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Strain-specific Differences in Modulatory Effects of Morphine on Peritoneal Inflammation in Mice*

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It has been previously shown that local administration of a high dose of morphine together with a proinflammatory agent has anti-inflammatory effects in several (but not all) strains of mice. In the present paper, behavioural and cellular consequences of morphine co-injection are compared between several strains of mice with zymosan-induced peritonitis. Males of C57C3H, Swiss, Balb/c, C57BL/6, and CBA strains were ip injected with Zymosan (40 mg/kg) and/or morphine at 0, 5, 10, 20 mg/kg without or with naltrexone pretreatment. Early stages of Zymosan-induced peritonitis were connected with intraperitoneal accumulation of leukocytes (mainly PMNs), pain symptoms (body writhes of strain-specific frequency: C57C3H>CBA>Swiss>>Balb/c~C57BL/6), and sedation (significant in Swiss and Balb/c). Morphine co-injection abolished pain symptoms at all doses in every investigated strain, and restored locomotor activity or induced hyperlocomotion in a dose- and strain-specific manner. The highest dose of morphine inhibited intraperitoneal accumulation of peritoneal leukocytes (PTLs) including polymorphonuclear cells (PMNs) in all but the CBA strain of mice. Anti-inflammatory and anti-nociceptive effects of morphine were reversed in naltrexone pre-treated animals. The pre-incubation of Swiss but not CBA leukocytes with morphine inhibited cell chemotaxis towards zymosan-activated serum. In conclusion, morphine exerts various strain-specific effects on peritonitis.

Key words: Peritonitis, pain symptoms, locomotor activity, CBA, Balb/c, C57BL/6, C57C3H, Swiss mice.

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It has been previously shown that the supplementation of a proinflammatory agent with a high dose of morphine not only attenuated pain but also inhibited influx of leukocytes to the focus of inflammation during Zymosan-induced peritonitis in mice (PLYTYCZ & NATORSKA 2002). Such antinociceptive and anti-inflammatory effects of local morphine administration might offer benefits during planned surgeries, thus the underlying mechanisms are worth of study. At the early stages of Zymosan-induced peritonitis, it was recorded that the intraperitoneal accumulation of endogenous opioids related to their influx from closely located lymph nodes and some brain areas (CHADZINSKA *et al.* 2003) and/or to their local synthesis/release by inflammatory leukocytes (CHADZINSKA *et al.* 2001), as evidenced also in other models of inflammation (CABOT *et al.* 1997; PRZEWLOCKI *et al.* 1992; STEIN *et al.* 2001). It seems that local administration of exogenous morphine might support and/or replace the anti-nociceptive action of endogenous opioids in the focus of inflammation and, as a consequence, might limit a chemotactic influx/accumulation of new leukocytes.

Anti-inflammatory effects of morphine were originally recorded in some strains of laboratory mice (PLYTYCZ & NATORSKA 2002; CHADZINSKA *et al.* 1999), in salmon (CHADZINSKA *et al.* 1999) and goldfish (CHADZINSKA *et al.* 2000), but in none of the investigated species of anuran amphibians (KOLACZKOWSKA *et al.* 2000). Such *in vivo* sensitivity or resistance to the anti-inflammatory action of morphine corresponded with the *in vitro* migratory activity of morphine-treated leukocytes towards some chemoattractants; pre-incubation with morphine inhibited migration of mouse and fish leukocytes but not of frog leukocytes to chemotactic factors present in Zymosan-activated se-

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rum (CHADZINSKA & PLYTYCZ 2004). During our ongoing series of experiments it turned out, however, that anti-inflammatory and anti-chemotactic effects of morphine are not shared even by the laboratory strains of mice, as they are absent in males of the CBA strain (STANKIEWICZ *et al.* 2001), perhaps due to the peculiar features of CBA mast cells (STANKIEWICZ *et al.* 2004). The aim of the present paper was to compare some behavioural and cellular effects of morphine co-injection on males of several strains of mice with Zymosan-induced peritonitis.

Material and Methods

Animals

Adult males (6-8 week-old) of Swiss, Balb/c, C57BL/6, and CBA strains were purchased from the Unit of Laboratory Animals (Collegium Medicum, Jagiellonian University, Kraków, Poland). Males of C57C3H mice were obtained from the departmental breeding colony, and originated from the random mating of F2 progeny of C57BL×C3H mice (SCISLOWSKA-CZARNECKA *et al.* 2004). The animals were kept in cages 20×13×18 cm under controlled temperature (22°C) and lighting (lights on 8:00-20:00), 4 individuals per cage. Food (mouse chow) and water were available *ad libitum*. The ethical guidelines by the local committee on animal care (license no. 16/OP/2001) were followed throughout the experiments.

