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Morphine-Modulated Mast Cell Migration and Proliferation during Early Stages of Zymosan-Induced Peritonitis in CBA Mice*

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We have previously shown that supplementation of inflammation-inducing zymosan with a high dose of morphine inhibits peritoneal influx of leukocytes in Swiss, C57C3H, Balb/c, and C57BL/6 strains but not in CBA mice. We have also reported that the different pattern of the response to morphine treatment might be, at least partially, due to the inter-strain differences in the peritoneal mast cell (P-MC) number (high in CBA mice versus other strains) and P-MC specific features (high sensitivity to degranulation upon morphine treatment in CBA mice). The aim of the present study was to investigate the mechanism of morphine action on P-MC in CBA mice. In particular, the effects of morphine on the proliferation and migration of P-MC in CBA mice with ongoing zymosan-induced peritonitis modulated by morphine were studied. Morphine alone acted as a strong chemoattractant for P-MC of CBA mice and this effect was opioid receptor-independent. Moreover, flow cytometric analysis showed that i.p. morphine injection induced significant proliferation of P-MC in CBA mice. Therefore, we conclude that the lack of anti-inflammatory effects of morphine during peritonitis in CBA mice might result not only from a unique sensitivity of CBA mast cells to morphine-induced degranulation but also from the fact that mast cell numbers increase at the inflammatory focus. The latter might be due to morphine-induced mast cell proliferation and/or migration.

Key words: Mast cell, peritonitis, morphine, proliferation, migration.

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Mast cells and their numerous mediators are considered to play an important role in many aspects of natural and acquired immunity (CRIVELLATO & RIBATTI 2010; STELEKATI *et al.* 2007). Experimental peritonitis induced by i.p. injection of a sterile stimulant such as zymosan represents a convenient model for studies on mast cell involvement in inflammation (KOLACZKOWSKA *et al.* 2008a; KOLACZKOWSKA *et al.* 2008b; PLYTYCZ & NATORSKA 2002; NATORSKA & PLYTYCZ 2005; STANKIEWICZ *et al.* 2004; KOLACZKOWSKA *et al.* 2001a). It was previously shown that peritoneal mast cells (P-MCs) are key effector cells in the initiation of zymosan-induced peritonitis and modulate its further course as confirmed by experiments conducted on genetically mast cell-deficient mice (KOLACZKOWSKA *et al.* 2001b).

It was also reported that the supplementation of zymosan with a high dose of morphine, besides its analgesic effects, inhibits influx of inflammatory leukocytes into peritoneum in four out of five investigated strains of mice (Swiss, C57C3H, Balb/c, and C57BL strains but not in CBA mice) (NATORSKA & PLYTYCZ 2005). Moreover, it was documented that the anti-inflammatory effects of morphine in the four strains of mice are connected with morphine-induced desensitization of leukocyte receptors for some chemotactic factors (SZABO *et al.* 2002). As stated above, in the CBA strain the influx of peritoneal leukocytes (PTLs) was not inhibited at any investigated time point

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and 8 hours after induction of peritonitis it was even enhanced in mice co-injected with morphine (NATORSKA & PLYTYCZ 2005). The different effects of morphine on CBA versus the other investigated murine strains might be linked to inter-strain differences in the number and characteristics of P-MCs. CBA mast cells, in contrast to Swiss mice, are much more numerous and highly prone to degranulation by morphine (STANKIEWICZ *et al*. 2004). Moreover, our preliminary studies revealed that the morphine treatment significantly increased P-MC accumulation despite their degranulation. These results prompted us to study the effects of morphine on peritoneal mast cell migration and proliferation in CBA mice during zymosan-induced peritonitis modulated by morphine.

Materials and Methods

Animals

The ethical guidelines of the local committee on animal care were followed throughout the experiments (license no. 23/OP/2005 and 11/2010).

Adult males of the CBA strain (4-6 week-old, 23-25 g) purchased from the Unit of Laboratory Animals (Collegium Medicum, Jagiellonian University, Kraków, Poland) were used in the present experiments. Mice were housed 5 per cage under strictly controlled conditions (at a room temperature of 20±2°C, 12h/12h light-dark cycle with food and water available *ad libitum).*

Inflammatory models and drug treatment

Peritoneal inflammation was induced according to DOHERTY *et al*. (1985). Zymosan A (Sigma-Aldrich, Co., London, UK) was freshly prepared (2 mg/ml) in sterile 0.9% w/v saline and 40 mg/kg b.w. (0.5 ml / 25 g b.w.) was administrated i.p. Animals were either injected with zymosan (Z), morphine sulphate (20 mg/kg b.w.; M) (Polfa, Kutno, Poland) or zymosan supplemented with morphine (ZM). One group of animals was left untreated (intact mice, INT). At the selected time points, animals were killed by cervical dislocation.

