



The influence of opioids on the humoral and cell-mediated immune responses in mice. The role of macrophages

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Abstract:

Background: Our experiments were aimed to test the influence of treatment with different opioids (morphine, fentanyl, methadone) on the humoral and cell-mediated immune responses.

Methods: Mice were treated intraperitoneally (*ip*) with opioids for several days and next either immunized with sheep red blood cells (SRBC) to test the antibody production or skin-sensitized with hapten picryl chloride (PCL) to induce contact hypersensitivity (CHS). In addition, the effects of opioids on the production of reactive oxygen intermediates (ROIs) and cytokines by peritoneal macrophages (Mf) and on the expression of surface markers on these cells and blood leukocytes were estimated.

Results: Opioids caused an enhancement of ROIs and cytokines production when macrophages were stimulated with zymosan or lipopolysaccharide (LPS) and reduced the expression of antigen presentation markers on Mf. Numbers of anti-SRBC plaque forming cells (PFC) and antibodies titres were lower in mice treated with all tested opioids. Depending on the use of particular opioid and the phase of allergic reaction, effects of the treatment on CHS were diverse. While morphine decreased the early and late phases of induction of CHS responses, methadone increased both reactions. In case of the effector phase of CHS, morphine and fentanyl increased both its early and late stages, while methadone decreased the late reaction. Treatment of recipients with opioids had diverse influence on the passive transfer of CHS in these animals.

Conclusions: Our experiments show that the action of opioids on the immune system is a complex phenomenon dependent on such variables as type of opioid, character of response (humoral *versus* cellular) and types of cells involved. Here Mf seem to play a significant role.

Key words:

opioids, macrophages, humoral response, contact hypersensitivity

Abbreviations: APCs – antigen presenting cells, CD – cluster of differentiation, CHS – contact hypersensitivity, DNFB – 1-fluoro-2,4-dinitrobenzene, DPBS – Dulbecco's phosphate buffered saline, FCS – fetal calf serum, FcγR – Fcγ receptor, FITC – fluorescein isothiocyanate, IL – interleukin, LPS –

lipopolysaccharide, Mf – macrophages, mAb – monoclonal antibody, NK – natural killer cells, NMDA – *N*-methyl-D-aspartate, Oil-Mf – oil-induced peritoneal macrophages, OPs – opioids, OX – oxazolone, PAMPs – pathogen associated molecular patterns, PCL – picryl chloride (1,3,5-trinitrophenyl

chloride), PE – phycoerythrin, PFC – plaque forming cells, R-Mf – resident peritoneal macrophages, ROIs – reactive oxygen intermediates, SRBC – sheep red blood cells, TGF – transforming growth factor, T-Mf – thioglycollate-induced peritoneal macrophages, TNBSA – trinitrobenzene sulfonic acid, TNF – tumor necrosis factor, TNP – 2,4,6-trinitrophenyl, Treg – T regulatory cells, *vs.* – *versus*

Introduction

It is commonly known that immune cells (macrophages, neutrophils and T lymphocytes) express different types of opioid receptors [5, 6, 33] and their signalization pathways may regulate immune response. Opioid receptors are generally classified into three distinct types: μ , δ and κ [11, 37, 38]. Each class of opioid receptors is activated by appropriate endogenous opioids such as endorphins, enkephalins and dynorphins. Additionally, they have some exogenous agonists; μ receptors bind morphine, fentanyl and methadone, which is also δ agonist, and κ receptors are activated by oxycodone [5, 6, 27].