In vivo experimental procedures

After one-week adaptation to the laboratory conditions, the animals were ip injected at 10:00 a.m. with 0.5 ml/25 g b.w. of 2% Zymosan A (40 mg/kg b.w.) (Sigma, London, UK) supplemented with morphine hydrochloride (0, 5, 10 or 20 mg/kg b.w., Polfa, Kutno, Poland) or with the same doses of morphine only, in some instances 20 min after ip injection with naltrexone hydrochloride (1 mg/kg b.w.) (Sigma, London, UK). Immediately after zymosan and/or morphine injection, each mouse was placed in a separate cage with a floor covered with a sheet of checked paper. Behaviour, namely body writhing (DOHERTY *et al.* 1987) and crossings over the transverse line (called crossovers) were scored for 45 minutes by a trained observer uninformed of the animals' experimental treatment. Then the animals were transferred to the original cages with a routine bedding, 4 per cage. Each mouse was used only once. The animals were sacrificed by cervical dislocation at time 0 (intact controls) or 4 hours after Zymosan injection (at 2:00 p.m.) and their peritoneal cavities were lavaged with 1 ml PBS. The retrieved exudatory leukocytes were stained with Turk's stain and counted in a haemocytometer. Experiments were repeated at least three times with 4 animals per each experimental group.

In vitro migratory assay

Bone marrow cells from the intact Swiss and CBA mice were collected in 2 ml of RPMI 1640 medium and the cell concentration was adjusted to $5x10^6$ cells/ml. Cells were incubated for 5 min in 10^{-8} M morphine in RPMI or in RPMI only (control). Serum from intact mice was activated by a 30-minute incubation with zymosan (20mg/ml, Sigma, London UK) at 37°C followed by centrifugation and heat-inactivation (56°C, 30 min). Heat-inactivated serum (without prior activation) or RPMI 1640 (Sigma, London, UK) served as a control.

A 48-well microchemotaxis chamber (Neuro Probe, Inc., Maryland, USA) was used to assess the leukocyte migratory activity (e.g. see CHADZINSKA & PLYTYCZ 2004). The lower wells of the apparatus were filled with 27 μl of Zymosan activated serum (ZAS) as a source of chemotactic factors, or heat-inactivated serum or medium, serving as controls. Then the bottom part of the apparatus was covered with nitro-cellulose filters (Nucleopore membrane, Neuro Probe INC., MD, USA), 5 μm pore size. The upper wells were filled with 50 μl of leukocyte suspension from the bone marrow. Chambers were incubated for 3 hrs at 37°C, and then the cells remaining on the upper surface of the filter were removed. Filters were fixed in 4% buffered formaline, stained with Harris's hematoxyline (40 min), washed in water (10 min) and cleared in xylene (15 min). Cells migrating through the filter were counted using a 40× objective. The results were expressed as the percentage of migratory cells = (mean number of cells migrating towards ZAS / mean number of cells migrating towards RPMI 1640) × 100%. Experiments were repeated three times with triplicate samples in each group.

Statistical analysis

All results were expressed as means (±SE) and analysed according to Tukey's test with a significance threshold at $P<0.05$.

Results and Discussion

Intact mice of all investigated strains possessed the lowest number of resident peritoneal leuko-

cytes (PTLs). A massive accumulation of exudatory PTLs, among them polimorphonuclear cells (PMNs), was evident in the animals of all strains investigated 4 hours after Zymosan injection. In CBA mice, the numbers of exudatory PTLs and PMNs were not affected by the morphine co-injection at any dose applied here. In contrast, the co-injection with the highest dose of morphine (20 mg/kg b.w.) caused a significant decrease of PTLs (among them PMNs) in the four remaining strains (see "A" *versus* "B" in Figs 1a,b). In C57BL/6 animals, the numbers of PMNs were already significantly decreased by the lower doses of morphine (5 and 10 mg/kg b.w.) (see "C" *versus* "B" in Fig. 1b).