Preparation of peritoneal leukocytes and fluids

The peritoneal cavity was lavaged with 1 ml of PBS, and after a 30-s gentle manual massage exudate was retrieved and centrifuged at 1200 rpm for 10 minutes. The cells were subsequently used for counting and mast cell separation.

Cell counts

Mast cell counts were done with a haemocytometer following staining with safranin O solution (0.1% safranine in 0.1% acetic acid) (GODFRAIND *et al*. 1998).

Cytokine content

The peritoneal exudate content of mouse KC (a murine CXC chemokine) was measured by application of an ELISA kit (R&D System, Minneapolis, USA). The assay was carried out as indicated by the manufacturer.

Mast cell separation

Mast cells were obtained in a high state of purity from mouse peritoneal washings by centrifugation in metrizamide density gradients (JOZAKI *et al*. 1990) (opioid receptor binding study) or using MACS (Magnetic Activated Cells Sorting) Technology (Miltenyi Biotec GmbH, Germany) according to the manufacturer's procedure. Briefly, in the latter method total peritoneal leukocytes collected from each mouse were counted, adjusted to $1x10^7$ cells per 100 μl and incubated with an antibody against c-kit (CD 117) receptor (present on mature MCs) conjugated with FITC. After washing, the unbound antibodies were removed and the cells were incubated with anti-FITC MicroBeads. Then the cells were washed and subjected to magnetic separation with MS (Middle Scale) Column. In the magnetic field, the bead-unlabeled cell fraction passed through. In contrast, magnetically labelled cells attached to the magnetic column and were flushed out from the column after removing the magnet. The pure MCs were used for the migration and proliferation assays.

β-Hexosaminidase assay

Release of the mast cell granule component β-hexosaminidase enzyme was used for detection of mast cells degranulation (DEMO *et al*. 1999). To determine β-hexosaminidase activity, cells were lysed on ice with Tyrode's buffer containing 0.1% (vol/vol) Triton X-100 for 5 min and then spun at 5000xg. The supernatant (100 μl) was collected and incubated with 2mM of the substrate solution (1.3 mg/ml of *p*-nitrophenyl-*N*-acetyl-b-D--glucosaminide (Sigma-Aldrich) in 100 μl of 40 m M citrate buffer (pH 4.5) for 15 min at 37°C. The reaction was terminated by the addition of 100 ml of 0.2 M NaOH/0.2 M glycine. Absorbance was read at 440 nm in an enzyme-linked immunosorbent assay reader, and the amount of exocytosis was expressed as the percentage of total β-hexosaminidase activity present in cells.

Opioid receptor binding

Metrizamide-purified peritoneal mast cells were washed in RPMI medium and incubated for 60 min with morphine (10^{-8} M) – a receptor opioid agonist; and the control samples were incubated with RPMI. In some experiments the samples were preincubated with RPMI or with antagonists of opioid receptors: for 60 min with *Pertussis toxin* (1 μg/ml, Sigma-Aldrich) (EMADI-KHIAV *et al*. 1995), 20 min with naltrexon (10^{-8} M, Sigma-Aldrich) (GRIMM *et al*. 1998a; GRIMM *et al*. 1998b) or 60 min with cromolyn – a mast cell membrane stabilizing agent (10^{-4} M, Sigma-Aldrich) (TANIZAKI *et al*. 1992). After the above incubation the samples were centrifuged (10 min at 400xg) and then cell degranulation was assessed morphologically according to LEVI-SCHAFFER *et al*. (2000) on safranine-stained cytospin preparations. Moreover, levels of histamine released to supernatants was assessed by enzyme-linked immunosorbent assay (ICM Pharmaceuticals, Inc., Cost Mesa, CA, USA) and calculated according to the VERBSKY *et al*. (1996) formula: % histamine released = (histamine released by the inducer/total histamine content) x 100.

