic and functional features of peritoneal mast cells. Like peritoneal mast cells, PCMC respond to IgG antibodies. IgG immune complex-induced responses depended on FcγRIIIA and were negatively regulated by FcγRIIB. It has been found that a moderate FcγRIIB-dependent negative regulation, due not to a higher FcγRIIIA/FcγRIIB ratio, but to a relatively inefficient use of the lipid phosphatase SHIP1, determines this property of PCMC. PCMC also respond to IgE antibodies. IgE-induced PCMC responses, however, differed quantitatively and qualitatively from BMMC responses. PCMC secreted no or much lower amounts of lipid mediators, chemokines and cytokines, but they contained and released much higher amounts of preformed granular mediators. PCMC, but not BMMC, also contained and released upon degranulation molecules with a potent proteolytic activity.

These properties make PCMC a useful new model for understanding the physiopathology of mast cells in IgE- and IgG-dependent tissue inflammation.

Further, as shown in the examples, mast cells are members of the innate immune system, but because they express Fc receptors (FcRs), they can be engaged in adaptive immunity by antibodies. Mast cell FcRs include immunoglobulin E (IgE) and IgG receptors and, among these, activating and inhibitory receptors. The engagement of mast cell IgG receptors by immune complexes may or may not trigger cell activation, depending on the type of mast cell. The coengagement of IgG and IgE receptors results in inhibition of mast cell activation. The Src homology-2 domain-containing inositol 5-phosphatase-1 is a major effector of negative regulation. Biological responses of mast cells depend on the balance between positive and negative signals that are generated in FcR complexes.

The exact contribution of human mast cell IgG receptors in allergies remains to be clarified. Increasing evidence indicates that mast cells play critical roles in IgG-dependent tissue-specific autoimmune diseases. Convincing evidence was obtained in murine models of multiple sclerosis, rheumatoid arthritis, bullous pemphigoid, and glomerulonephritis. In these models, the intensity of lesions depended on the relative engagement of activating and inhibitory IgG receptors. In vitro models of mature tissue-specific murine mast cells are needed to investigate the roles of mast cells in these diseases. One such model may allow unraveling unique differentiation/maturation-dependent biological responses of serosal-type mast cells.

One distinctive property of PCMCs is their ability to respond to stimulation by IgG antibodies. IgG immune complex-induced PCMC activation depended on FcγRIIIA, and, as expected, it was negatively regulated by FcγRIIB. Surprisingly, the differential responses of PCMCs and BMMCs to IgG immune complexes could not be accounted for by a different ratio of activating/inhibitory FcγRs. FcγRIIIA-dependent cell activation was as efficient in both cells, but FcγRIIB-dependent negative regulation was more efficient in BMMCs than in PCMCs, revealing that previously unsuspected mechanisms may control FcγRIIB-dependent negative regulation. This difference could be accounted for by an inefficient use of SHIP1 by PCMCs. Indeed, the deletion of SHIP1 differentially affected the responses of BMMCs and PCMCs to IgE and IgG antibodies. These results indicate that differentiation/maturation-dependent regulatory mechanisms that control SHIP1-mediated negative regulation of cell activation determine the ability of mast cells to respond to IgG immune complexes. This mechanism may apply to human basophils.

Another major difference between the two cell types is in their secretory responses to FcεRI aggregation: early responses are more robust than late responses in PCMCs, whereas late responses are more robust than early responses in BMMCs. Although the

two groups of cells released comparable percentages of granular mediators, PCMCs contained at least 100-fold higher amounts of histamine than BMMCs. Consequently, PCMCs released more granular mediators than BMMCs within the first minutes of activation by FcεRI. Notably, PCMCs, but not BMMCs, contained and released a highly efficient proteolytic activity upon stimulation by FcεRI. By contrast, PCMCs produced much lower amounts of lipid mediators during the first half hour of stimulation and no macrophage inflammatory protein-1α during the first hours. PCMCs also secreted much less TNF-α and synthesized fewer cytokines than BMMCs.

The properties of PCMCs may be informative in studies on the role of serosal-type mature mast cells in tissue inflammation. Mast cells that reside in tissues involved in allergies and inflammatory diseases are indeed of the serosal type, and skin mast cells and synovial mast cells play critical roles in IgE-dependent skin allergies and in IgG-induced arthritis, respectively. The massive amounts of vasoactive mediators and proteases released by serosal-type mast cells within minutes should greatly facilitate the constitution of a local inflammatory infiltrate. Histamine was shown to mediate IgG immune complex-induced, FcγRIIIA-dependent inflammation in K/B×N serum-induced arthritis. Mast cell proteases induce a variety of biological effects, most of which are triggered by the activation of protease-activated receptor-2 (PAR-2). PAR-2 activation is involved in the control of blood pressure and plasma extravasation, in neutrophil infiltration and proliferation, in the induction of pain, and by stimulating the phagocytosis of melanosomes by keratinocytes, in the control of skin pigmentation. PAR-2 also induces keratinocytes to proliferate and to secrete cytokines. Interestingly, PAR-2 is upregulated in asthma and rheumatoid arthritis.

Thus, the present invention provides a role for PCMCs as a model for studying the role of serosal-type mature mast cells in tissue inflammation arising from allergy and/or inflammatory diseases. In particular, the present invention provides a role for PCMCs as a model for studying the role of serosal-type mature mast cells in multiple sclerosis and rheumatoid arthritis. Thus, the present invention provides a model system to study the physiopathology of inflammatory pathologies induced by IgE and IgG.

The invention therefore provides a method of preparing an in vitro model system to study the role of serosal-type mature mast cells in tissue inflammation arising from allergy and/or inflammatory diseases comprising preparing a culture of peritoneal cell-derived mast cells by culturing peritoneal cells with SCF, and activating said peritoneal cell-derived mast cells with an IgG or an IgE antibody. The peritoneal cells are cultured with SCF for a time and under conditions suitable to expand peritoneal cell-derived mast cells,

for example according to any one of the methods described in the above paragraph entitled "Cells producing heparin".

In another embodiment of the present invention and based on the foregoing, it is an object of the present invention to provide a model system to study anti-inflammatory effect of therapeutic drugs. In this model, the anti-inflammatory effect of candidate therapeutic drugs can be assessed by monitoring the effect of IgE and/or IgG, preferably IgG, activation of the peritoneal mast cells in the presence of candidate drugs as compared to the effect of activation of the peritoneal mast cells in the absence of candidate drugs. This model system finds an application as a method for drug evaluations together or before clinical assays. For example, it may be used in screening assays for identifying anti-inflammatory compounds and/or in functional cell-based assays for characterizing a potential drug during preclinical trials

Such a model system may for example be based on a method of identifying the anti-inflammatory effect of a candidate therapeutic drug comprising the steps of:

- a) preparing a culture of peritoneal cell-derived mast cells by culturing peritoneal cells with SCF;
- b) splitting the culture of peritoneal cell-derived mast cells into two sub-cultures;
- c) activating said peritoneal cell-derived mast cells in one sub-culture with an IgG or an IgE antibody in the presence of said candidate therapeutic drug;
- d) activating said peritoneal cell-derived mast cells in the other sub-culture with an IgG or an IgE antibody in the absence of said candidate therapeutic drug; and
- e) comparing the effect of IgG or IgE activation of the peritoneal cell-derived mast cells in the presence of said candidate therapeutic drug as to the effect of activation of the cell-derived peritoneal mast cells in the absence of said candidate therapeutic drug.

In this method, a reduced activation of the peritoneal cell-derived mast cells in the presence of said candidate therapeutic drug as compared to the activation of the peritoneal cell-derived mast cells in the presence of said candidate therapeutic drug indicates that said candidate therapeutic drug has an anti-inflammatory effect.

The effect of IgG or IgE activation of the peritoneal cell-derived mast cells may be assessed by any method well-known in the art. It may for example be assessed by measuring β -hexamidase release, histamine release, LTC₄ production, MIP-1 α secretion or TNF- α secretion as described in Example 1.

The peritoneal cell-derived mast cells may be activated with an IgG or an IgE antibody. Since the ability to be activated by an IgG antibody is a characteristic feature of PCMCs as compared to BMMCs, the mast cells are preferably activated with an IgG antibody. Most preferably, the antibody is an IgG1, an IgG2a or an IgG2b antibody.

At step (a), the peritoneal cells are cultured with SCF for a time and under conditions suitable to expand peritoneal cell-derived mast cells, for example according to any one of the methods described in the above paragraph entitled "Cells producing heparin".

The peritoneal cell-derived mast cells for use as mast cell models are preferably of murine origin.

Use of the heparin-producing cells for producing heparin

It has been found that PCMCs prepared as described hereabove contain massive amounts of heparin. More specifically, upon staining with alcian blue/safranin, PCMC granules were much more intensely colored in red than BMMC granules (see Fig. 1A), indicating a higher heparin content. When examined by electron microscopy, more numerous granules were seen in PCMC. They were larger, more homogenous, and had a higher density than BMMC granules (see Fig. 1B).