Animals with peritonitis (but not their intact counterparts) exhibited characteristic body writhes (consisting of a contraction of the abdominal muscles together with a stretching of hind limbs) considered to be pain symptoms (SCISLOWSKA-CZARNECKA *et al.* 2004; DOHERTY *et al.* 1987; RIBEIRO *et al.* 2000). The number of such writhes was high during the first half-an-hour after Zymosan injection, while thereafter these symptoms were sporadic only (SCISLOWSKA-CZARNECKA *et al.* 2004). For statistical comparisons the cumulative numbers of writhes occurring between 0 and 45 min after zymosan injection were used. The frequency of the body writhes was in the order: C57C3H>CBA>Swiss>>Balb/c~C57BL/6 (Fig. 2), thus did not correspond with the pattern of intraperitoneal leukocyte accumulation, including PMNs, which at 4 hrs after zymosan injection was in the order: C57C3H>Swiss~Balb/c>C57BL/6>CBA (Fig. 1). Morphine co-injection at all doses applied here

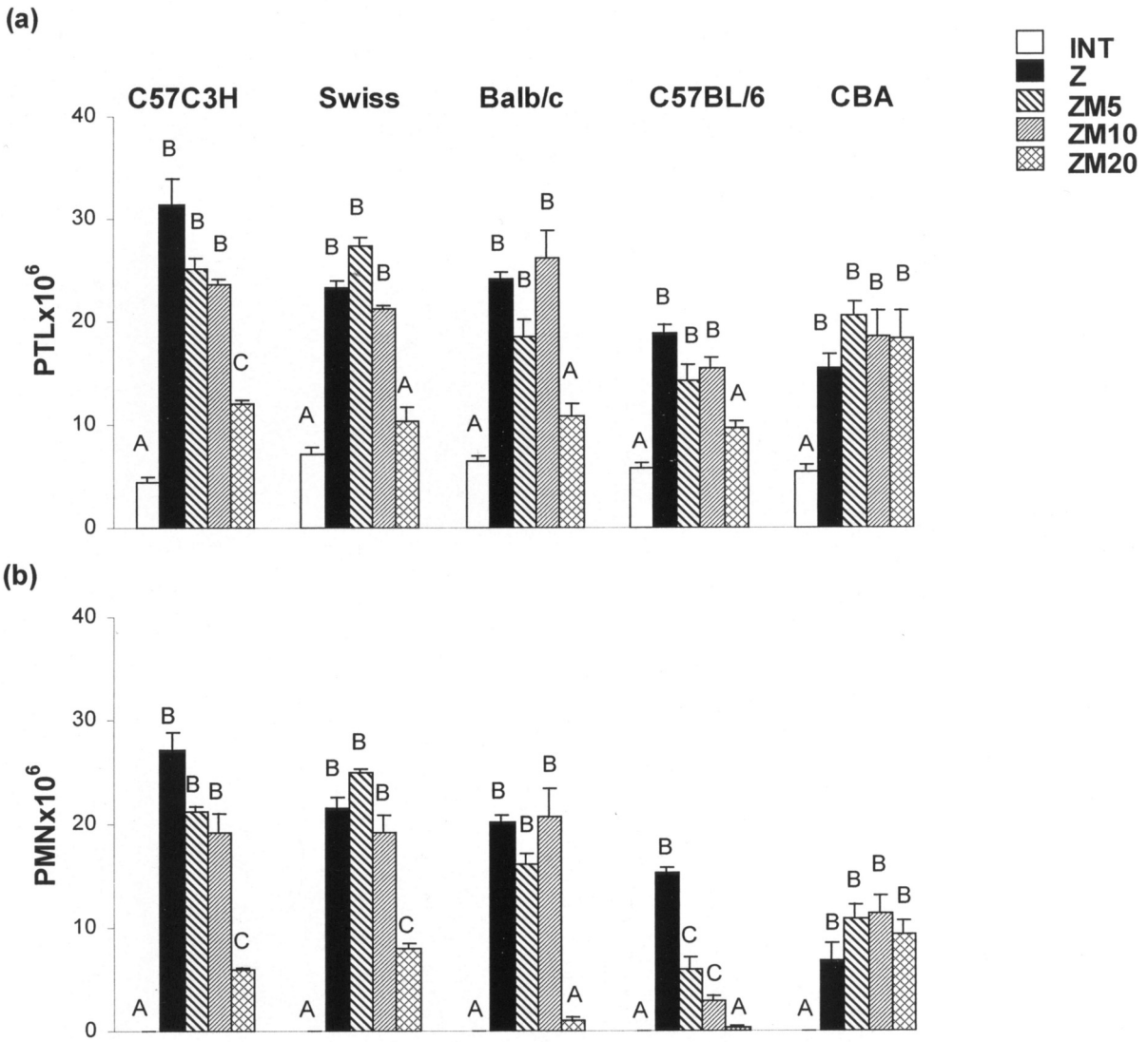

Fig. 1. Effects of morphine co-injection on Zymosan-induced peritonitis in males of C57C3H, Swiss, BALB/c, C57BL/6, and CBA mice. Z, ZM – animals ip injected with Zymosan (40 mg/kg b.w.) supplemented with morphine (5, 10, 20 mg/kg b.w.); INT – intact animals. Means ± SE (n=8-12). Means with different letters vary significantly according to Tukey's test ($P<0.05$). (a) Numbers of peritoneal leukocytes (PTLs), and (b) polymorphonuclear leukocytes (PMNs), 4 hrs after ip injection.

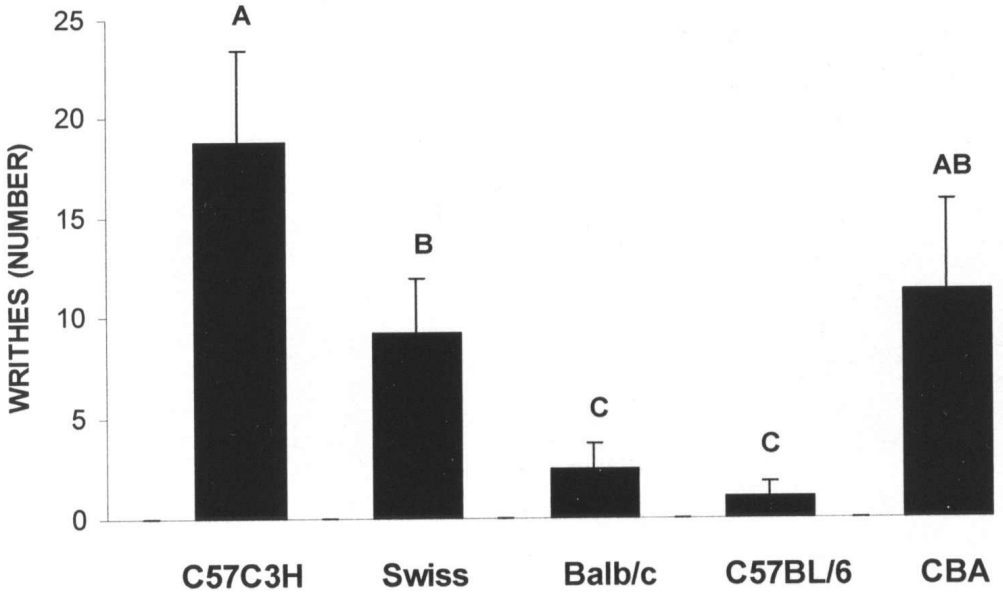

Fig. 2. Pain symptoms (cumulative numbers of body writhes/45 min) during Zymosan-induced peritonitis in males of C57C3H, Swiss, BALB/c, C57BL/6, and CBA mice. Means ± SE (n=8-12). Means with different letters vary significantly according to Tukey's test (P<0.05).