Mast cell migration assay

P-MC migratory activity was assessed by using a 48-well microchemotaxis chamber (Neuro Probe, Inc., Maryland, USA). The lower wells of the apparatus were filled with either 27 μl of morphine (10^{-8} M) dissolved in culture medium (RPMI) or RPMI alone (as a control of random migration) and then covered with nitro-cellulose filters (10 μm pore size; Nucleopore membrane, Neuro Probe Inc.). The upper wells were filled with 50 μl of peritoneal mast cell suspension ($5x10^5$ cells/ml) separated on the MACS Technology. Some cells were exposed to 5 minutes of preincubation with two different concentrations of naltrexon (either 10^{-3} M or 10^{-8} M); the controls were preincubated with RPMI. After 3-hour incubation at 37^{o}C the cells remaining on the upper surface of the filter were removed. The filters were fixed in 4% buffered formalin, stained with Harris's haematoxylin (40 min), washed in water (10 min) and cleared in xylene (15 min) (all from POCh, Gliwice, Poland). The cells migrating through the filter were counted using a 40x objective on three to four levels and pooled. The counting procedure was repeated in three independent fields, and the mean value was calculated for each well. The mean values from triplicate wells of each sample (within the same filter) were used for statistical analysis.

Mast cell proliferation

For analysis of cell proliferation by flow cytometry, the separated mast cells were adjusted to a concentration of $5x10^5$ cells/ml and incubated with propidium iodide (PI) (Bender MedSystem, Vienna, Austria) according to BLACKLIDGE and BIDWELL (1993). After 10 minutes the samples were analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry System, USA) to assess the cell cycle DNA profile. Orange emission from PI was collected through the FL-2 channel. Intensity of propidium iodide-derived FL-2 fluorescence is proportional to cell DNA content. The resulting files of DNA histograms were analysed using WinMDI 2.8 software (Joe Trotter, http://facs.scripps.edu).

Statistical analysis

All data were expressed as means ± SE from three independent experiments. The numbers of cells and kinetic changes of each parameter were compared by analysis of variance (ANOVA) followed by *post hoc* Tukey's test. The differences were considered statistically significant at $p<0.05$.

Results and Discussion

It has been previously shown that the supplementation of a proinflammatory agent with a high dose of morphine (20 mg/kg of body weight) not only attenuated pain but also inhibited influx of polymorphonuclear leukocytes (PMNs) to the focus of inflammation during zymosan-induced peritonitis in mice (PLYTYCZ & NATORSKA 2002). These anti-inflammatory effects of morphine were recorded in most strains of mice (C57C3H, Swiss, Balb/c, C57BL/6) (PLYTYCZ & NATORSKA 2002) except CBA (NATORSKA & PLYTYCZ 2005). The latter strain revealed high levels of PMN accumulation both after zymosan and zymosan-supplemented with morphine i.p. co-injection (Fig. 1).

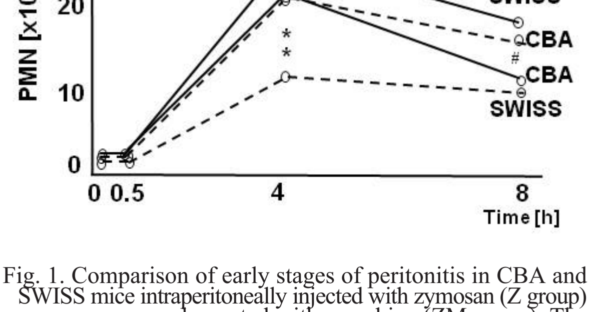

Fig. 1. Comparison of early stages of peritonitis in CBA and SWISS mice intraperitoneally injected with zymosan (Z group) or zymosan supplemented with morphine (ZM group). The number of peritoneal polymorphonuclear leukocytes (PMNs) at time 0 (controls) or 0.5h, 4 h and 8 h after injection. Data presented as mean +/- SE (n=4-6). Values significantly different between the groups at $P<0.05$, $^{**}P<0.01$ (SWISS mice), $\#P<0.05$ (CBA mice) according to Student's *t* test.