Noticeably, PCMC contained about 8-fold more β -hexosaminidase and about 100 to 400-fold more histamine than BMMC (Fig. 2, left panel). As a consequence, when sensitized with IgE and challenged for 10 min with an antigen, PCMC released much higher absolute amounts of β -hexosaminidase and even higher amounts of histamine than BMMC (Fig. 2, right panel). Histamine being linked to the granules, it is likely that it reflects the important granule content of PCMCs. It is therefore likely that PCMC contains about 100 to 400-fold more heparin than BMMC.

Thus PCMC contain more numerous granules, which have higher granule content, than BMMC. Therefore, the PCMC prepared as described herein can advantageously be used for producing heparin.

The invention is thus directed to a method of producing heparin comprising producing a heparin-producing cell according to any of the methods described in the above paragraph entitled "Cells producing heparin", and recovering said heparin from the culture containing said heparin. This method may further comprise the step of purifying said heparin.

The invention is also directed to a method for producing a low molecular weight heparin comprising producing heparin by the above method of producing heparin and chemical or enzymatic depolymerizing said heparin to produce said low molecular

weight heparin having a weight-average molecular weight ranging from 1,000 to 10,000 daltons.

Recovering and purification of heparin

5 The heparin may be recovered from the culture and/or purified by any method well-known in the art.

For example, the cells can be harvested and separated from the culture medium, generally by centrifugation or filtration. Various centrifugation systems can be used. As a non-limiting example, mention will be made to those described by Vogel and Todaro
10 (Fermentation and Biochemical Engineering Handbook, 2nd Edition, Noyes Publication, Westwood, N.J., USA). Alternatively or in combination with centrifugation, the separation may be carried out by tangential microfiltration using membranes the porosity of which is less than the average diameter of the cells (5 to 20 μm) while at the same time allowing the other compounds in solution/suspension to pass through. The rate of
15 tangential flow and the pressure applied to the membrane will be chosen so as to generate little shear force (Reynolds number less than 5 000 sec^{-1}) in order to reduce clogging of the membranes and to preserve the integrity of the cells during the separating operation. Various membranes can be used, for example spiral membranes (AMICON, MILLIPORE), flat membranes or hollow fibers (AMICON, MILLIPORE,
20 SARTORIUS, PALL, GF). It is also possible to choose membranes the porosity, the charge or the grafting of which makes it possible to perform a separation and a first purification with respect to possible contaminants which may be present in the culture medium, such as cell proteins, DNA, viruses, or other macromolecules.

The heparin is preferably recovered from the culture by a method which keeps the
25 mast cells granules, which contain heparin, intact. This can for example be made by adding water, thereby inducing an osmotic shock. The intact heparin-containing granules can then be separated from the sample by any method well-known in the art. The methods described e.g. in Krüger et al. (Experimental Cell Research Volume 129, Issue 1, September 1980, pages 83-93) and in Lagunoff and Rickard (American Journal of
30 Pathology, Vol. 154, No. 5, May 1999) may for example be used.

The heparin can be harvested from the culture medium after lysis or degranulation of the cells. When the heparin has been released from the intracellular content, by degranulation or lysis of all or some of the mast cells, and is present in the culture medium at the time of the separation step, the use of membranes with a smaller porosity may also
35 be envisaged. In this case, the cell separation is combined with a step consisting of ultrafiltration on one or more membranes, the organization and the porosity of which make

it possible to concentrate the heparin and to separate it from the other species present in the medium, as a function of the size and the molecular weight and, optionally, of the electrical charge, or of the biological properties. In the context of this embodiment, the cutoff threshold of the membranes is preferably between 1000 and 5 kDa. Use may be made of membrane systems similar to those used for microfiltration, for example spiral membranes, flat membranes or hollow fibers. Use may advantageously be made of membranes which make it possible to separate and purify the heparin due to their charge properties or their properties of grafting of ligands exhibiting affinity for heparin (for example antibodies, ATIII, lectin, peptides, nucleotides, etc.). The degranulation may be caused by the binding of specific ligands to the receptors present at the surface of the mast cells, for example IgG or IgE, or by other agents including but not limited to cytotoxic agents, enzymes, polysaccharides, lectins, anaphylatoxins, basic compounds (compound 48/80, substance P, etc.), calcium (A23187 ionophore, ionomycin, etc.). The mast-cell lysis can be induced, for example, by osmotic shock using hypotonic or hypertonic solutions, by thermal shock (freezing/thawing), by mechanical shock (for example sonication or pressure variation), by the action of chemical agents (NaOH, THESIT.TM., NP40.TM., TWEEN 20.TM., BRIJ-58.TM., TRITON X.TM.-100, etc.) or by enzyme lysis (papain, trypsin, etc.), or by a combination of two or more of these methods.

To extract and purify the heparin from the cell lysate and to separate the heparin chains from the other GAGs present in the extraction medium, the skilled in the art may use methods similar to those used in the context of the extraction and purification of heparin from animal tissues, which are known in themselves, and described in general works such as the manual by Duclos ("L'héparine: fabrication, structure, propriétés, analyse", Ed. Masson, Paris, 1984).

By way of non-limiting examples, in order to separate the heparin from the nucleic acids and from the cell proteins, and to solubilize it, i.e. to break the bonds with the serglycine core:

- the cell lysate can be subjected to one or more enzyme digestions (pronase, trypsin, papain, etc.);
- the heparin-protein bonds can be hydrolyzed in alkali medium, in the presence of sulfates or chlorides;
- a treatment in acid medium (for example with trichloroacetic acid under cold conditions) can be carried out in order to destroy the nucleic acids and the proteins originating from the cells, to which is added the use of an ionic solution which makes it possible to dissociate the GAG-protein interactions; and

- an extraction with guanidine, after enzyme hydrolysis, can be carried out to purify the solubilized heparin. It is for example possible to precipitate it with potassium acetate, with a quaternary ammonium, or with acetone.

These purification steps may optionally be followed or replaced with one or more chromatography steps, in particular an anion exchange chromatography step and/or an affinity chromatography step. For example, the purification may be carried out as described by Volpi (J Chromatogr B Biomed Appl. 1996 Oct 11;685(1):27-34).

Use of heparin as a therapeutic compound

Heparin, which is a highly sulfated glycosaminoglycan, finds significant utility in treatment regimens for cardiovascular and/or hematological diseases, as well as in surgical applications. Heparin is widely used as an injectable anticoagulant and has the highest negative charge density of any known biological molecule. It also may be used to form an inner anticoagulant surface on various experimental and medical devices such as test tubes and renal dialysis machines. In an aspect of the present invention, the heparin producing-cells (or heparin by itself) are used as a part of treatment regimens for treating cardiovascular and/or hematological diseases (see above). Further, the heparin producing-cells (or heparin by itself) may also be used in surgical-related applications (see above). In addition, in the present invention the heparin may be isolated and/or purified from the cells producing the same and used in the isolated and/or purified form.

Additional applications for which heparin finds application, include:

- Anticoagulant therapy in prophylaxis and treatment of venous thrombosis and its extension (e.g., deep-vein thrombosis).
- In a low-dose regimen for prevention of post-operative deep venous thrombosis and pulmonary embolism in patients undergoing major abdomino-thoracic surgery or who for other reasons are at risk of developing thromboembolic disease.
- Prophylaxis and treatment of pulmonary embolism.
- Atrial fibrillation with embolization.
- Diagnosis and treatment of acute and chronic consumptive coagulopathies (disseminated intravascular coagulation).
- Prevention of clotting in arterial and cardiac surgery.
- Prophylaxis and treatment of peripheral arterial embolism.
- As an anticoagulant in blood transfusions, extracorporeal circulation, dialysis procedures and in blood samples for laboratory purposes.
- Acute coronary syndrome (e.g., myocardial infarction).

- Treatment of interstitial cystitis.

The key structural unit of heparin is thought to be a unique pentasaccharide sequence (below). This sequence consists of three D-glucosamine and two uronic acid residues. The central D-glucosamine residue contains a unique 3-O-sulfate moiety that is rare outside of this sequence. Four sulfate groups on the D-glucosamines are found to be critical for retaining high anticoagulant activity. Elimination of any one of them results in a dramatic reduction in the anticoagulant activity. Removal of the unique 3-O-sulfate group results in complete loss of the anticoagulant activity. Removal of sulfate groups other than the critical ones seems to not affect the anticoagulant activity.