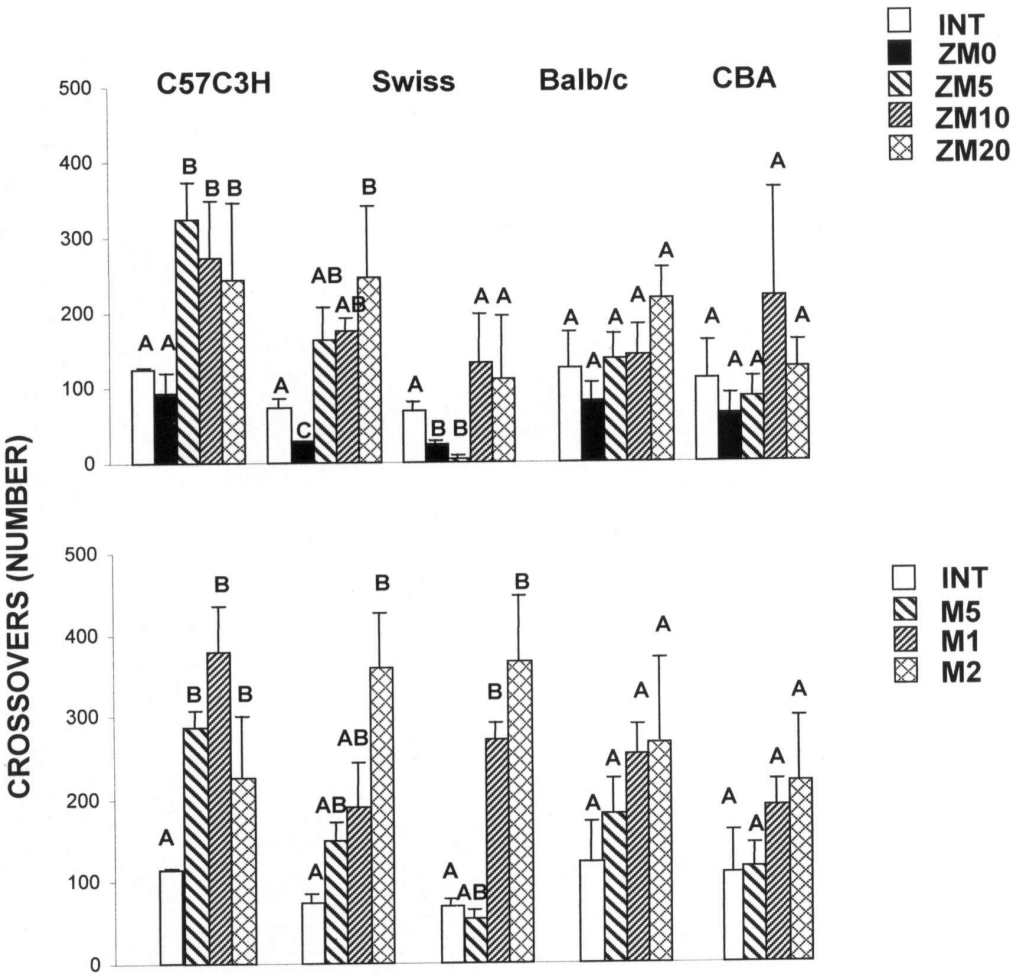

Fig. 3. Effects of morphine on locomotor activity (cumulative numbers of crossovers/45 min) in males of C57C3H, Swiss, BALB/c, C57BL/6, and CBA mice with morphine-modulated Zymosan-induced peritonitis (top) and their counterparts treated with morphine only (bottom). ZM – animals ip injected with Zymosan (40 mg/kg b.w.) supplemented with morphine (0, 5, 10, 20 mg/kg b.w.); M – animals ip injected with with morphine only (5, 10, 20 mg/kg b.w.); INT – intact animals. Means ± SE (n=8-12). Means with different letters vary significantly according to Tukey's test (P<0.05).