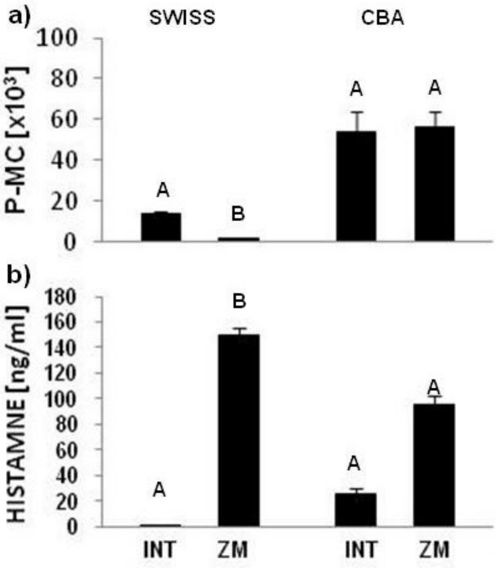

Fig. 2. Effects of i.p. injection of morphine on the number of peritoneal mast cells (P-MCs) (a) and histamine level in peritoneal fluid (b) at 0.5 h after i.p. zymosan injection into SWISS and CBA mice. Animals were either untreated (INT) or received i.p. zymosan supplemented with morphine (ZM). Data presented as mean +/- SE (n/4/6). Mean values not sharing letters are statistically different according to ANOVA.

STANKIEWICZ and co-writers (2004) formulated a hypothesis that different responses to supplementation of peritonitis-inducing agent with morphine may depend, at least partially, on the inter-strain differences in peritoneal MC (P-MC) number and their characteristics. This was based on observation that in contrast to Swiss mice, the CBA P-MCs are not only much more numerous (STANKIEWICZ *et al.* 2001) but also very sensitive to morphine-induced degranulation and histamine release leading to induction of inflammatory response (STANKIEWICZ *et al.* 2004).

In current experiments, in Swiss mice, thirty minutes after morphine co-injection with zymosan, P-MC numbers diminished (Fig. 2a) and a concomitant increase in histamine levels in the peritoneal fluid suggests that this was due to their degranulation (Fig. 2b). Interestingly, in CBA mice the P-MC numbers did not change although some histamine release was observed (Fig. 2b).

Because of this we decided to extend our observation of P-MCs for the next few hours of peritonitis (Fig. 3). We detected that in the CBA strain, in contrast to Swiss mice, mast cell numbers significantly increased within the first 4 hours of peritonitis (Fig. 3a). This increase of P-MCs was connected with their degranulation quantified by

