Effects of Macrophage Depletion on Peritoneal Inflammation in Swiss Mice, Edible Frogs and Goldfish*

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SWISS mice, edible frogs and goldfish i.p. injected with zymosan (Z groups) develop peritoneal inflammation connected with a massive intraperitoneal accumulation of leukocytes, which is significantly diminished in mice and fish (but not frogs) by supplementation of zymosan with morphine (ZM groups). In order to check the putative role of resident peritoneal macrophages in morphine-modulated zymosan-induced peritonitis, some animals were depleted of resident macrophages by repeated i.p. injections of clodronate-liposomes (CL) followed by Z or ZM injection. In SWISS mice such CL-induced removal of Mac-3-positive cells (macrophages) resulted in an enhanced influx and prolonged accumulation of polymorphonuclear leukocytes (PMNs) in CL-Z and CL-ZM groups in comparison with their counterparts with intact macrophages. Nevertheless, supplementation of zymosan with morphine inhibited the early stages of peritonitis in CL-treated animals as it did in untreated mice. This indicates that intact peritoneal macrophages of SWISS mice are important for limiting PMN accumulation, perhaps mainly through the release of IL-10, but are not critical for the induction of anti-inflammatory effects of morphine during the early stages of peritonitis. Unexpectedly, macrophage depletion in CL-treated frogs and fish resulted in a lack of a typical peritonitis in both Z and ZM groups of these ectothermic animals.

Key words: Clodronate-liposomes, peritonitis, Rana esculenta, Carassius auratus, IL-10.

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Peritoneal inflammation is easily induced and followed in various vertebrates. Previously, its course was investigated in representatives of fish (CHADZINSKA et al. 1999; CHADZINSKA et al. 2000; GRUCA et al. 1996), anuran amphibians (KOLACZKOWSKA et al. 2000; MENASZEK et al. 1999), and in several strains of mice (KOLACZKOWSKA et al. 2001a; KOLACZKOWSKA et al. 2002; PLYTYCZ & NATORSKA 2002; STANKIEWICZ et al. 2001). The course of peritonitis may be modulated by several endogenous and exogenous factors, such as season (MENASZEK et al. 1999), ambient temperature (MENASZEK et al. 1999), stress (CHADZINSKA et al. 2002; NATORSKA et al. 2001; SCISLOWSKA-CZARNECKA et al. 2004), and pharmacological factors, e.g. morphine (CHADZINSKA et al. 1999; CHADZINSKA et al. 2000; KOLACZKOWSKA et al. 2001a; PLYTYCZ & NATORSKA 2002).

In a series of experiments on the modulatory effects of morphine in experimental peritonitis, animals were intraperitoneally injected with a single irritant (e.g. thioglycollate broth, sephadex or zymosan) or an irritant supplemented with morphine. Morphine co-injection significantly impaired peritonitis in the Atlantic salmon (CHADZINSKA et al. 1999), goldfish (CHADZINSKA et al. 2000), and several strains of mice (C57C3H, SWISS, Balb/c, C57BL/6, CB6) (CHADZINSKA et al. 1999; KOLACZKOWSKA et al. 2001a; PLYTYCZ & NATORSKA 2002; NATORSKA & PLYTYCZ 2004), but did not impair peritonitis in the CBA mouse strain (NATORSKA & PLYTYCZ 2004) nor in frogs and

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MICE seem to be responsible for the lack of anti-
morphine-dependent degranulation in CBA mice.