Heparin, contains a unique five-residue sequence

GlcNAc/NS(6S)-GlcA-GlcNS(3S,6S)-IdoA(2S)-GlcNS(6S)

- **GlcNAc** = 2-deoxy-2-acetamido- α -D-glucopyranosyl
- **GlcA** = β -L-glucuronic acid
- **GlcNS(3S,6S)** = 2-deoxy-3,6-di-O-sulfo-2-(sulfoamino)- α -D-glucopyranosyl
- **IdoA(2S)** = 2-O-sulfo- α -L-iduronic acid
- **GlcNS(6S)** = 2-deoxy-2-sulfamido- α -D-glucopyranosyl-6-O-sulfate

which is recognized by and forms a high-affinity complex with antithrombin (e.g., antithrombin III). Upon binding heparin, antithrombin III undergoes a conformational change, which results in its active site being exposed. The activated antithrombin III resulting from the formation of antithrombin - heparin complex becomes a more rapid acting inhibitor of thrombin, factor X, and several other coagulation enzymes (IX, XI & XII).

The formation of antithrombin - heparin complex greatly increases the rate of inhibition of two principle procoagulant proteases, factor Xa and thrombin. The normally slow rate of inhibition of factor Xa and thrombin ($\sim 10^3 - 10^4 \text{ M}^{-1}\text{s}^{-1}$) by antithrombin alone is increased about a 1,000-fold by heparin (Bjork I, Lindahl U. (1982). "Mechanism of the anticoagulant action of heparin". Mol. Cell. Biochem. 48: 161-182).

The conformational change in antithrombin III on heparin binding mediates its inhibition of factor Xa. For thrombin inhibition however, thrombin must also bind to the heparin polymer at a site proximal to the pentasaccharide. The high negative charge density of heparin contributing to its very strong electrostatic interaction with thrombin (Cox, M.; Nelson D. (2004). *Lehninger, Principles of Biochemistry*. Freeman, 1100). The formation of a ternary complex between antithrombin III, thrombin and heparin results in the inactivation of thrombin. For this reason heparin's activity against thrombin is size dependent, the ternary complex requiring at least 18 saccharide units for efficient formation (Petitou M, Herault JP, Bernat A, Driguez PA, et al. (1999). "Synthesis of

Thrombin inhibiting Heparin mimetics without side effects". *Nature* 398: 417-422). In contrast anti factor Xa activity only requires the pentasaccharide binding site.

Accelerated inactivation of both the active forms of proteases prevents the subsequent conversion of fibrinogen to fibrin that is crucial for clot formation. With the foregoing in mind, heparin acts to prevent the formation of clots and extension of existing
5 clots within the blood. However, heparin does not break down clots that have already formed (the tissue plasminogen activator will), it allows the body's natural clot lysis mechanisms to work normally to break down clots that have already formed.

Because of its highly acidic sulfate groups, heparin exists as the anion at
10 physiologic pH and is usually administered as the sodium salt. However, the present invention is not limited to the sodium salt. Other salt forms, including but not limited to the lithium salt and ammonium salt, may also be used.

Heparin may be administered parenterally. Alternatively, heparin may be administered by intravenous injection or subcutaneous (under the skin) injection.
15 Intramuscular injections (into muscle) are generally avoided because of the potential for forming hematomas. However, the present invention also contemplates intramuscular injection as a means of delivery. Topical administration may also be employed.

Because of its short biologic half-life of approximately one hour, it is desired that heparin be administered frequently or as a continuous infusion. As an example of
20 continuous infusion, intravenous administration is mentioned.

The dosage of heparin sodium should be adjusted according to the patient's coagulation test results. For example, when heparin sodium is given by continuous intravenous infusion, the coagulation time may be determined approximately every four hours in the early stages of treatment. When the drug is administered intermittently by
25 intravenous injection coagulation tests may be performed before each injection during the early stages of treatment and at appropriate intervals thereafter.

Dosage is considered adequate when the activated partial thromboplastin time (APTT) is 1.5 to 2 times normal or when the whole blood clotting time is elevated approximately 2.5 to 3 times the control value. After deep subcutaneous (intrafat)
30 injections tests for adequacy of dosage are best performed on samples drawn four to six hours after the injections.

Periodic platelet counts, hematocrits and tests for occult blood in stool are recommended during the entire course of heparin therapy, regardless of the route of administration.

Although dosage must be adjusted for the individual patient according to the results of suitable laboratory tests, the dosage schedules of the following table may be used as guidelines.

METHOD OF ADMINISTRATION	FREQUENCY	RECOMMENDED DOSE*
Deep Subcutaneous (Intrafat) Injection	Initial Dose	5,000 Units by IV injection followed by 10,000 to 20,000 Units of a concentrated solution, subcutaneously
A different site should be used for each injection to prevent the development of massive hematoma	Every 8 hours	8,000 to 10,000 Units of a concentrated solution
	or	
	Every 8 hours	15,000 to 20,000 Units of a concentrated solution
Intermittent Intravenous Injection	Initial Dose	10,000 Units, either undiluted or in 50 to 100 mL of 0.9% Sodium Chloride Injection, USP
	Every 4 to 6 hours	5,000 to 10,000 Units, either undiluted or in 50 to 100 mL of 0.9% Sodium Chloride Injection, USP
Continuous Intravenous Infusion	Initial Dose	5,000 Units by IV injection
	Continuous	20,000 to 40,000 Units/ 24 hours in 1,000 mL of 0.9% Sodium Chloride Injection USP (or in any compatible solution for infusion)

5 * Based on 150 lb. (68 kg) patient. Dosage should scale relatively according to the mass of the patient.

Heparin referred to above relates to that directly obtained from mastocytes. In a preferred embodiment, the heparin-producing cells are used directly. In another preferred
10 embodiment, the heparin produced by these cells is isolated and/or purified by conventional techniques.

It is noted that the effects of natural, or *unfractionated*, heparin can be difficult to control dosing and administration due to the required constant monitoring resulting from the relatively short half-life. Thus, after a standard dose of unfractionated heparin,
15 coagulation parameters must be monitored very closely to prevent over- or under-anticoagulation.

Low molecular weight heparins, in contrast, consist of only short chains of polysaccharide. Low-molecular-weight heparin is derived from standard heparin through either chemical or enzymatic depolymerization. Whereas standard heparin has a weight-
20 average molecular weight of 5,000 to 40,000 daltons, low-molecular-weight heparin

ranges from 1,000 to 10,000 daltons, resulting in properties that are distinct from those of traditional heparin. The following methods of production of low-molecular-weight heparin from standard heparin have been used in the art and may be used in the present invention:

- 5 • Oxidative depolymerisation with hydrogen peroxide.
- Deaminative cleavage with isoamyl nitrite.
- Alkaline beta-eliminative cleavage of the benzyl ester of heparin.
- Oxidative depolymerisation with Cu^{2+} and hydrogen peroxide.
- Beta-eliminative cleavage by the heparinase enzyme.
- 10 • Deaminative cleavage with nitrous acid.

For example, processes for depolymerizing heparin and obtaining low molecular weight heparin are disclosed in WO 2006/011179, EP 0014184, EP 0040144, EP 0076279 and EP 0121067.

15 With respect to the distinct properties of low-molecular-weight heparin from those of traditional heparin, the following is mentioned. Low-molecular-weight heparin binds less strongly to protein, has enhanced bioavailability, interacts less with platelets and yields a very predictable dose response, eliminating the need to monitor the aPPT. Low-molecular-weight heparin, like standard heparin, binds to antithrombin III; however, low-molecular-weight heparin inhibits thrombin to a lesser degree (and Factor Xa to a greater
20 degree) than standard heparin (Hirsch J, Raschke R, Warkentin TE, Dalen JE, Deykin D, Poller L. Heparin: mechanism of action, pharmacokinetics, dosing considerations, monitoring, efficacy and safety. Chest 1995;108(4 suppl): 258-75).

 The clinical advantages of low-molecular-weight heparin include predictability, dose-dependent plasma levels, a long half-life and less bleeding for a given antithrombotic
25 effect (Warkentin TE, Levine MN, Hirsch J, Horsewood P, Roberts RS, Gent M, et al. Heparin-induced thrombocytopenia in patients treated with low-molecular-weight heparin or unfractionated heparin. N Engl J Med 1995;332:1330-5). Furthermore, immune-mediated thrombocytopenia is not associated with short-term use of low-molecular-weight heparin (Warkentin et al), and the risk of heparin-induced osteoporosis may be lower than
30 the risk with the use of standard heparin. Low-molecular-weight heparin is administered according to body weight once or twice daily. Thus, continued monitoring is not necessary.

 With respect to dosing and administration, as well as the disorders to be treated with the low-molecular-weight heparin, reference is made to the foregoing discussion
35 relating to the natural heparin. However, it is also noted that the dosing of low-molecular-

weight heparin may be modified as appropriate for the age and condition of the patient, as well as for the disorder to be treated. In view of the increased stability of the low-molecular-weight heparin, in a preferred embodiment, the low-molecular-weight heparin is administered to a subject in need of such treatment one to six times daily, preferably one
5 or two times daily, with a total daily dosage ranging from 30 to 150 mg per day, preferably from 45 to 125 mg per day. In a preferred dosage regimen, the amount to be administered is calculated as ranging from 0.3 to 2 mg/kg per day, which may be administered in divided dosages. A more preferred amount is calculated as ranging from 0.5 to 1.5 mg/kg per day, and most preferred as 1 mg/kg per day.