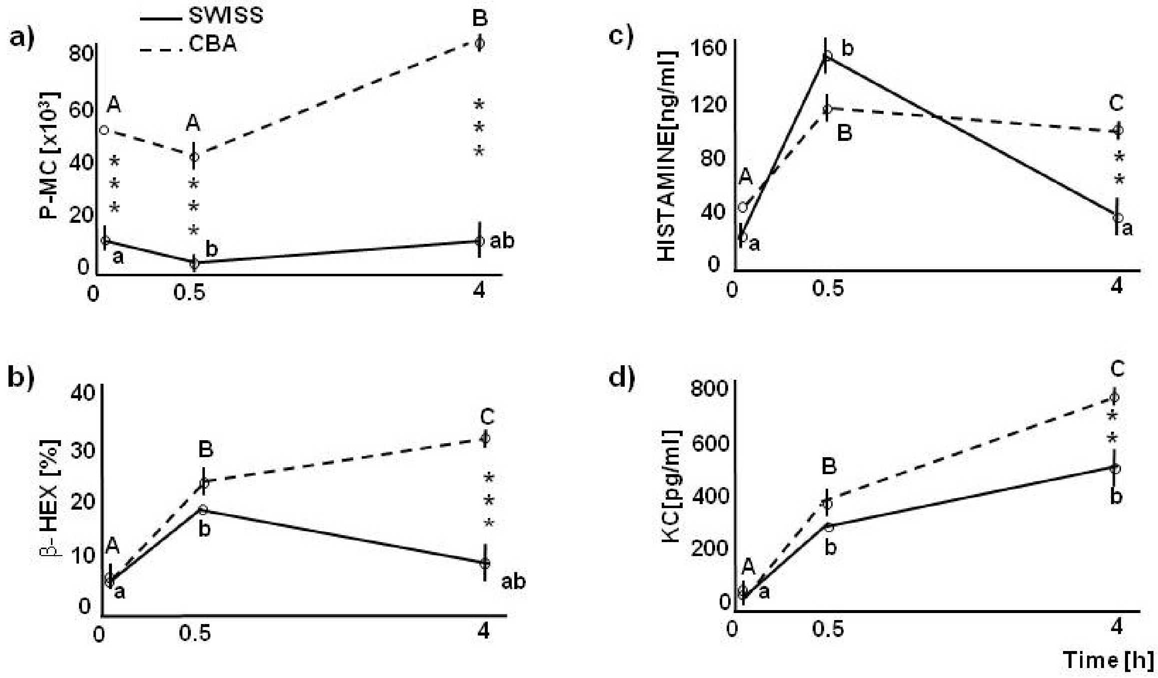

Fig. 3. Effects of i.p. injection of morphine on the number of peritoneal mast cells (P-MCs) (a), β-hexosaminidase release (β-HEX) (b), histamine (c) and chemokine KC (d) levels in peritoneal fluid at 0.5 h and 4 h after i.p. zymosan injection into SWISS and CBA mice. Animals were either untreated (INT) or received i.p. zymosan supplemented with morphine (ZM). Data presented as mean +/- SE (n/4/6). Mean values not sharing letters are statistically different according to ANOVA.

β-hexosaminidase release and histamine secretion (Fig. 3b and 3c, respectively). Moreover, a murine CXC chemokine KC (equivalent to human CXCL8/IL-8) was detected in zymosan exudates both in Swiss and CBA mice but its level was significantly higher in CBA than Swiss mice at 4 hours of peritonitis (Fig. 3d). It was previously reported that resident peritoneal MCs play a central role in the production of the KC and mMCP-1 chemokines (mainly for inflammatory neutrophiles and monocytes, respectively) during the acute inflammatory response induced by *i.p.* injection of zymosan (AJUEBOR *et al.* 1999). It is known that chemokines and opiates have the capacity to desensitize chemokine receptors on leukocytes (GRIMM *et al.* 1998a; GRIMM *et al.* 1998b) resulting in a significant inhibition of leukocyte infiltration to the focus of inflammation (CHADZINSKA *et al.* 1999). Therefore, in the present study the high number of degranulating P-MCs associated with the high-level of KC secretion may suggest that desensitization of chemokine receptors would be even stronger in CBA than in Swiss mice. However, in CBA animals KC production significantly increased from the 4th hour of morphine-modulated peritonitis (most probably as a consequence of high P-MC numbers and their sustained degranulation), leading to continuous leukocyte accumulation from this time on. Therefore even if morphine could have initially impaired neutrophile accumulation, prolonged KC production by continuously migrating and/or proliferating mast cells evoked constant leukocyte influx and counterbalanced the otherwise anti-inflammatory effects of morphine.

Mast cell accumulation within inflamed tissue has been described in a number of diseases such as asthma (DOUGHERTY *et al.* 2010) or rheumatoid arthritis (SHIN *et al.* 2009) but mechanisms of this action are still unclear. An increase of MC could occur by recruitment of mast cell precursors from the circulation followed by their local maturation, local proliferation of resident mast cells, or migration of mature mast cells from adjacent tissues (OKAYAMA & KAWAKAMI 2006; GODFRAIND *et al.* 1998). In the latter case this could be caused by MC-derived mediators such as histamine (THURMOND *et al.* 2004), LTB_4 (WELLER *et al.* 2005) or PGE_2 (WELLER *et al.* 2007). Thus in order to verify the cause of P-MC accumulation during morphine-modulated peritonitis we investigated the migratory activity and proliferation of separated P-MC (from the whole population of PTLs) of CBA mice in response to morphine. Morphine alone acted as a strong chemoattractant for P-MC and this effect was not reversed by a specific antagonist of opioid receptors (naltrexon) (Fig. 4) suggesting the opioid-independent action of morphine. Previously, CHADZINSKA *et al.* (1999) showed that migration of mouse bone marrow cells towards plasma of intact or thioglycollate-treated CB6 mice was enhanced after its *in vitro* supplementation with morphine sulfate (10^{-8} M) in comparison to treatments without the opioid. Moreover, it was observed that opiate compounds, including met-enkephalin and morphine acted as strong chemoattractants for monocytes and neutrophiles and this action was inhibited by the opiate receptor antagonist naloxone (GRIMM *et al.* 1999a; GRIMM *et al.* 1998b). The present experiments showed that opioid receptors expressed on CBA mast cells are insensitive to naltrexone, indicating opioid receptor-independence.