Dance of peritoneal mast cells and their sensitivity
in WBB6F1-W/Wν and Balb/c mice induced by
narcotics has shown that a lack of mast cells sig-
ificantly inhibited the early stages of peritonitis
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events participating in the inflammatory process,
e.g. mast cells and/or resident peritoneal macro-
phages (AJUEBOR et al. 1999; KOLACZKOWSKA
et al. 2001a, b; KOLACZKOWSKA et al. 2002;
STANKIEWICZ et al. 2001). Mast cell-knockout
animals may be used for investigations on the role
of mast cells in inflammatory processes (WBB6F1-W/W*)
(KOLACZKOWSKA et al. 2001a, b; KOLACZKOWSKA
et al. 2002). Animals pre-injected with a
well-known mast cell degranulator, compound
48/80 (AJUEBOR et al. 1999; KOLACZKOWSKA
et al. 2001a, b; KOLACZKOWSKA et al. 2002;
STANKIEWICZ et al. 2001), may also be used. Such ap-
proaches have shown that a lack of mast cells sig-
nificantly inhibited the early stages of peritonitis in
WBB6F1-W/W* and Balb/c mice induced by
zymosan only (KOLACZKOWSKA et al. 2001a, b;
KOLACZKOWSKA et al. 2002; STANKIEWICZ et al.
2001) or by zymosan supplemented with mor-
phine (KOLACZKOWSKA et al. 2001a). It should be
underlined that the effects of manipulation with
mast cell numbers are strain specific. In particular,
pre-treatment with the compound 48/80 has en-
hancing effects on the subsequent zymosan-
duced peritonitis in SWISS mice while the same
procedure is inhibitory in CBA mice (STANKIE-
WICZ et al. 2001). On the other hand, the abundance
of peritoneal mast cells and their sensitivity to
morphine-dependent degranulation in CBA mice
seem to be responsible for the lack of anti-
inflammatory effects of morphine on this strain of
mice (in preparation).

A procedure commonly used for macrophage de-
pletion is the injection of a liposome-encapsulated
apoptosis-inducing drug, clodronate (CL) (AJUEBOR
et al. 1999; ESPENES et al. 1997; KOLACZKOWSKA
et al. 2002; MIKAMI et al. 2003; VAN ROOIJEN
et al. 1996).

The aim of the present experiments was to inves-
tigate the effects of animal pre-treatment with
clodronate-liposomes on the subsequent peritoniti-
tis induced by zymosan alone or zymosan supple-
mented with morphine in phylogenetically distant
animals: SWISS mice, frogs, and fish. Macro-
phage depletion enhanced subsequent peritonitis
in SWISS mice but abolished it completely in the
frog and fish.

Material and Methods

Animals

Goldfish (Carassius auratus) and edible frogs (Rana esculenta) from commercial suppliers were
kept in aquaria at room temperature. Body mass of
the goldfish and frogs at the time of experiments
were 10-20 g and 17-25 g, respectively.

Adult male SWISS mice, purchased from a
commercial supplier (Breeding of Laboratory Animals,
Collegium Medicum, Kraków, Poland), were kept
in 20x13x18 cm cages (four mice per cage) in a
room with controlled temperature (22°C) and pho-
toperiod (lights on 8:00-20:00). Food (standard
mouse laboratory chow) and water were available
ad libitum. Animals were 6-8 weeks of age (25-30
g body mass) at the beginning of investigations.
The experiments were conducted according to license
do. 16/OP/2001 from the Local Ethical Committee.

Treatments

Macrophage depletion

Multilamellar liposomes containing clodronate
dichloromethylene diphosphonate, Cl2MDP-lipo-
somes, CL) and control PBS-containing lipo-
somes (L) were prepared as described previously
(VAN ROOIJEN & SANDERS 1994). CL2MDP was a
gift of Roche Diagnostics GmbH, Mannheim,
Germany. The animals were either untreated or i.p.
injected with CL or L for three consecutive days, in
a volume of 100 μl for mice (according to AJUEBOR
et al. 1999; KOLACZKOWSKA et al. 2002), 50 μl
for fish, and 200 μl for frogs (on the basis of pre-
liminary experiments on depletion of peritoneal
macrophages).
Induction of peritonitis

Twenty-four hours after the last CL or L injection the animals were either left untreated or i.p. injected with freshly prepared zymosan (Z groups) (2 mg/ml, 0.5 ml/25 g b.w.) (Zymosan A, Sigma, St. Louis, MO, USA) in sterile PBS (220 mOsM for frogs, 280 mOsM/fish and 320 mOsM/mice), or with zymosan supplemented with morphine hydrochloride (20mg/kg b.w.; ZM groups, Polfa, Kutno, Poland). Animals were killed at selected time points and their peritoneal cavities were lavaged with 1 ml of PBS. Exudatory cells were Turk-stained and counted with a hemocytometer or on cytopsin preparations stained with MGG. Peritoneal fluid was stored at -20°C for future cytokine assays.
Immunocytochemistry

For macrophage identification, cytospin preparations were fixed with a methanol:acetone mixture (1:1), stored at -20°C and treated with rat anti-mouse anti-Mac-3 monoclonal antibodies (Pharmingen, San Diego, USA) followed by mouse anti-rat biotin-conjugated anti-IgG1 secondary antibodies (Pharmingen, San Diego, USA). Thereafter, the preparations were incubated with a streptavidin-peroxidase complex (Pharmingen, San Diego, USA) and the products of reaction were visualised with 3',3'-diaminobenzidine tetrahydrochloride – DAB (ICN, Aurora, USA).