Macrophages (Mf) are engaged in nonspecific innate immunity (phagocytosis and neutralization of antigens) and in specific immune responses. They participate in the induction stage as antigen presenting cells (APCs) and as effector cells in delayed-type hypersensitivity reactions including contact hypersensitivity (CHS) [1–4]. Although Mf may act as APCs, typical antigen presentation, especially in CHS, is a complex phenomenon generally mediated by other cells, like skin dendritic cells, epidermal Langerhans cells or keratinocytes [12]. Defence functions mediated by Mf are modulated by opioid therapy [13], in most cases leading to their inhibition or reduction (phagocytosis, antibody formation). Similarly, in the presence of opioids (OPs), various immune mechanisms like activation of NK and lymphocyte proliferation under the influence of mitogens are reduced, but sometimes, although rarely, OPs (like morphine) stimulate CHS and the synthesis of interferon- γ [18–20]. These effects are enabled by the presence of cell surface opioid μ -receptors on immunocytes [6, 32, 38], which are selectively antagonized by naloxone [37]. Since Mf possess μ -opioid receptors, they potentially may be influenced by OPs [8, 24, 33, 34, 36]. Although all tested by us OPs (morphine, fentanyl and methadone) are able to activate opioid μ receptors, such action on Mf was previously shown

only for morphine [20, 33, 34, 36]. All mentioned OPs vary with respect to solubility; morphine is hydrophilic while fentanyl and methadone are both lipophilic, and also differ in their pharmacokinetics [32, 37]. Fentanyl is a semisynthetic opioid which has analgesic activity 75–100 times stronger than morphine [38]. Methadone, apart from the ability to activate μ receptors, stimulates opioid δ receptors and is a NMDA-receptor agonist as well [37]. Its analgesic action depends also on the presynaptic blocking of the re-uptake of monoamines (serotonin and noradrenaline). The clinical observations confirming these particular properties of methadone lead to establishing this opioid as a basic medicament in the management of neuropathic pain [37]. Because of its lipophilic property, methadone has a long half-life time in plasma and accumulates in the tissues [38] leading to different pharmacokinetic properties and perhaps various immunomodulatory influences on immunocytes, mainly macrophages.

The aim of the present study was to examine the impact of the therapy with methadone, compared to morphine and fentanyl, on the immune functions of macrophages and other immunocytes expressing opioid receptors, in humoral and cellular immunity (contact hypersensitivity model).

Materials and Methods

Animals

Eight- to 10 week-old inbred male CBA/J mice (23 ± 2 g) from the Department of Immunology, College of Medicine, Jagiellonian University, Kraków, Poland were used. Mice had free access to standard food and water. All experiments were conducted according to the guidelines of Animal Use and Care Committee of Jagiellonian University College of Medicine (102/2009).

Reagents

The following reagents were used: lucigenin (bis-N-methylacridinium nitrate), zymosan A, hydrogen peroxide, heparin sodium salt, o-phenylenediamine, 2-mercaptoethanol (2-ME), recombinant murine TNF- α (all from Sigma, St. Louis, MO, USA); RPMI 1640

cell culture medium, fetal calf serum (FCS), thioglycollate medium (Gibco Life Technologies, Grand Island, NY); 2,4,6-trinitrobenzene sulfonic acid (TNBSA) (Eastman Kodak, Rochester, NY, USA); twice recrystallized picryl chloride [2,4,6-trinitrophenyl (TNP) chloride, PCL] (Chemica Alta, Edmonton, Alberta, Canada); recombinant mouse IL-6 (PeproTech, Rocky Hill, NY, USA); paraffin oil Marcol 152 (Exxon Corp., Houston TX, USA); horseradish peroxidase, streptavidin (Vector Laboratories, Burlingame, CA, USA); granulated milk (Marvel, Chivers Ltd., Coolock, Ireland); sheep red blood cells (SRBC) from Department of Breeding Sheep and Goats, University of Agriculture (Kraków, Poland). For cell cultures Nunc lab ware (Roskilde, Denmark) was used.

Antibodies

Rat anti-mouse IL-6 mAb (MP5-20F3), biotinylated rat anti-mouse IL-6 mAb (MP5-32C11), rat anti-mouse FcγRII/III mAb (2.4G2), rat anti-mouse TNF-α mAb (G281-2626), biotinylated rat anti-mouse TNF-α mAb (MP6-XT3), IL-10 OptEIA™ ELISA Set; FITC conjugated rat anti-mouse CD4, anti-CD45, anti-NK11 and anti-Mac3 mAbs, PE-conjugated anti-mouse anti-CD8, anti-CD14, anti-CD19, anti-CD80, anti-CD86, anti-H2k, APC-conjugated anti-mouse CD3 mAbs (all from BD Pharmingen, San Diego, CA, USA).