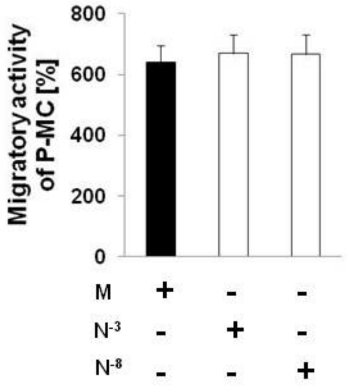

Fig. 4. Migration of peritoneal mast cells (P-MCs) of CBA mice towards morphine (M, 10^{-8}M). P-MCs were separated from peritoneum of healthy unstimulated mice using MACS Technology and subjected to chemotaxis assay. Some cells were exposed to 5 minutes preincubation with two different concentrations of naltrexon: 10^{-3} M , 10^{-8} M (forming group: N^{-3}, N^{-8}, respectively) or with RPMI only (M group). The cells were then placed in upper wells of the chemotaxis chamber. The results are shown as % of control cells migrating towards RPMI only (100%). Data are presented as mean +/- SE from three experiments.

Our *in vitro* experiments utilizing the specific opiate inhibitors *Pertussis toxin* (antagonist of G protein receptors), naltrexone (antagonist of opioid receptors) and cromolyn (stabilizer of the mast cell membrane) confirm the hypothesis of receptor-independent mast cell activation by morphine in CBA mice (Fig. 5). In particular, none of the components reversed mast cell degranulation (Fig. 5a) nor histamine release (Fig. 5b) induced by morphine. It is known that opiates (including morphine) act on MCs either through opioid receptors that are connected with G proteins (SHEEN *et al.* 2007; KLINKER *et al.* 1997) or like some other substances (compound 48/80, substance P, transcription factor E2F80) may activate MC through receptor-independent G-protein manner (BUNDOC & SEIFERT 1997; HAGEMANN *et al.* 2007; MOUSLI *et al.* 1990) but the molecular mechanisms behind these processes are still unresolved. It was suggested that morphine, cocaine and methadone might become activated into free radicals which produce membrane lipid perturbation and histamine release (DI BELLO *et al.* 1998). It was also proposed that substance P and compound 48/80 may stimulate G-proteins by mimicking part of the

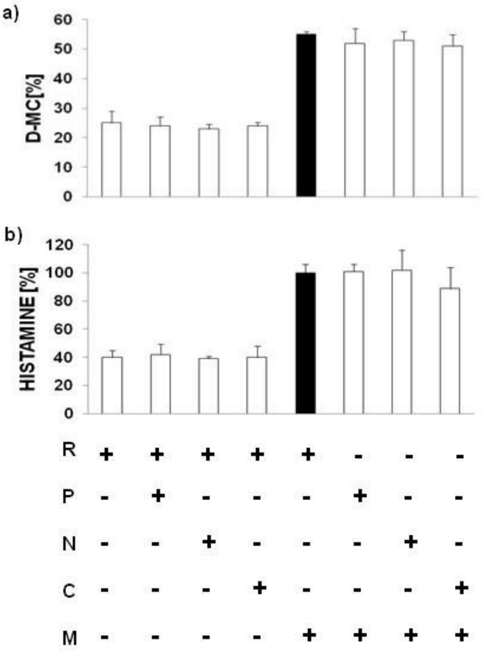

Fig. 5. Effects of opioid receptors antagonists and/or agonists on peritoneal mast cell degranulation (D-MC) (a) and histamine release (b) in CBA mice. P-MCs were separated from the peritoneum of healthy unstimulated mice by centrifugation in metrizamide density gradients. The cells were incubated with or without morphine (M, 10^{-8} M) or medium (RPMI, R). Some cells were preincubated with antagonists of opioid receptors: *Pertussis toxin* (P, 1 μg/ml) and naltrexon (N, 10^{-8} M) or with mast cell membrane stabilizing agent – cromolyn (C, 10^{-4} M). Data are presented as mean +/- SE from three independent experiments.

intracellular loop of G-protein-coupled receptors (MOUSLI *et al.* 1990). Therefore, we hypothesize that the sensitivity of opioid receptors to naltrexone is strain-dependent and perhaps related to the characteristics of the local cell subpopulations and/or their maturation.