10 The invention further contemplates a method of treating or preventing a disorder selected from the group consisting of venous thrombosis and its extension, post-operative deep venous thrombosis and pulmonary embolism in patients undergoing major abdomino-thoracic surgery and/or are at risk of developing thromboembolic disease, pulmonary embolism, atrial fibrillation with embolization, acute and chronic consumptive
15 coagulopathies, clotting in arterial and cardiac surgery, peripheral arterial embolism, acute coronary syndrome, and interstitial cystitis comprising administering heparin or low molecular weight heparin obtained according to the methods of the invention to a subject.

In this method, an effective amount of heparin and/or of low molecular weight heparin is preferably administered to the subject in need thereof. With respect to dosing
20 and administration, reference is made to the foregoing discussion.

The subject in need thereof may for example suffer from, or be at risk of suffering from, a disorder selected from the group consisting of venous thrombosis and its extension, post-operative deep venous thrombosis and pulmonary embolism in patients undergoing major abdomino-thoracic surgery and/or are at risk of developing
25 thromboembolic disease, pulmonary embolism, atrial fibrillation with embolization, acute and chronic consumptive coagulopathies, clotting in arterial and cardiac surgery, peripheral arterial embolism, acute coronary syndrome, and interstitial cystitis.

As used herein, the term "venous thrombosis and its extension" refers to the disorders described in López et al. (Hematology (2004) pages 439-456).

30 The invention also pertains to heparin and/or low molecular weight heparin obtainable by the methods according to the invention for use in the treatment or prevention of a disorder selected from the group consisting of venous thrombosis and its extension, post-operative deep venous thrombosis and pulmonary embolism in patients undergoing major abdomino-thoracic surgery and/or are at risk of developing
35 thromboembolic disease, pulmonary embolism, atrial fibrillation with embolization, acute

and chronic consumptive coagulopathies, clotting in arterial and cardiac surgery, peripheral arterial embolism, acute coronary syndrome, and interstitial cystitis.

All references cited herein, including journal articles or abstracts, published or unpublished patent application, issued patents or any other references, are entirely incorporated by reference herein, including all data, tables, figures and text presented in the cited references.

Although having distinct meanings, the terms “comprising”, “having” and “consisting of” have been used interchangeably throughout this specification and may be replaced with one another.

The invention will be further evaluated in view of the following examples and figures.

DESCRIPTION OF THE FIGURES

Figure 1: Characterization of PCMC. (A) Morphology of cultured mast cells. PCMCs and BMCMs were cytocentrifuged, stained with alcian blue/safranin and observed under the microscope. (B) Ultrastructure of cultured mast cells. PCMC and BMCM were observed by electron microscopy.

Figure 2: FcεRI-dependent β-hexosaminidase and histamine release. PCMC and BMCM sensitized with mouse IgE anti-DNP, were challenged with the indicated concentrations of DNP-BSA for 10 min at 37°C. 1×10^5 cells were used for β-hexosaminidase release and 1×10^6 cells were used for histamine release. β-hexosaminidase and histamine were measured in supernatants and in cell lysates. Left panels show the mean ± SD of values measured in BMCM and PCMC lysates. Right panels show the relative (percentage) and the absolute amounts of β-hexosaminidase and histamine released by individual cell populations. The insert shows histamine released by BMCM with an expanded vertical scale.

EXAMPLES

Example 1: Materials and Methods

Mice. C57BL/6 mice, purchased from IFFA-CREDO (Saint-Germain-sur-L'Arbresle, France) or from Charles River Laboratories (L'Arbresle, France), were used as donors of bone marrow and peritoneal cells. BALB/c mice, purchased from IFFA-CREDO, were used for immunizations. SHIP1^{-/-} mice, generated by Dr. Gerald Krystal (The Terry Fox Laboratories, Vancouver, Canada), and SHIP1^{+/-} littermate controls were kindly provided by Dr. Michael Huber (Max Plank Institut für Immunbiologie, Freiburg, Germany). Bone

marrow and peritoneal cells from RFc γ IIb^{-/-}, RFc γ IIIA^{-/-} and wt littermate controls mice (generation 8 on C57BL/6 background) were kindly provided by Dr. Jeffrey V. Ravetch (The Rockefeller University, New York, NY). Mice were used at 6-9 weeks of age.

Cells. Femoral bone marrow cells were collected and cultured in Opti-MEM supplemented with 10% Fetal Calf Serum (FCS), 100 IU/ml penicillin, 100 μ g/ml streptomycin (complete Opti-MEM) and 4% supernatant of X63 transfectants secreting murine IL-3. Cultures were passaged every 3 days by resuspending pelleted cell in fresh culture medium at a concentration of 3×10^5 /ml. Peritoneal cells were collected from the same mice injected with 2 ml RPMI intraperitoneally. They were seeded at 1×10^6 /ml in complete Opti-MEM supplemented with 4% supernatant of CHO transfectants secreting murine SCF. Twenty four hours later, non-adherent cells were removed and fresh culture medium was added to adherent cells. Three days later, non-adherent cells and adherent cells recovered with trypsin-EDTA, were harvested, pelleted and resuspended in fresh culture medium at a concentration of 3×10^5 /ml. The same procedure was repeated twice a week. Age-matched cultures (3-9 week-old) were used for experiments. Culture reagents were from Invitrogen (Paisley, Scotland, UK).

Antibodies and antigens. The rat anti-mouse Fc γ RIIB/IIIA mAb 2.4G2 was affinity-purified from culture supernatants on Protein G-sepharose (Amersham Little Chalfont, UK). The mouse anti-Ly-17.2 mAb K9.361 (Holmes et al. 1985. Proc. Natl. Acad. Sci. USA 82:7706-7710) and the mouse IgE anti-DNP mAb 2682-I (Liu et al. 1980. J. Immunol. 124:2728-2737) were used as culture supernatants. K9.361, which recognizes the Ly-17.2 alloantigen, encoded by the Ly-17b allele of the *fcgr2b* gene, was demonstrated as being an Fc γ RIIB-specific mAb with no cross-reactivities to other Fc γ Rs, including Fc γ RIIIA (Schiller et al. Eur J Immunol 30:481-490). The IgE concentration in 2682-I supernatant was 10 μ g/ml as titrated by ELISA. Allophycocyanin (APC)-labeled anti CD117 antibodies, Phycoerythrin (PE)-labeled anti-CD19 antibodies, PE-labeled anti-GR1 antibodies and PE-labeled anti-Mac1 antibodies were from BD-Pharmingen (Le Pont de Claix, France). Fluorescein (FITC)-labeled anti Fc ϵ R1 antibodies were from e-biosciences (San Diego, CA). FITC-labeled Mouse anti-Rat (MAR) F(ab')₂ fragments, FITC-labeled Goat anti-Mouse (GAM) F(ab')₂ fragments, FITC-labeled Goat anti-Rabbit (GAR) F(ab')₂ fragments, Rabbit anti-Mouse (RAM) F(ab')₂ fragments and intact IgG antibodies were from Jackson ImmunoResearch (Westgrove, PA). Bovine Serum Albumin (BSA), from Sigma Aldrich (Lyon, France), was dinitrophenylated with dinitrobenzene-sulfonic acid (Eastman Kodak, Rochester, NY). DNP₁₅-BSA was obtained. Mouse anti-Glutathione S-Transferase (GST) serum was raised in BALB/c mice injected once with purified GST in complete Freund's adjuvant and twice in incomplete Freund's adjuvant intraperitoneally. IgG were affinity-

purified from serum on Protein G-sepharose. Phycoerythrin (PE)-labeled anti-IL-6 and anti-IL-10 antibodies, and biotinylated anti-TGF β 1 antibodies were from Beckton Dickinson (Franklin Lakes, NJ). PE-labeled anti-IFN γ , anti-TNF- α , and anti-IL-4 antibodies were from Sérotec (Cergy Saint-Christophe, France). Biotinylated anti-IL-13 antibodies were from R&D Systems (Lille, France). FITC-labeled streptavidine was from Molecular Probes (Carlsbad, CA). The mouse anti-FcR γ mAb JRK was the one described in Rivera et al. (Molecular Immunology, formerly known as Immunochemistry, Volume 25, Issue 7, Pages 647-661. July 1988)). Rabbit anti-Lyn, anti-SHP-1, anti-SHP-2, anti-Gab2, anti-Sos and anti-PLC- γ 1 antibodies were from Upstate biotechnology (Waltham, MA), as well as mouse anti-Vav antibodies. Rabbit anti-Grb2, anti-PLC- γ 2 and anti-SHIP1 were from Santa Cruz (Santa Cruz, CA). Mouse anti-Fyn antibodies were from Transduction laboratories (Lexington, KY). Rabbit anti-Erk and anti-Akt antibodies were from Cell signaling (Beverly, MA). Horse Radish Peroxidase (HRP)-labeled Goat anti-Rabbit (GAR) and Goat anti-Mouse (GAM) were from Santa Cruz.