IL-10 detection

IL-10 levels were estimated by ELISA according to manufacturer instructions (BiouSource, Camarillo, USA).

Fig. 3. Effects of macrophage depletion by clodronate liposomes (CL) on the number of exudatory polymorphonuclear leukocytes (PMNs) (top) and IL-10 levels (bottom) in SWISS mice at the 6th hour of peritoneal inflammation induced by zymosan only (Z) or zymosan supplemented with morphine (ZM). INT – intact animals. X ± SE (n=4-8). Values with different letters (A, B, C) vary significantly according to Tukey’s test at P<0.05.

Fig. 4. Effects of morphine supplementation (ZM) on zymosan-induced peritonitis (Z) in SWISS mice pre-treated with clodronate-liposomes (CL) for macrophage depletion. X ± SE (n=4-8). Top: PTL – peritoneal leukocytes; Bottom: PMN – polymorphonuclear leukocytes; Asterisks – statistically significant differences between CL-Z and CL-ZM at P<0.05.

Fig. 5. Effects of macrophage depletion by clodronate liposomes (CL) on exudatory polymorphonuclear leukocytes (PMNs) in the edible frog Rana esculenta (top) and goldfish Carassius auratus (bottom) at 24 hours of peritoneal inflammation induced by zymosan only (Z) or zymosan supplemented with morphine (ZM). INT – intact animals. X ± SE (n=4-8). Values with different letters (A, B, C) vary significantly according to Tukey’s test at P<0.05.
Results and Discussion

In all investigated species i.p. zymosan injection (Z groups) induced peritoneal inflammation connected with intraperitoneal accumulation of exudatory leukocytes (Fig. 1, top). Among the total pool of peritoneal leukocytes (PTLs) of mice and frog, polymorphonuclear neutrophils (PMNs) were easy to distinguish and count both by hemocytometer and on cyto spin preparations. They were apparently absent in intact animals and appeared quickly at the early stages of peritonitis (Fig. 1, bottom). In contrast, goldfish heterophiles can be distinguished only at the level of transmission electron microscopy (BIELEK et al. 1999), therefore for the present purpose they were pooled with macrophages as phagocytic cells (PCs) (Fig. 1, bottom right). Kinetics of the intraperitoneal accumulation of leukocytes were species-specific, but in each case the number of exudatory leukocytes that accumulated in the focus of inflammation 24 hours after injection was higher than that in the respective controls (time 0) (Fig. 1). Figure 1 also shows that the intraperitoneal influx of leukocytes, among them PMNs/PCs, was significantly impaired by morphine supplementation of the irritant in SWISS mice and goldfish, but not in the edible frogs. This fully confirms our previous results on zymosan-induced inflammation in SWISS mice (PLYTYCZ & NATORSKA 2002), on thiglycollate-induced inflammation in the goldfish (CHADZINSKA et al. 2000), and sephadex-induced frog peritonitis (KOLACZKOWSKA et al. 2000).

Anti-inflammatory effects of morphine are antagonized by naltrexone and correspond to decreased levels of chemoattractants in the focus of inflammation and in blood (CHADZINSKA et al. 1999; CHADZINSKA et al. 2000). In the case of mice, morphine effects are achieved by a relatively high dose (20 mg/kg b.w.), while 5 mg/kg b.w. of morphine efficiently elicits the well-known anti-nociceptive effects of this drug. In our model of peritoneal inflammation, zymosan-induced pain is manifested by the characteristic body writhes that are completely attenuated in the animals co-injected with morphine (PLYTYCZ & NATORSKA 2002; NATORSKA & PLYTYCZ 2004).