The increased number of MCs during the inflammatory process may also be induced by their local proliferation regulated by inflammatory cytokines (HU *et al.* 2007), the presence of c-kit ligand-SCF required for mast cell maturation (OKAYAMA & KAWAKAMI, 2006), IL-3 (AVANZI *et al.* 1991), IL-6 (MISIAK-TLOCZEK & BRZEZINSKA-BLASZCZYK 2009) and TNF (BRZEZINSKA-BLASZCZYK & MISIAK-TLOCZEK 2007). Here we demonstrate that in CBA mice MCs collected from peritoneum 4 hours after stimulation with either morphine (Fig. 6b) or zymosan co-injected with morphine (Fig. 6c) show a significant higher proliferation rate in comparison to the untreated mice (Fig. 6a and d).

Altogether the results reveal that in CBA mice morphine acts as a chemotactic factor for P-MCs and induces their proliferation. Moreover, it was previously reported that in CBA mice morphine acts as a strong pro-inflammatory agent inducing P-MC degranulation and histamine release (STANKIEWICZ *et al.* 2004). Surprisingly, upon morphine treatment the number of P-MCs increases significantly in the focus of inflammation despite their degranulation. Thus, we postulate the

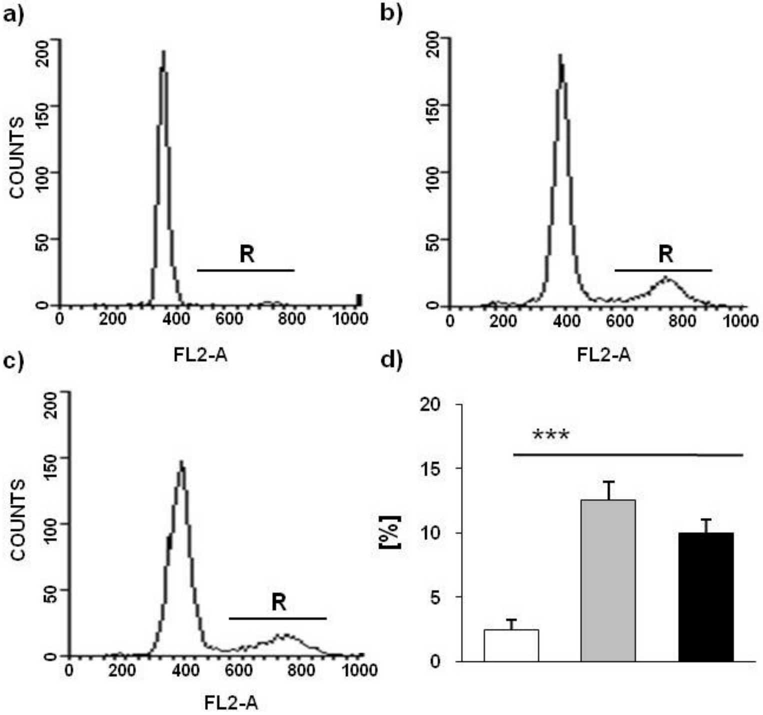

Fig. 6. Proliferation of peritoneal mast cells (P-MCs) detected by flow cytometric DNA analysis in CBA mice. P-MCs were separated from the peritoneum of healthy unstimulated mice (control, INT) or at the 4th hour after injection of morphine (M) or zymosan supplemented with morphine (ZM) and then stained with propidium iodide (PI). Orange emission from PI was collected through FL-2 channel. (a-c) Representative histograms from three independent experiments showing the percentage of proliferating P-MCs in INT (a), M (b) and ZM (c) groups, i.e. those at S+G2+M parts of the cell cycle (peaks under R lines). (d) Percentage (means +/- SE) of proliferating P-MCs (calculated from R peaks on flow cytometric profiles in the samples retrieved from INT mice (white bar), M (grey bar) or ZM group (black bar). Data are presented as means from three experiments, ***P<0.001 when compared with the control animals.

following scenario: CBA mast cells degranulate and secrete chemotactic factors (mainly KC acting on inflammatory PMNs and MCP-1 affecting macrophage migration (Ajuebor *et al*. 1999) but simultaneously their number increases due to MC proliferation. We also cannot exclude that some mast cells may migrate to the focus of inflammation in response to morphine treatment. Chemotactic factors (e.g. KC) secreted by numerous peritoneal mast cells cause continuous leukocyte infiltration to the peritoneum cavity which impairs the anti-inflammatory effect of morphine in CBA mice.

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