Direct immunofluorescence. Cells were incubated for 5 min at 0°C with 10 μ g/ml 2.4G2. They were then incubated for 15 min at 0°C with 10 μ g/ml FITC-labeled anti-Fc ϵ R1 antibodies and APC- or PE-labeled antibodies. Cells were washed and fluorescence was analyzed by flow cytometry using a FACScalibur (Becton Dickinson).

Alcian blue/safranin staining. Cells were cytocentrifuged, air-dried, incubated for 20 min with 0.5% alcian blue in 0.3% acetic acid, rinsed in water, and incubated for 20 min with 0.1% safranin in 1% acetic acid. Cells were examined with a Nikon Eclipse TE 2000-U microscope.

Electron microscopy. Cells were fixed with 2.5% glutaraldehyde in 0.01 M PBS pH 7.4 for 1 hr at 4°C, post-fixed with 2% osmium tetr oxide for 1 hr, dehydrated with ethanol, and embedded in Epon epoxy resin. Ultra-thin sections (80-100 nm) were stained with uranyl acetate and lead citrate, and examined at 80 kV using a JEOL (JEM-1005) electron microscope.

RT-PCR analysis of mMCP and SHIP1 transcripts. Total RNA was extracted from cultured mast cells or from cells recovered by peritoneal washing using Trizol (Invitrogen). Reverse transcription was performed on 1 μ g RNA using the Prostar First-Strand RT-PCR kit (Stratagene Europe, Amsterdam, The Netherlands). Transcripts were detected by PCR using the oligonucleotides listed in the table below. PCR products were electrophoresed in 1.5% agarose gel containing ethidium bromide and visualized under UV light.

24

Transcript	SEQ ID NO: of the sense primer	SEQ ID NO: of the antisense primer
mMCP-1	1	2
mMCP-2	3	4
mMCP-4	5	6
mMCP-5	7	8
mMCP-6	9	10
mMCP-7	11	12
mMCP-8	13	14
mMCP-9	15	16
mMCP-10	17	18
SHIP1	19	20
α 2-microglobulin	21	22

Peritoneal mast cell cloning in soft agar. Mast cells, identified by their morphology under the microscope, were individually picked-up from peritoneal cells, resuspended at 37°C in soft agar dissolved in SCF-containing medium and layered over medium-containing soft agar previously layered over adherent SCF-secreting CHO transfectants.

5 *β -hexosaminidase release.* Mast cells, sensitized with IgE anti-DNP, were challenged for 10 min at 37°C with indicated reagents. Non sensitized mast cells were challenged for 10 min at 37°C with preformed immune complexes made by incubating serum anti-GST or affinity-purified IgG anti-GST with GST at the indicated dilutions or concentrations for 15 min at 37°C immediately before use. Reactions were stopped on
10 ice. Five μ l supernatant were mixed with 45 μ l β -hexosaminidase substrate (Sigma Aldrich) and incubated for 2 hr at 37°C. 0.2 M glycine pH 10 was added, and absorbance at 405 nm was measured.

Indirect immunofluorescence. A) Surface labeling. Cells were incubated for 1 hr at 0°C with K9.361 supernatant or 10 μ g/ml 2.4G2 in medium containing 5% FCS, washed
15 and stained with 50 μ g/ml FITC-GAM or MAR F(ab')₂ for 30 min at 0°C. Fluorescence was analyzed by flow cytometry using a FACScalibur (Becton Dickinson). *B) Intracellular labeling.* Cells were fixed for 20 min with 3% paraformaldehyde in PBS, permeabilized for 15 min with 0.5% saponin in 2% BSA-PBS, and incubated for 30 min at 0°C with the indicated antibodies in saponin-containing BSA-PBS. Cells were washed in PBS and
20 stained with the indicated labeled antibodies in saponin-containing BSA-PBS for 30 min at 0°C. Fluorescence was analyzed by flow cytometry.

LTC₄ production. Mast cells, sensitized with IgE anti-DNP, were challenged with DNP-BSA for 20 min at 37°C. LTC₄ was titrated in supernatants by competitive ELISA (Neogen corporation, Lexington, KY).

25 *MIP-1 α secretion.* Mast cells, sensitized with IgE anti-DNP, were challenged with DNP-BSA for indicated periods of time at 37°C. MIP-1 α was titrated in supernatants by ELISA (R&D Systems, Lille, France).

TNF- α secretion. Mast cells, sensitized with IgE anti-DNP, were challenged for various periods of time at 37°C with DNP-BSA. TNF- α was titrated in supernatants by a cytotoxicity assay on L929 cells as described (Latour et al. 1992. J. Immunol. 149:2155-2162).

5 *Morphological assay for mast cell degranulation.* Cultured mast cells or peritoneal cells, sensitized with mouse IgE, were challenged for 5 min at 37°C with RAM F(ab')₂. Reactions were stopped on ice, and cells were stained with Toluidine Blue.

10 *Histamine release.* Mast cells, sensitized with IgE anti-DNP, were challenged for 10 min at 37°C with DNP-BSA in serum-free medium. Reactions were stopped on ice. Histamine was measured in cells and supernatants using a high sampling-rate automated continuous flow fluorometric technique (Siraganian 1974. Anal Biochem 57:383-394).

15 *Western blot analysis.* Mast cells were lysed in lysis buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 1% TX100, 1 mM Na₃VO₄, 5 mM NaF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin and 1 mM PMSF. Proteins were quantified using a Biorad protein assay (Hercules, CA). Twenty µg proteins were electrophoresed and Western blotted with indicated antibodies followed by HRP-GAR or HRP-GAM. Labeled antibodies were detected using an enhanced chemo-luminescence kit (Amersham). When indicated, cells were lysed by being boiled for 5 min at 95°C in 10 mM Tris pH 7.4 containing 1% SDS. Lysates were passaged 6 times through a gauge-26 needle, centrifuged at 12,000 rpm for 10 min at 4°C and immediately electrophoresed.

20 *Protease activity secretion.* Mast cells, sensitized with IgE anti-DNP, were challenged for the indicated periods of time at 37°C with DNP-BSA. Reactions were stopped on ice. Proteolytic activity was measured in supernatants using an enzymatic assay. Briefly, 100 µl were incubated for 30 min at 37°C with 10 µl 0.2 M Tris pH 7.8, 0.02 M CaCl₂ and 10 µl 0.4% resorufin-labeled casein (Roche Diagnostic, Penzberg, Germany). 100 µl 5% trichloroacetic acid were added and plates were incubated for 10 min at 37°C before being centrifuged for 5 min. 80 µl supernatants were mixed with 120 µl 0.5 M Tris pH 8.8. The absorbance was read at 570 nm.

30 **Example 2: High numbers of homogenous mature serosal-type mast cells
can be generated in culture from mouse peritoneal cells.**

35 Peritoneal cells from two C57BL/6 mice were cultured in SCF-containing medium as described in *Materials & Methods*. BMMC were generated in parallel from bone marrow cells of the same two mice cultured in IL-3-containing medium. One month-old cultures of

both types consisted of FcεRI⁺, Kit⁺, CD19⁻, GR1⁻, Mac1⁻ homogenous cells. This phenotype is characteristic of mast cells. About 1×10^{10} and 1×10^8 mast cells could be recovered, after one month, from bone marrow and from peritoneal cell cultures, respectively. Large numbers of pure mast cells can therefore be generated by culturing mouse peritoneal cells with SCF. These cells will be referred to as Peritoneal Cell-derived Mast Cells (PCMC).

PCMC and BMMC contained granules stained with alcian blue/safranin. PCMC granules were much more intensely colored in red than BMMC granules (Fig. 1A), indicating a higher heparin content. When examined by electron microscopy, more numerous granules were seen in PCMC. They were larger, more homogenous, and had a higher density than BMMC granules (Fig. 1B). These staining and morphological properties are characteristic features of mature mast cells. PCMC and BMMC contained mast cell-specific protease transcripts. Both expressed mMCP-2, mMCP-4, mMCP-5, mMCP-6, mMCP-7 and mMCP-8. BMMC, but not PCMC, expressed mMCP-9 and mMCP-10. The same mMCP transcripts as in PCMC, except mMCP-2, were found in peritoneal cells. PCMC therefore retain most properties of mature serosal-type peritoneal mast cells.