Experimental design

The summary of several experiments performed is shown in Figure 1a. Mice donors treated with opioids (see OPs administration) were the source of blood cells (for FACS analysis of cell surface markers expression), sera (for anti-SRBC antibodies estimation) and spleens (for PFA). Opioid-treated mice were also donors of T-Mf (for SRBC feeding followed by transfer into naive recipients), mediating humoral response later tested in PFA. The influence of OPs treatment was also tested in the induction and effector phases of CHS to PCL (see Fig. 1B). PCL-stimulated lymph nodes lymphocytes and splenocytes were adoptively transferred into naive or OPs-treated recipients, in whom CHS response was tested (see Fig. 1C). OPs-treated mice were also stimulated with mineral oil by *ip* injection and thus induced Oil-Mf were tested for ROIs production, cell surface markers expression in FACS analysis, or were cultured and cytokine release into the supernatants was measured by ELISA. Fi-

nally, Oil-Mf were labelled with TNP and used as APCs, when transferred into naive recipients to elicit CHS reaction (see Fig. 1D).

OPs administration

Mice were treated with OPs for five days as follows: morphine 20 mg/kg in two doses per 24 h, fentanyl 10 mg/kg in two doses per 24 h, and methadone 30 mg/kg once a day. In case of naloxone, the antagonist of morphine, a daily treatment was established on the level of 0.5 mg/kg. All medicaments were administered *ip*.

Harvesting of peritoneal oil-, thioglycollate-induced or resident Mf [2, 3]

Mf were collected from peritoneal cavities of CHS tested control mice (resident Mf, R-Mf) or from opioid treated mice (morphine, fentanyl, methadone, see OPs administration) injected on day 5, *ip* with 1 ml of Marcol 152 mineral oil (Oil-Mf) or with 2 ml of thioglycollate medium (T-Mf). Mf stimulated differently display particular properties and so are used in distinct assays. R-Mf from CHS tested mice and Oil-Mf were used for total ROIs activation assessment, and T-Mf were stimulated for phagocytosis activation. Three to 6 mice were used per group. Mf were washed out with 5 ml of DPBS containing 5 U heparin/ml. Oil-induced peritoneal cells contained over 90 percent of Mf (FcR- and esterase-positive cells) and were not purified further. R-Mf with Mf content below 60% were purified from granulocytes by discontinuous gradient centrifugation (30 min, 700 × g, 20°C) on the Ficoll-Paque medium ($\rho = 1.077 \text{ g/cm}^3$) and collected from the upper interface, which contained over 90% Mf and some lymphocytes and was used for chemiluminescence assay.

Hemolytic plaque (PFC) and direct hemagglutination assays [7, 17]

OPs-treated mice and untreated controls were immunized *iv* with 5×10^7 sheep red blood cells (SRBC). Alternatively, thioglycollate-induced peritoneal macrophages from naive or OPs-treated donors were incubated for 30 min in 37°C with SRBC, in ratio 1:10, followed by osmotic shock for lysis of not engulfed SRBC and then were injected *ip* in dose 4×10^6 per naive recipient. In both cases humoral response was

measured a week later. Mice were individually sacrificed and blood and spleens were collected. Anti-SRBC antibodies levels were estimated by direct hemagglutination assay (HA) and results are expressed as log 2 of titres. Twofold serial dilutions of the tested sera in DPBS were incubated with 1% SRBC for 90 min in 37°C. The highest dilution of the serum causing the hemagglutination effect was considered the titer of combined IgM and IgG anti-SRBC antibodies. Anti-SRBC IgM antibodies were eliminated by treatment of sera with 0.15M 2-ME, which allowed the assessment of IgM and IgG levels individually. From harvested spleens single cell suspensions were prepared in RPMI 1640 medium and numbers of cells producing anti-SRBC antibodies were estimated in hemolytic plaque assay by microscope slide technique in the presence of guinea pig complement. The numbers of PFC were individually calculated for each mouse and are expressed as numbers of PFC per spleen (PFC/spl).