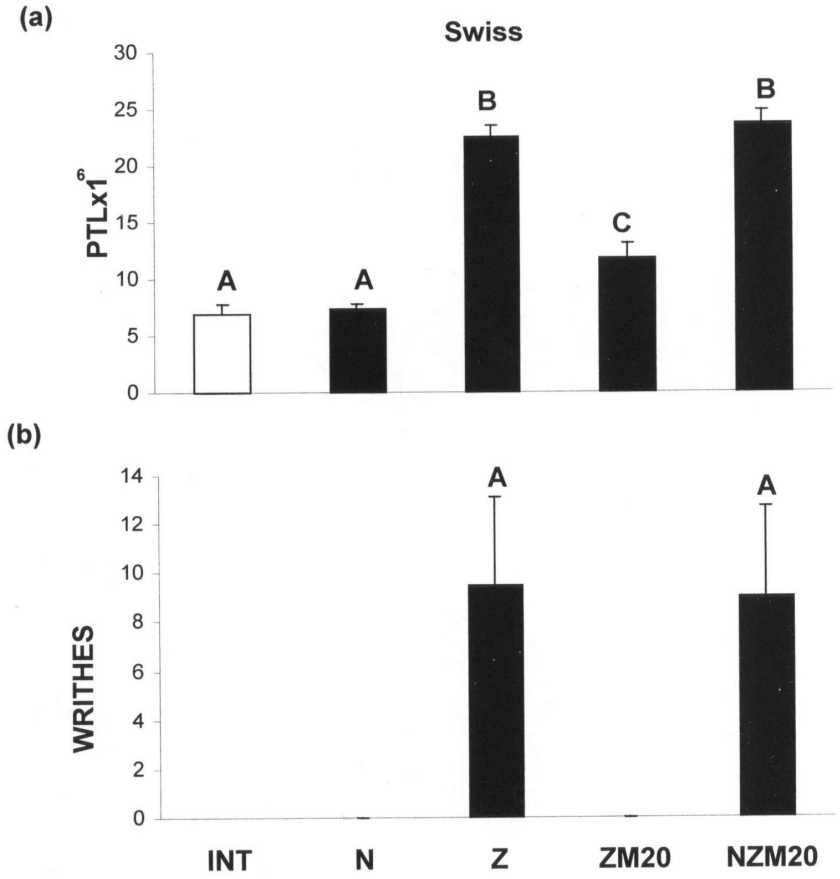

Fig. 4. Effects of morphine and naltrexone injection on Zymosan-induced peritonitis in males of Swiss mice. (a) Numbers of peritoneal leukocytes (PTLs) at 4th hour after Z or ZM injection. (b) Pain symptoms (cumulative numbers of body writhes/45 min). ZM20 – animals ip injected with Zymosan (40 mg/kg b.w.) supplemented with morphine (20 mg/kg b.w.); N – animals ip injected with naltrexone (1 mg/kg b.w.); NZM20 – animals ip injected with naltrexone (1 mg/kg b.w.) 20 minutes before injection of Zymosan with morphine; INT – intact animals. Means ± SE (n=8-12). Means with different letters vary significantly according to Tukey's test ($P<0.05$).

abolished the pain symptoms almost completely in all investigated strains (data not shown).

In all strains the inflammation-related pain symptoms corresponded with the suppression of animal mobility when compared with that of the intact mice exposed to the novel environment (PAOLONE *et al.* 2003). Such inflammation-related hypolocomotion was significant in Swiss and Balb/c (Fig. 3). In all strains, sedation characteristic for the early stages of ongoing peritonitis was reversed by the morphine co-injection. In this respect morphine was efficient already at the lowest dose in all but the Balb/c strain, where higher doses were necessary for anti-sedative effects. Moreover, morphine co-injection with Zymosan induced hyperlocomotion of C57C3H and Swiss animals measured by intensive animal crossovers (Fig. 3, top).

Injection of animals only with morphine induced hyperlocomotion in five investigated strains, which was significant in three of them: C57C3H, Swiss, and Balb/c (Fig. 3, bottom). Such morphine-induced changes of animal locomotor activity are well known in rodents (COOK & BEARDSLEY 2003; MANZANEDO *et al.* 2004). Present results show that morphine-induced hyperactivity is little affected by the ongoing inflammatory process in C57C3H and Swiss mice, while it is inhibited in Balb/c, C57BL/6 and CBA animals (Fig. 3 top and bottom). In conclusion, morphine affects the locomotor activity of animals with peritonitis in a strain-specific manner.

In animals injected with naltrexone 20 minutes before ZM ip injection, both anti-nociceptive and anti-inflammatory effects of morphine were completely reversed while naltrexone itself induced neither PTL accumulation nor pain symptoms, as exemplified in Swiss mice on Fig. 4. These results prove that both anti-nociceptive and anti-inflammatory effects of morphine are mediated through opioid receptor binding.

Bone marrow leukocytes from CBA mice were much more mobile in the presence of a chemoattractant than their counterparts from Swiss animals (Fig. 5). Preincubation with morphine did not affect migration of CBA cells towards chemotac-