BMMC and PCMC proliferated at similar rates during the first three weeks. Differing from BMMC, however, which grew steadily for 2-3 months, PCMC started to proliferate more slowly during the fourth week of culture, and they stopped proliferating after about one month. Noticeably, typical mast cells which were observed in small numbers at the onset of cultures, rapidly increased in numbers within the first days of culture, suggesting that PCMC could result from an expansion of differentiated peritoneal mast cells. To test this possibility, individual mast cells, identified by their size and morphology among the peritoneal cells harvested from C57BL/6 mice, were isolated by micromanipulation and cultured in soft agar layered over adherent SCF-producing CHO transfectants used as feeder cells. Clones developed within one week from single cells. Differentiated peritoneal mast cells can therefore proliferate in the presence of SCF.

Example 3: PCMC respond not only to IgE and antigen, but also to IgG immune complexes.

BMMC and PCMC expressed comparable levels of FcεRI. When sensitized with IgE anti-DNP and challenged with DNP-BSA for 10 min, PCMC and BMMC released similar percentages of β-hexosaminidase. Inhibition observed in excess of antigen was however more pronounced in BMMC than in PCMC.

When challenged with preformed immune complexes, non-sensitized PCMC dose-dependently released β -hexosaminidase. Under the same conditions, BMMC did not respond or very poorly. Immune complex-induced β -hexosaminidase release was enhanced in Fc γ RIIB^{-/-} PCMC. The deletion of Fc γ RIIB enabled BMMC to respond to immune complexes and the responses of Fc γ RIIB^{-/-} BMMC were of the same magnitude as those of Fc γ RIIB^{-/-} PCMC. β -hexosaminidase release was abrogated in Fc γ RIIA^{-/-} BMMC and PCMC (Fig. 2B, right panel). Noticeably, wt BMMC and PCMC expressed comparable levels of Fc γ R as assessed by immunofluorescence with the anti-Fc γ RIIB+IIIA mAb 2.4G2, and comparable levels of Fc γ RIIB as assessed with the anti-allotypic mAb K9.361. Fc γ RIIB could therefore negatively regulate Fc γ RIIA-dependent cell activation in both BMMC and PCMC, but no difference in the relative expression of activating and inhibitory receptors could explain why it prevented BMMC, but not PCMC, from responding to IgG immune complexes.

To further assess Fc γ RIIB-dependent negative regulation, its ability to inhibit Fc ϵ RI-dependent mast cell activation was investigated in the two cell types. BMMC and PCMC sensitized with mouse IgE were challenged either with RAM F(ab')₂ fragments, to aggregate Fc ϵ RI, or with intact RAM IgG, to co-aggregate Fc ϵ RI with Fc γ RIIB in wt cells. Fc γ RIIB-dependent inhibition was more pronounced in BMMC than in PCMC.

SHIP1 is the main intracellular effector of Fc γ RIIB-dependent negative regulation. It also controls Fc ϵ RI signaling. To investigate the role of SHIP1 in the two types of mast cells, BMMC and PCMC were generated from SHIP1^{-/-} and from wt littermate controls. The deletion of SHIP1 increased immune complex-induced β -hexosaminidase release in BMMC but, surprisingly, not in PCMC. The deletion of SHIP1, however, abrogated RAM IgG-induced Fc γ RIIB-dependent negative regulation of Fc ϵ RI-dependent β -hexosaminidase release in both BMMC and PCMC. Inhibition was again more marked in BMMC than in PCMC. As expected, the deletion of SHIP1 increased antigen-induced β -hexosaminidase release by IgE-sensitized BMMC and, as previously reported (Gimborn et al. 2005. J Immunol 174:507-516), it abrogated inhibition observed in excess of antigen. It however did not detectably affect the response of PCMC challenged under the same conditions. The deletion of SHIP1 therefore markedly affected both IgE- and IgG-induced β -hexosaminidase release in BMMC, but not detectably in PCMC, suggesting that SHIP1-dependent negative regulation was more efficient in BMMC than in PCMC. Indeed, Fc γ RIIB-dependent negative regulation, which was abrogated by the deletion of SHIP1 in both cell types, was more efficient in BMMC than in PCMC. This functional difference between the two types of mast cells was not accounted for by a difference in the content

of SHIP1. BMMC and PCMC indeed contained comparable amounts of SHIP1 transcripts, as assessed by RT-PCR, and comparable amounts of the SHIP1 protein, as assessed by intracellular immunofluorescence.

Example 4: PCMC secrete small amounts of newly formed

lipid mediators, chemokines and cytokines in response to IgE and antigen.

When sensitized with IgE anti-DNP and challenged with DNP-BSA for 20 min, BMMC secreted about 30 times more LTC₄ than PCMC, as assessed by ELISA. When sensitized with IgE anti-DNP and challenged with DNP-BSA for longer periods of time, BMMC, but not PCMC, secreted MIP-1 α , as assessed by ELISA. When sensitized with IgE anti-DNP and challenged with DNP-BSA for various periods of time, BMMC secreted TNF- α , as assessed by a cytotoxicity assay. PCMC, however, secreted much less TNF- α than BMMC. No TNF- α was detected at 10 min in supernatants from either cell type. Secretion became detectable at 30 min, peaked at 2 hr and started to decline at 24 hr in BMMC. A moderate secretion was detectable at 2 hr only in PCMC.

PCMC containing intracellular TNF- α were also detected by immunofluorescence following stimulation by PMA + ionomycin for 2 hr. They were less numerous than BMMC containing TNF- α observed following the same treatment. PMA + ionomycin-treated PCMC and BMMC also contained IL-6, and lower numbers of cells contained, IL-13, but not IL-4, IL-10, TGF- β 1 or IFN γ .

PCMC, therefore secreted no or much lower amounts of LTC₄, MIP-1 α and TNF- α . PCMC could however synthesized the same set of cytokines as BMMC in response to PMA + ionomycin.

Example 5: PCMC release large amounts of preformed granular mediators

in response to IgE and antigen.

When sensitized with IgE and challenged for 5 min with RAM F(ab')₂ fragments, a robust degranulation was observed in PCMC and in peritoneal mast cells, as assessed morphologically following toluidine blue staining. Degranulated PCMC resembled degranulated peritoneal mast cells. Under the same conditions, BMMC degranulated only weakly.

Noticeably, PCMC contained about 8-fold more β -hexosaminidase and about 100-fold more histamine than BMMC (Fig. 2, left panel). As a consequence, when sensitized with IgE and challenged for 10 min with antigen, PCMC released much higher absolute amounts of β -hexosaminidase and even higher amounts of histamine than BMMC (Fig. 2,

right panel), even though both mast cells released comparable percentages of these mediators (Fig. 2, middle panel). Noticeably, both cells released a higher percentage of histamine than of β -hexosaminidase, when challenged identically in the same experiment.

When analyzing TX100 lysates from the two cells, a whole set of high-mw intracellular proteins, including SHIP1, failed to be detected by Western blotting. The discordance between this unexpected observation and the immunofluorescence data shown in Example 3 could be best explained if proteolysis occurred during cell lysis in spite of protease inhibitors present in lysis buffer. To investigate this possibility, equal numbers of PCMC and BMMC were mixed before they were lysed in TX100-containing buffer. The presence of PCMC abrogated the detection of BMMC SHIP1, but not that of molecules observed in PCMC lysates such as Lyn. PCMC, but not BMMC, therefore contain a highly efficient protease activity that is released upon cell lysis and hydrolyzes several high-mw intracellular proteins. Proteolysis could however be prevented if PCMC were lysed in SDS-containing buffer and immediately boiled before electrophoresis. Under these conditions, SHIP1, but also other molecules not seen in PCMC TX100 lysates, such as Akt, were readily detectable, and in similar amounts as in BMMC.

On the basis of this observation, it was further investigated whether proteolytic enzymes contained in PCMC could be released and hydrolyze an exogenous substrate such as casein. Proteolytic activity was indeed detected in supernatants of PCMC sensitized with IgE anti-DNP and challenged with DNP-BSA for 10 min, but not in supernatants of IgE-sensitized, but not challenged PCMC. Under the same conditions, no proteolytic activity was detected in supernatants from IgE-sensitized BMMC, whether they were or not challenged with antigen. Proteolytic activity was released from IgE-sensitized PCMC with the same kinetics as β -hexosaminidase, *i.e.* within the first 10 min after antigen challenge, and it did not increase thereafter. Proteolytic enzymes are therefore likely to be contained in PCMC granules and to be released upon degranulation.

Example 6: Discussion of the results

PCMCs were characterized, and it was shown that PCMC is a new model of cultured mouse mast cells which markedly differ from BMMC. Indeed, PCMCs consist of mature differentiated mast cells which retain most of the properties of serosal-type peritoneal mast cells. This makes PCMC useful for studying immunopathological processes. Serosal-type mast cells are indeed present in tissues involved in allergies and inflammatory diseases. Skin mast cells and synovial mast cells, for instance, both of the serosal type, play critical roles in skin allergies and in the murine model of IgG-induced autoimmune rheumatoid arthritis recently described in K/BxN mice, respectively.