Active sensitization and measurement of contact sensitivity *in vivo* [23, 30]

Mice were actively sensitized by a topical application of 0.15 ml of 5% PCL in acetone-ethanol mixture (1:3, v/v) to the shaved abdomen and hind feet. Five days later (see Fig. 1b), mice were challenged on both sides of ear skin with 10 µl of 0.4% PCL in olive oil-acetone mixture (1:1, v/v). Experimental groups of mice were treated with distinct opioids (morphine ± naloxone, fentanyl or methadone) for 5 days. The administration of OPs in case of induction phase testing began 2 days before sensitization with hapten and 2 days before challenge in case of effector phase measurement (see Fig. 1b). The positive control group consisted of animals which were skin-sensitized without OPs therapy. The subsequent increase in ear thickness was measured 2 h and 24 h after challenge with an engineer's micrometer (Mitutoyo, Tokyo, Japan). The background increase in ear thickness (~2 units at 24 h) of non-sensitized littermate animals that were similarly challenged, was subtracted from positive control group and each of the experimental groups to yield a net ear swelling. Each experimental and control group consisted of 5 mice.

Adoptive cell transfer of CHS [29]

CBA/J mice were skin sensitized topically as described above. On day 4, single cell suspensions were prepared from the harvested inguinal and axillary lymph nodes and spleens. Then, the mixtures of 7×10^7 immune lymph node and spleen cells in 0.25 ml of DPBS were adoptively transferred *iv* into syngeneic naive recipients that were subsequently ear challenged with PCL and 24 h later tested for CHS as described above. Some groups of recipients had been injected with appropriate opioid (morphine ± naloxone, fentanyl or methadone) for 5 days before CHS measurement (see Fig. 1C).

Transfer of *in vivo* OPs-treated, *in vitro* TNP-labelled Oil-Mf to naive recipients [2]

Mice treated with OPs for 6 days were source of Oil-Mf, induced by *ip* injection of 1 ml of mineral oil at day 0 into each mouse donor (see Fig. 1D). Five days later, Mf were harvested and labelled with TNBSA (at ratio 2 mg TNBSA per 10^8 Mf in DPBS for 10 min in darkness) and were transferred to naive recipients. A week later background measurement was done, followed by 0.4% PCL challenge. CHS reactions were measured 2 h (early component) and 24 h (late component) after challenge. For more information see Active sensitization and measurement of contact sensitivity *in vivo*.

Flow cytometry [1]

Peritoneal Oil-Mf or blood PCL-sensitized immune cells from OPs-treated donors were resuspended in DPBS supplemented with 1% FCS and their FcγR were blocked with 2.4G2 mAb. Then, blood leukocytes were stained with appropriately diluted FITC-conjugated anti-CD3 or anti-CD4 mAbs or with PE-conjugated anti-CD8, anti-CD19, anti-CD14, anti-NK1.1. Oil-Mf were stained with appropriately diluted FITC-conjugated anti-Mac3 and PE-conjugated anti-CD14, anti-CD80, anti-CD86, or anti-MHC class II (DR) mAbs. Cell surface immunophenotypic analysis was performed by cytofluorography using Ortho CytronAbsolute flow cytometer collecting 1×10^4 cells (Ortho Diagnostic System, Raritan, NJ, USA). Immunocount II software was used for data acquisition and analysis.