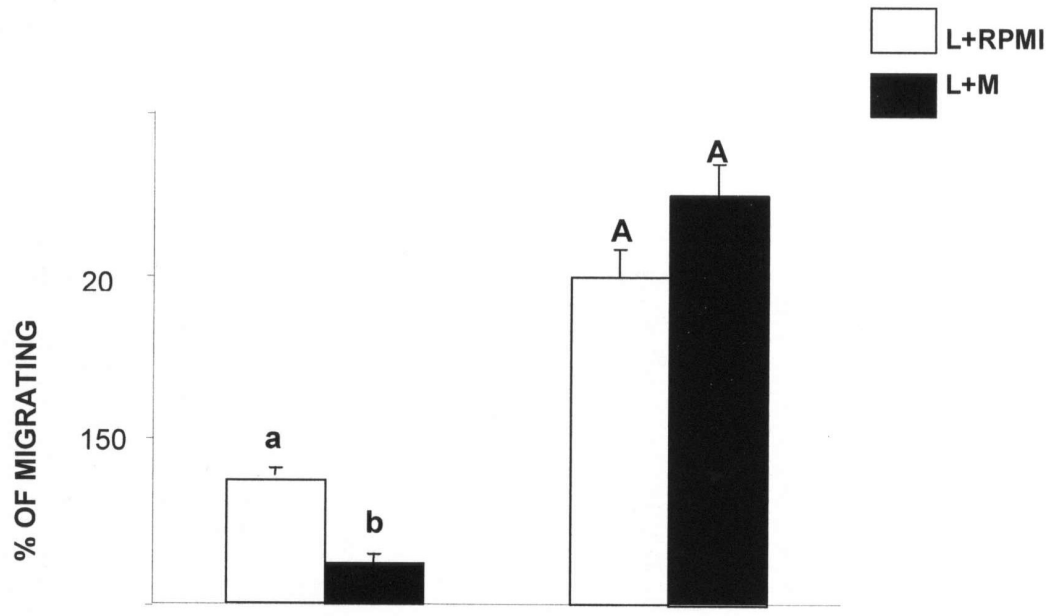

Fig. 5. Effects of morphine preincubation on migration of Swiss and CBA bone marrow leukocytes towards Zymosan-activated serum (ZAS). Leukocytes pre-incubated with medium (L+RPMI) or with morphine (L+M). Means ± SE (n=6). Percentage of migrating cells = (cells migrating towards ZAS / cells migrating towards medium) x 100%. Means with different letters vary significantly according to Tukey's test ($P<0.05$).

tic factors present in Zymosan-activated serum, while the same pre-treatment significantly inhibited migration of Swiss leukocytes towards the same chemoattractants (Fig. 5), the latter already evidenced in a previous paper (CHADZINSKA & PLYTYCZ 2004). The inhibition of Swiss leukocyte chemotactic activity might be connected with morphine-induced desensitisation of leukocyte receptors for some chemotactic factors (ROGERS *et al.* 2000; CHADZINSKA & PLYTYCZ 2004). The lack of morphine-induced inhibition of CBA leukocyte migration to some chemoattractants present *in vivo* in the focus of inflammation might be at least partly responsible for the absence of anti-inflammatory effects of morphine in this strain of mice. The lack of cross-desensitisation of chemokine receptors in CBA mice might be connected with the lower expression of opioid receptors in this particular strain of mice (NATORSKA *et al.* in progress).

It has been shown previously that the peritoneal cavity of CBA mice is inhabited by an exceptionally rich population of mast cells, which are much more numerous than those in other investigated strains of mice (CBA>Balb/c>C57BL>OUTBRED>>Swiss) (STANKIEWICZ *et al.* 2001). Moreover, CBA mast cells degranulate and release histamine upon morphine treatment, while such morphine-induced degranulation and histamine release are not evident in the Swiss mast cells (STANKIEWICZ *et al.* 2004). Corollary, morphine-induced degranulation of a rich peritoneal CBA mast cell population undoubtedly contributes to the lack of anti-inflammatory effects of the drug in this particular strain of mice (STANKIEWICZ *et al.* 2004). In sum, the peculiarity of CBA strain resistance to anti-inflammatory effects of morphine is based on the lack of morphine-induced inhibition of chemotaxis and on the abundance of morphine-sensitive mast cells.

Summarising, the early stages of Zymosan-induced peritonitis in male mice were accompanied by pain manifested by body writhes of strain-specific frequency, corresponding with animal hypoactivity. These symptoms were reversed by the morphine co-injection. In some (but not all) strains the ongoing peritonitis did not abolish the strain-specific morphine-induced hyperlocomotion. In addition to anti-nociceptive action, the high dose of morphine added to the pro-inflammatory agent inhibited accumulation of exudatory PTLs (among them PMNs) in males of some but not all strains of mice. The lack of anti-inflammatory effects of morphine in the CBA strain may be related to the abundant population of peritoneal mast cells sensitive to morphine-induced degranulation and to the lack of morphine-induced inhibition of leukocyte migration towards inflamed peritoneal cavity. Other factors contributing to the lack of morphine-induced inhibition of peritonitis in CBA strain are being currently elucidated. In conclusion, local administration of morphine together with the inflammation-inducing agent exerts in mice several dissociated modulatory effects that are strain-specific.

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