Mast cells can be obtained by fractionation techniques from mouse peritoneal cells (Sterk and Ishizaka. 1982. J Immunol 128:838-843). No more than 1×10^5 mast cells can however be obtained per mouse, which greatly limits investigations. A few hundred million homogenous mast cells can be readily generated from the peritoneal cells of two mice and kept in culture for at least two months. These cells are typical mast cells as judged by their expression of FcεRI and Kit, their morphology, their histamine content and their functional features. They are mature mast cells as judged by their intense staining with alcian blue/safranin, and by the high number and the dense structure of their granules. They are serosal-type mast cells as judged by their ability to degranulate in response to compound 48/80, by their high content of granular mediators and by the mMCP transcripts they contain (Miller and Pemberton. 2002. Immunology 105:375-390). These PCMC are likely to result from the expansion of differentiated mast cells present in the peritoneal cavity of normal mice, rather than from the differentiation of Kit⁺ mast cell progenitors, possibly present in peritoneal cells. The number of mast cells rapidly increased in a few days, so that the majority of cells in culture had a mast cell morphology after one week. About 1×10^7 cells were obtained after 2 weeks, and these already consisted of a single population of FcεRI⁺ cells (not shown). If, as indicated by the initial slope of growth curves, cells underwent two divisions/week, they originated from about 5×10^5 cells. This number is compatible with numbers of mast cells contained in peritoneal washings from two adult mice (3-5% of $1-1.5 \times 10^7$ cells). Supporting this assumption, individual peritoneal mast cells could form clones in SCF-containing soft agar. Likewise, individual mouse peritoneal mast cells were previously reported to form colonies when cultured with SCF and IL-3 in methylcellulose (Takagi et al. 1992. J Immunol 148:3446-3453). Because PCMC were generated in medium containing supernatant from SCF-secreting CHO transfectants, it was investigated whether SCF would be sufficient to generate PCMC. Indeed, a single-cell population of FcεRI⁺ cells which released β-hexosaminidase when challenged with preformed GST-IgG anti-GST immune complexes was obtained when culturing peritoneal cells with recombinant SCF as the sole source of added growth factor. Several groups previously reported the *in vitro* generation of alternatives to BMMC. Mast cells with some degree of differentiation toward the connective tissue-type were obtained by culturing mouse bone marrow cells with SCF and a high concentration of IL-4 (Karimi et al. 1999. Exp Hematol 27:654-662). More interestingly, a few million mast cells were generated from fetal skin, which shared several properties with PCMC (Yamada et al. 2003. J Invest Dermatol 121:1425-1432). Differing from PCMC, however, these mast cells apparently differentiated in cultures from mast cell precursors.

One distinctive property of PCMC is their ability to respond to IgG immune complexes. Importantly, this property is shared with peritoneal mast cells. Peritoneal mast cells have indeed long been known to degranulate in response to IgG antibodies (Vaz and Prouvost-Danon. 1969. *Progr. Allergy* 13:111-173). Active antibodies were found in IgG1, rather than IgG2 fractions of polyclonal mouse antibodies (Ovary, Z. 1965. *Fed. Proc.* 24:94-97). All monoclonal IgG1 tested and some IgG2a (Daëron et al. 1982. *Cell. Immunol.* 70:27-40) or IgG2b (Hirayama et al. 1982. *Proc. Natl. Acad. Sci. USA* 79:613-615) could activate peritoneal mouse mast cells. As previously observed for peritoneal mast cells (Hazenbos et al. 1996. *Immunity* 5:181-188), immune complex-induced PCMC activation depended on FcγRIIIA and, as expected, it was negatively regulated by FcγRIIB. Surprisingly, the differential responses of the two cells to IgG immune complexes could not be accounted for by a different ratio of activating/inhibitory FcγRs, revealing that previously unsuspected mechanisms may control FcγRIIB-dependent negative regulation. FcγRIIIA-dependent cell activation was indeed as efficient in both cells, since responses of similar magnitudes were induced by immune complexes in FcγRIIB^{-/-} BMMC and PCMC. FcγRIIB-dependent negative regulation, however, was efficient enough to prevent BMMC, but not PCMC, from responding to immune complexes.

One likely reason for this difference is that PCMC use less efficiently the lipid phosphatase SHIP1 than BMMC. Indeed, the deletion of SHIP1 differentially affected BMMC and PCMC although both cell types contained similar amounts of this phosphatase. SHIP1 was shown to negatively regulate FcεRI signaling and, recently, to account for the inhibition of IgE-induced responses of BMMC in excess of antigen. Little or no inhibition of β-hexosaminidase or histamine release was observed in IgE-sensitized PCMC stimulated by an excess of antigen. As expected, the deletion of SHIP1 increased both IgE- and immune complex-induced β-hexosaminidase release, and abrogated inhibition in excess of antigen in BMMC. Noticeably, however, SHIP1 deletion had no detectable effect on IgE- or immune complex-induced β-hexosaminidase release in PCMC. The finding that SHIP1 deletion did not affect immune complex-induced responses in PCMC is intriguing. Immune complexes indeed coengage FcγRIIIA and FcγRIIB in PCMC since they induced a higher release of β-hexosaminidase in FcγRIIB^{-/-} than in wt PCMC. The possibility that FcγRIIB-dependent negative regulation might not depend on SHIP1 in PCMC was excluded by the observation that, like in BMMC, the deletion of SHIP1 abrogated FcγRIIB-dependent negative regulation of IgE-induced release of β-hexosaminidase in PCMC. SHIP1 is therefore also used by FcγRIIB in PCMC, although, for an unknown reason, less efficiently than in BMMC. Whatever the reason, these results

indicate that differentiation-dependent regulatory mechanisms which control FcγRIIB-dependent SHIP1-mediated negative regulation of cell activation determine the ability of mast cells to respond to IgG immune complexes. This may apply to human basophils which express FcγRIIA and FcγRIIB and which do not respond to FcγRII aggregation.

Supporting the possibility that a differential use of SHIP1 may determine biological responses of basophils, anti-IgE-induced histamine release by basophils from different donors was inversely correlated with the extent of SHIP1 phosphorylation, although basophils from non responders, moderate responders and high responders contained similar amounts of SHIP1. Noticeably, it was found that SHIP1 phosphorylation was of a lower magnitude in PCMC than in BMMC, when challenged with IgE and antigen.

Another distinctive feature of PCMC is that their biological responses differ quantitatively and qualitatively, from BMMC responses. With a few exceptions, FcεRI aggregation triggered the same responses in BMMC and PCMC. These responses, however, markedly differed by their relative intensities. BMMC and PCMC released comparable percentages of β-hexosaminidase and comparable percentages of histamine. PCMC, however, contained almost 10-fold higher amounts of β-hexosaminidase and, like peritoneal mast cells, about 100-fold higher amounts of histamine than BMMC. Consequently, PCMC released much more granular mediators than BMMC within the first minutes of activation *via* FcεRI. By contrast, PCMC produced much lower amounts of lipid mediators during the first half hour of stimulation, and no MIP-1α during the first hours. They also secreted much less TNF-α than BMMC. Noticeably, no TNF-α was detected in supernatants of either cell type at 10 min, when degranulation was completed, and no TNF-α was detected by intracellular immunofluorescence in non stimulated cells, indicating that this cytokine is not stored in BMMC or PCMC granules. TNF-α, however, became detectable intracellularly, several hours following stimulation. Fewer numbers of PCMC than BMMC contained intracellular TNF-α, even following PMA+ionomycin stimulation, indicating that PCMC not only secreted, but also synthesized less cytokines than BMMC. Altogether these data indicate that early responses are more robust than late responses in PCMC, whereas late responses are more robust than early responses in BMMC.

Another biological response was unique to PCMC. Indeed, FcεRI aggregation triggered a release of proteolytic activity in PCMC, but not in BMMC. The responsible proteases were not identified. They hydrolyzed cleavage sites that are rare in low-mw proteins such as casein, and more frequent in high-mw proteins such as SHIP1. They are present in resting cells since proteolysis was observed in PCMC lysates before

stimulation. Noticeably, proteases released in PCMC supernatants did not account for the low amounts of TNF- α found in these supernatants. The amount of TNF- α secreted by IgE-sensitized BMMC in response to antigen was indeed not lower when these were mixed with equals numbers of IgE-sensitized PCMC (not shown). Proteases were released concomitantly with β -hexosaminidase and histamine upon Fc ϵ RI aggregation, and their concentration in supernatants did not increase with time after 10 min. These data altogether suggest that PCMC-specific proteases are preformed and released upon degranulation. A variety of proteases were reported to be stored in mast cell granules and their expression pattern to vary with the tissue where mast cells are located (Miller and Pemberton. 2002. Immunology 105:375-390). PCMC expressed several mMCP transcripts. None of these, however, was selectively expressed in PCMC.