At the early stages of zymosan-induced peritonitis, the intraperitoneal accumulation of endogenous opioids was recorded. These were derived from closely located lymph nodes and some brain areas (CHADZINSKA et al. 2003) and/or produced and released locally by inflammatory leukocytes (CHADZINSKA et al. 2001) as evidenced also in other models of inflammation (CABOT et al. 2001; RITTNER et al. 2001; PRZEWLOCKI et al. 1992). It seems that local administration of exogenous morphine may support or replace the anti-nociceptive action of endogenous opioids in the focus of inflammation and can limit the chemotactic influx/accumulation of new-coming leukocytes (PLYTYCZ & NATORSKA 2002).

In order to check the role of macrophages in morphine-modulated peritonitis, some animals were selectively depleted of peritoneal macrophages by a 3-day treatment with liposomes encapsulating the apoptosis-inducing clodronate (CL). On the 4th day, animals were injected only with zymosan (CL-Z groups) or zymosan supplemented with morphine (CL-ZM groups). No differences were observed between animals treated with PBS-containing liposomes (L) and those left untreated. In the case of mice, an efficiency of clodronate-liposomes treatment in the removal of macrophages was evidenced by the lack of Mac-3-positive cells in CL-treated groups, while they were numerous in the respective groups of animals untreated with CLs (data not shown).

Figure 2 shows that in SWISS mice an intraperitoneal influx and accumulation of PTLs (including PMNs) during the first 24 hours of inflammation was significantly higher in macrophage-depleted CL-Z and CL-ZM groups of animals than in the respective Z and ZM counterparts with the intact population of macrophages.

Figure 3 shows the PMN (top) and IL-10 (bottom) accumulation in exudatory fluid at the 6th hour of peritonitis in SWISS mice with intact macrophages (left parts) and their CL-treated counterparts (right parts). The IL-10 is significantly elevated in Z and ZM groups of animals with intact macrophages, while it is at the control level in all CL-pretreated mice. The latter corresponds with a significant increase of PMNs in macrophage-depleted animals versus respective controls (CL-ZM versus ZM; CL-Z versus Z) (Fig. 3). Similar enhancement of PMN influx in macrophage-depleted SWISS mice, corresponding to a marked reduction of IL-10 levels in the lavage fluid, was described previously by AJUEBOR et al. (1999). Therefore our results confirmed the assumption that macrophage-derived IL-10 plays a pivotal role in a down-regulation of PMN accumulation in the focus of inflammation as its lack leads to enhanced accumulation of PMNs.

Interestingly, despite the macrophage depletion, morphine supplementation inhibited influx and prolonged local accumulation of leukocytes (int-
including PMNs) in the focus of inflammation in CL-ZM versus CL-Z groups of animals (Fig. 4), in a manner similar to that in the animals with intact macrophages (compare Fig. 4 with the left panel of Fig. 1). Therefore it seems that intact macrophages are not critical for the development of inhibitory effects of morphine in the early stages of inflammation, at least in SWISS mice, whereas the role of mast cells may be more crucial (KOLACZKOWSKA et al. 2001a; STANKIEWICZ et al., in press).

Surprisingly, macrophage depletion in the goldfish and edible frogs resulted in a lack of inflammatory cell influx in CL-treated animals injected with Z or ZM. The results of experiments performed so far on fish and frogs depleted of macrophages are not conclusive. The CL-treatment alone induced a significant intraperitoneal accumulation of PMNs in the frog (Fig. 5, top) and phagocytes (perhaps heterophiles) in the goldfish (Fig. 5, bottom) when compared to the respective intact controls. In contrast to animals with intact macrophages, the number of peritoneal leukocytes in CL-treated frogs and fish was not further increased by injection with either Z or ZM (Fig. 5). It seems that in frogs and fish the clodronate-liposomes themselves induced moderate but relatively long-lasting peritoneal inflammation with local accumulation of exudatory PMNs/heterophiles. It is known that in rodents CLs induce a minor inflammatory response that is quickly resolved and absent by day 4 when further experiments on macrophage-depleted animals are conducted (VAN ROOIJEN & SANDERS 1994). In ecotermic vertebrates with a low metabolic rate, such CL-induced inflammation may last longer, thus the starting point for Z or ZM injections should be postponed. Nevertheless, the possibility that intact macrophages are crucial for the proper development of peritonitis in fish and frogs cannot be excluded at present.

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