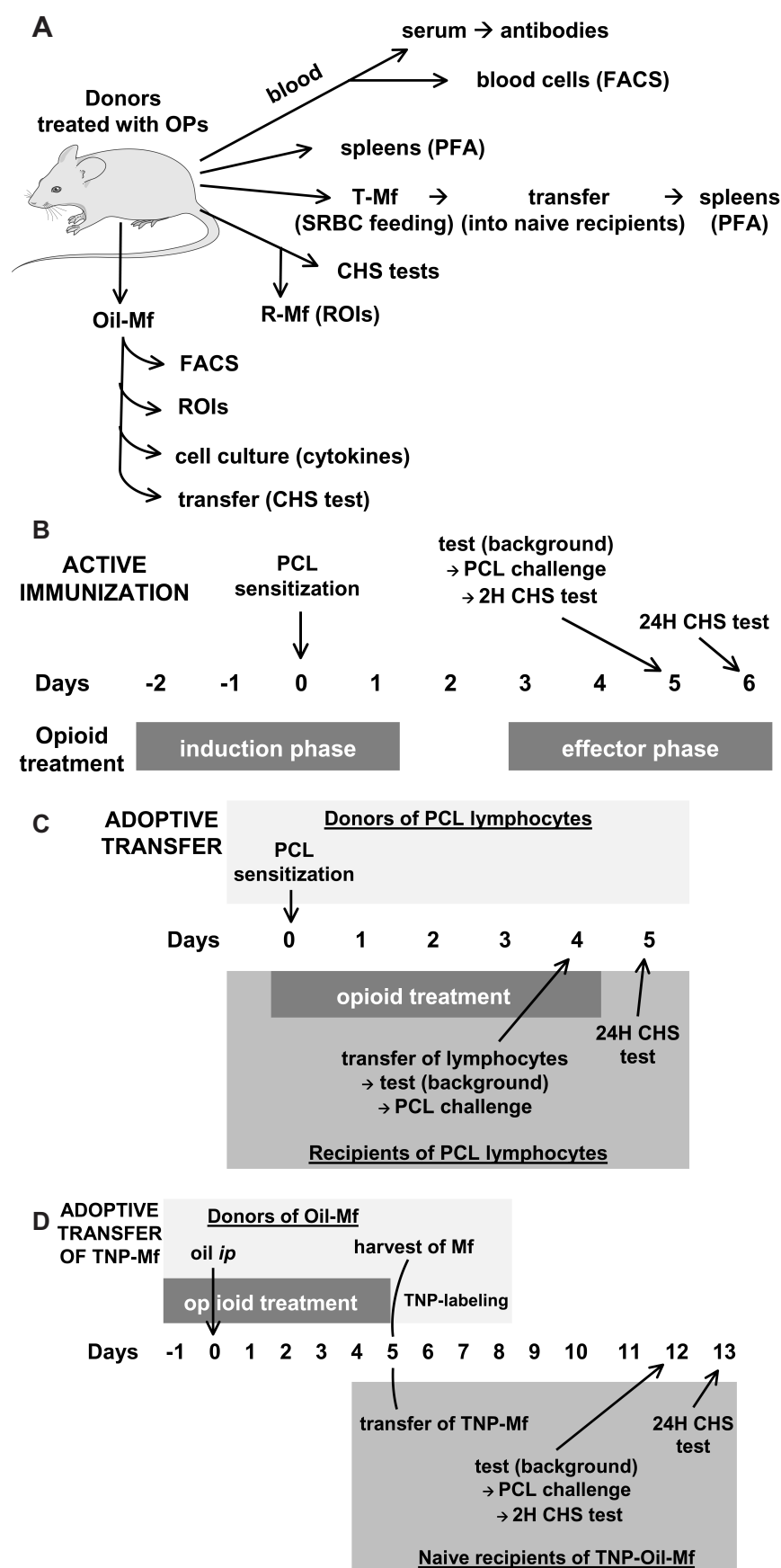


Fig. 1. Figure (A) shows the scheme of several experiments which were planned to be performed. Mice donors treated with opioids (see OPs administration) were the source of blood cells (for cell surface markers expression in FACS analysis) or sera (for anti-SRBC antibodies estimation) and spleens (for PFA). OPs-treated mice served as donors of T-Mf, which were then fed with SRBC and transferred into naive recipients to activate humoral response, later tested in PFA. The influence of OPs treatment was tested in the induction or in the effector phase of CHS to PCL (B) and simultaneously the experimental mice were the source of R-Mf (for ROIs production). In the adoptive transfer, PCL stimulated lymph nodes lymphocytes and splenocytes were injected into naive or OPs treated recipients to test their CHS-transferring activity (C). OPs treated mice were also stimulated with mineral oil by *ip* injection to obtain Oil-Mf, then tested for ROIs production, cell surface markers expression in FACS analysis, or cultured for cytokine release measurement into the supernatants by ELISA. Finally, Oil-Mf were labelled with TNP and transferred into the naive recipients for CHS reaction mediated by Mf (D)