The various molecules that are sequentially produced and released by activated mast cells are thought, altogether, to account for the clinical expression pattern of allergies and inflammatory diseases. Vasoactive granular mediators, especially histamine, account for the local and systemic manifestations of the immediate allergic reaction. Histamine was also shown to mediate IgG immune complex-induced, Fc γ RIIIA-dependent inflammation in the K/BxN model of autoimmune arthritis. It was recently reported to act as a T cell chemotactic factor *via* Type 1 histamine receptors. Mast cell proteases induce a variety of biological effects, most of which are triggered *via* the activation of Protease-Activated Receptor-2 (PAR-2). PAR-2 is expressed by neutrophils, endothelial cells, vascular smooth muscle cells, neurons and glial cells, enterocytes, keratinocytes and many tumor cells. PAR-2 activation is involved in the control of blood pressure and plasma extravasation, in neutrophil infiltration and proliferation, in the induction of pain and, by stimulating the phagocytosis of melanosomes by keratinocytes, in the control of skin pigmentation. PAR-2 also induces keratinocytes to proliferate and to secrete cytokines. Interestingly, PAR-2 is up-regulated in asthma and rheumatoid arthritis. Lipid mediators account for the late-phase reaction which develops locally. Cytokines and chemokines account for the chronic inflammatory reaction which is responsible for most of the long-lasting clinical symptoms of allergic diseases. Among other cytokines, TNF- α , which induces bronchial hyperresponsiveness, airway infiltration by neutrophils and eosinophils, activation of airway smooth muscles and myofibroblasts, and which upregulates the expression of adhesion molecules, was recognized as playing a major role in asthma-associated remodeling and pulmonary inflammation, especially in asthma refractory to corticosteroid therapy. TNF- α also critically contributes to the pathogenesis of rheumatoid arthritis.

Although poor releasers of granular mediators, BMMC are potent secretors of pro-inflammatory chemokines and cytokines. Physiological BMMC equivalents may exist in the bone marrow and, transiently, in the circulation, but not in peripheral tissues. They therefore cannot account for tissue inflammation. If, as discussed above, PCMC result from an expansion of preexisting peritoneal mast cells and are representative of serosal-type mast cells, these mast cells cannot either account themselves for tissue inflammation. They are indeed poor secretors of cytokines. Other cells, which are known to converge to allergic sites and which infiltrate tissues are required for inflammation to be generated. The massive amounts of vasoactive mediators and proteases that are released by PCMC within minutes should greatly facilitate the subsequent constitution of an inflammatory infiltrate. One may therefore speculate that serosal-type mast cells function as promoters rather than as effectors of inflammation in allergies and autoimmune diseases.

CLAIMS

1. A method of producing heparin comprising the steps of:

- 5 a) producing a heparin-producing cell by a method comprising culturing
 peritoneal mast cells in the presence of Stem Cell Factor (SCF); and
 b) recovering said heparin from the culture containing said heparin.

10 2. The method according to claim 1, further comprising the step of purifying said
 heparin.

 3. The method according to claim 1 or 2, wherein said peritoneal mast cells are
 murine, porcine or human peritoneal mast cells.

15 4. The method according to claim 3, wherein said peritoneal mast cells are murine
 peritoneal mast cells.

 5. The method according to any one of claims 1 to 4, wherein said peritoneal mast
 cells are cultured for at least two weeks.

20 6. The method according to claim 5, wherein said peritoneal mast cells are
 cultured for at least one month.

 7. The method according to any one of claims 1 to 6, wherein said Stem Cell
 Factor is produced by CHO transfectants secreting SCF.

25 8. The method according to claim 7, wherein said SCF is murine SCF.

 9. The method according to any one of claims 1 to 8, wherein said method of
 producing a heparin-producing cell comprises the steps of:

- 30 i) providing peritoneal cells;
 ii) seeding said peritoneal cells in a culture medium supplemented with a
 supernatant obtained from a culture of CHO transfectants secreting
 murine SCF;
 iii) twelve to thirty-six hours later, removing non-adherent cells and adding
35 fresh culture medium to the adherent cells;

- iv) two to four days later, recovering non-adherent and adherent cells, and harvesting, pelleting and resuspending said non-adherent and adherent cells in fresh culture medium; and
- v) repeating step (iv) twice a week.

5

10. The method according to any one of claims 1 to 9, wherein at least 1×10^8 heparin-producing mast cells are recovered after one month of culture.

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11. A method for producing a low molecular weight heparin comprising producing heparin according to the method as defined in any one of claims 1 to 10 and chemically or enzymatically depolymerizing said heparin to produce said low molecular weight heparin having a weight-average molecular weight ranging from 1,000 to 10,000 daltons.

15

12. A method of treating or preventing a disorder selected from the group consisting of venous thrombosis, post-operative deep venous thrombosis and pulmonary embolism in patients undergoing major abdomino-thoracic surgery and/or are at risk of developing thromboembolic disease, pulmonary embolism, atrial fibrillation with embolization, acute and chronic consumptive coagulopathies, clotting in arterial and cardiac surgery, peripheral arterial embolism, acute coronary syndrome, and interstitial cystitis comprising the steps of:

20

- a) producing heparin according to the method as defined in any one of claims 1 to 10; and
- b) administering said heparin to a subject.

25

13. A method of treating or preventing a disorder selected from the group consisting of venous thrombosis, post-operative deep venous thrombosis and pulmonary embolism in patients undergoing major abdomino-thoracic surgery and/or are at risk of developing thromboembolic disease, pulmonary embolism, atrial fibrillation with embolization, acute and chronic consumptive coagulopathies, clotting in arterial and cardiac surgery, peripheral arterial embolism, acute coronary syndrome, and interstitial cystitis comprising the steps of:

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- a) producing low molecular weight heparin according to the method as defined in claim 11; and
- b) administering said low molecular weight heparin to a subject.

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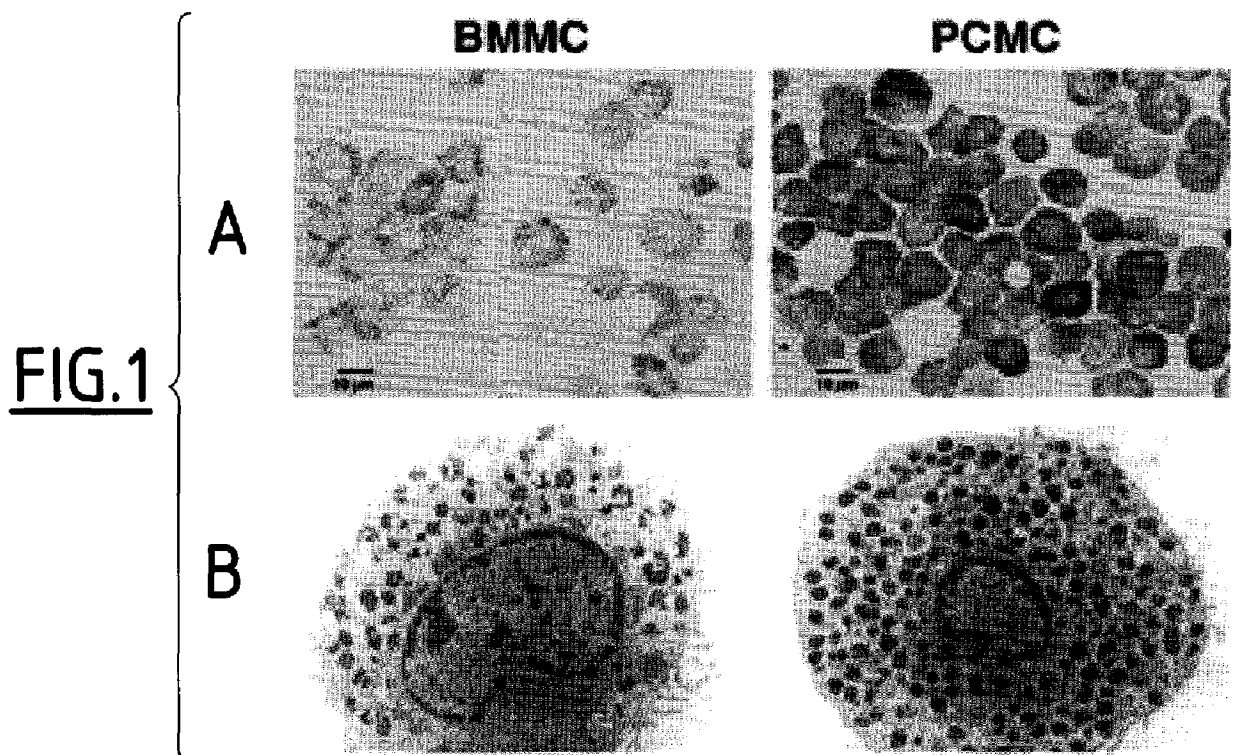


FIG.2