Lucigenin-dependent chemiluminescence [1, 4]

Oil- and R-Mf from OPs-treated or non-treated mice were distributed in 96-well flat bottom dark plates (Nunc, Roskilde, Denmark) at a concentration of 5×10^5 viable cells/well in 0.2 ml RPMI 1640 medium supplemented with 10% FCS. After that, 10 μ M lucigenin was added and cells were incubated for 15 min at 37°C in darkness. Some groups of Mf were stimulated with zymosan opsonized with mouse serum (at ratio 10 particles per cell). Plates were immediately transferred to a Lucy 1 luminometer (Anthos, Salzburg, Austria) and the photon emission was measured for 75 to 100 min. Each experiment was run in duplicate.

Cytokine immunoassays [1, 2]

Oil-Mf of naive or OPs-treated mice were cultured in 24-well flat bottom plates at a concentration of 5×10^5 /ml in RPMI 1640 medium supplemented with 5% FCS in 5% CO₂ at 37°C. Some groups were stimulated with 100 ng/ml of LPS. Supernatants for TNF- α and IL-6 measurements were collected after 24 h and for IL-10 after 48 h, followed by storing at -80°C until further use. Concentrations of IL-6 and TNF- α were measured in capture ELISA tests, using 96-well flat Corning Easy Wash plates (Corning, Corning, NY, USA). The concentration of IL-10 was determined by using the IL-10 OptEIA™ ELISA kit as recommended by the manufacturer. The details of the procedure were published previously.

Statistical analysis

All experiments were carried out two to four times. An analysis of variance (ANOVA) was used to assess the significance of the results. To evaluate the differences between the groups the *post-hoc* Tukey's test was used and in case of the comparison between the control and tested groups Dunnett's test was employed. In some cases the double-tailed Student's *t*-test was also used to assess the significance of differences between only the control and tested group. In all cases $p < 0.05$ was taken as a minimum level of significance.

Results

Influence of OPs treatment on anti-SRBC antibody response in mice estimated in the plaque-forming assay (PFA)

The humoral response in mice treated with different OPs and immunized with 5×10^7 SRBC is expressed as the number of PFC per spleen (Fig. 2A). In two independent experiments, morphine caused a statistically significant decrease in PFC numbers (62% of control response, group B) while fentanyl and methadone were less inhibitory (respectively 75 and 74% of control level, for groups D and E, respectively). Administration of naloxone to morphine-treated animals resulted in the PFC numbers almost equal to those observed in the control group (Fig. 2A, groups C vs. A).

Anti-SRBC humoral response induced by SRBC fed T-Mf from OPs treated mice, estimated in PFA

Humoral response to corpuscular antigens like SRBC requires antigen engulfment (phagocytosis) and then its presentation by Mf, which stimulates B lymphocytes to produce specific anti-SRBC antibodies.

T-Mf were obtained from opioids treated mice and *in vitro* fed with SRBC in ratio 1:10 followed by removal of non-phagocytosed SRBC and *ip* injection of 4×10^6 cells into naive recipients. Humoral response was estimated as described in Figure 2A and is expressed as the number of PFC per spleen. Our experiments showed significant reduction in PFC numbers in mice treated with morphine (group B, 72% of positive control), fentanyl (group D, about 70% of control response) and methadone (group E, about 43% of control response). Results are shown in Figure 2B. In case of morphine treatment, this effect can be reversed by the administration of the antagonist of μ -opioid receptor, naloxone (Fig. 2B groups C vs. B).

Anti-SRBC antibody titres in sera of mice treated with OPs

Anti-SRBC immunoglobulin levels in mice sera (class IgG and IgM), measured in the direct hemagglutination test, showed moderate but statistically significant decrease in the anti-SRBC antibody titres in animals treated with morphine (7.8 ± 0.45) in comparison to the control group of mice (8.8 ± 0.45). The

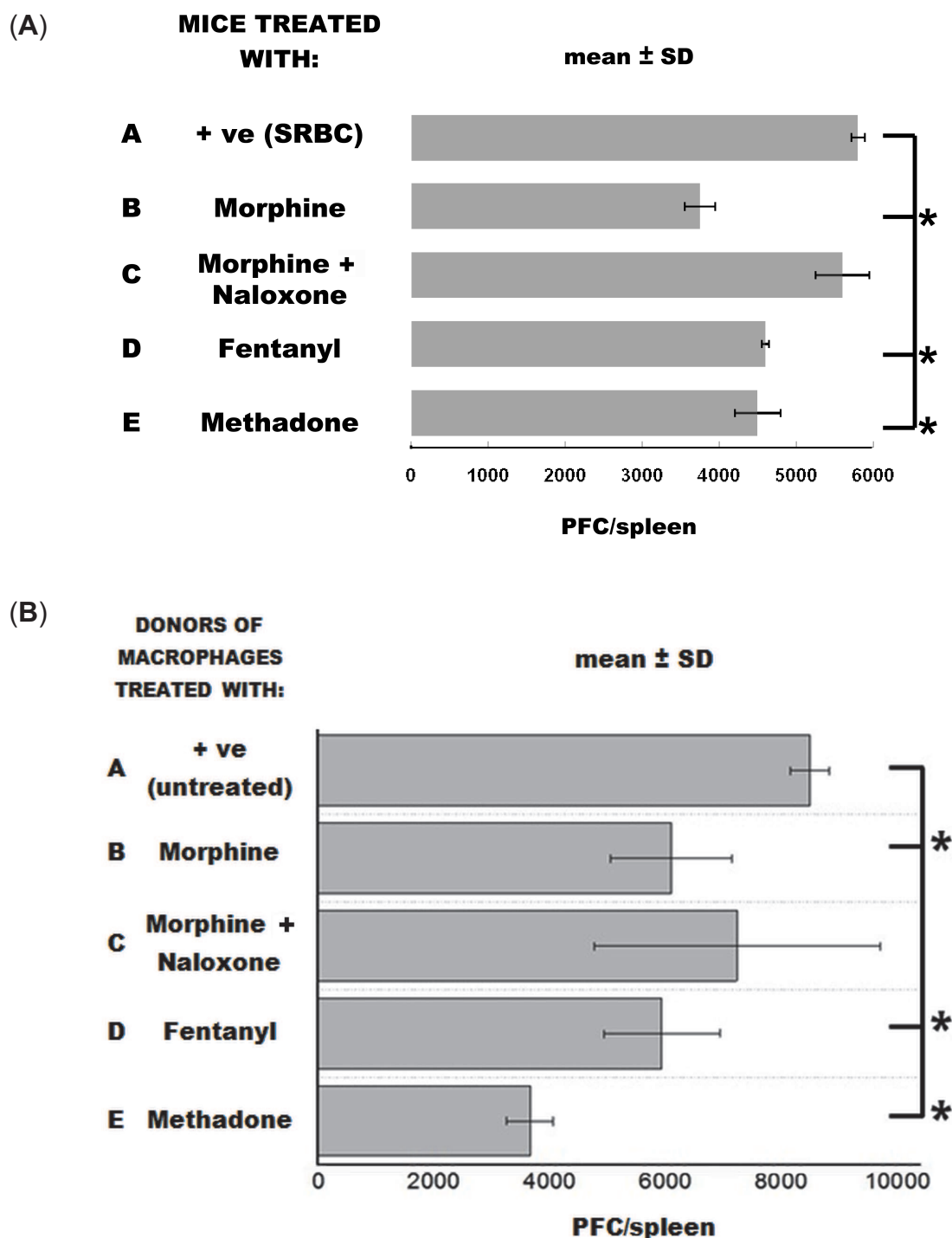


Fig. 2. (A) Anti-SRBC antibody response in mice treated with different opioids estimated in plaque forming assay (PFA). Naive animals (group **A**) or mice treated with OPs for 5 days (morphine – group **B**; morphine and naloxone – group **C**, fentanyl – group **D**, and methadone – group **E**) were injected *iv* on day 2nd with 5×10^7 SRBC. Seven days later the mice were sacrificed, sera were collected to estimate the level of anti-SRBC antibodies (see Fig. 3), and spleen cell suspensions were obtained to estimate the number of plaque forming cells by microscope slide technique (for details see Materials and Methods). Results are expressed as the mean $\pm$ SD of PFC/spleen. Statistics: PFC/spl: groups **A** vs. **B**, **D** and **E** $p < 0.001$; **A** vs. **C** NS. Asterisks (*), point out $p < 0.05$ as a level of statistical significance. **(B)** The ability of thioglycollate-induced peritoneal macrophages (T-Mf) from donors treated with OPs to induce humoral response estimated by plaque forming assay (PFA). Naive animals (group **A**) or mice treated with different opioids for 5 days (groups **B–E**) on day 2nd of OPs administration were injected *ip* with thioglycollate medium (groups **A–E**). Four days later T-Mf were collected and incubated for 30 min at 37°C with SRBC in ratio 1:10, followed by osmotic shock for lysis of not engulfed SRBC, and then were injected *ip* in a dose of 4×10^6 cells per recipient. Seven days later spleen cells suspensions were used for PFA (for details see Materials and Methods). Results are expressed as the mean $\pm$ SD of PFC/spleen. Statistics: PFC/spl: groups **A** vs. **B**, **D** and **E** $p < 0.01$; **A** vs. **C** NS. Asterisks (*), point out $p < 0.05$ as a level of statistical significance. + ve = positive

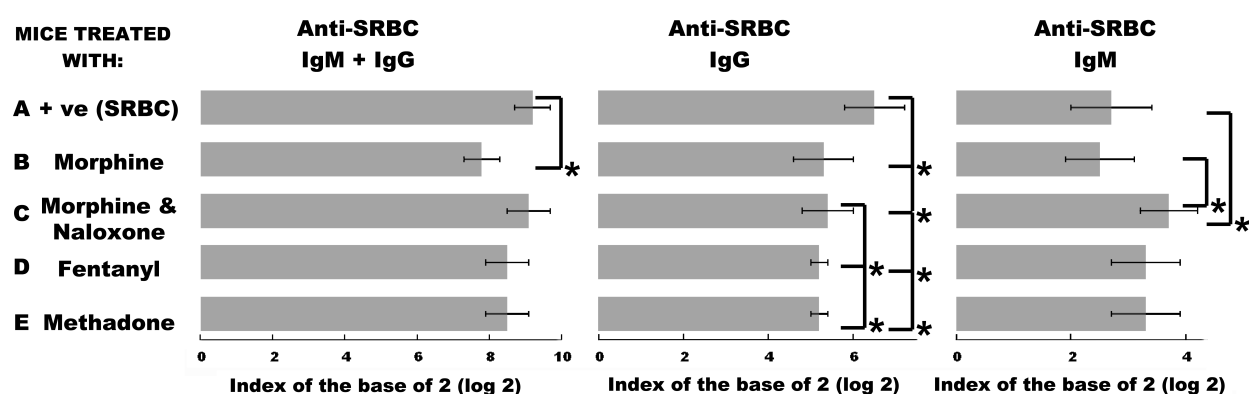


Fig. 3. Production of IgG and IgM isotypes of anti-SRBC antibodies in OPs-treated mice estimated in direct hemagglutination assay. Titers of anti-SRBC antibodies in sera of mice treated with different OPs (see description to Fig. 2a) were estimated in direct hemagglutination assay and are shown in column 1 – total (IgG + IgM), column 2 – IgG, column 3 – IgM. Titers are expressed as index of base of two of titers $\pm$ SD. IgM titer is calculated as the difference between the titer of the whole serum and the titer of sera treated with 0.15 M 2-mercaptoethanol. Statistics: anti-SRBC IgG and IgM: group A vs. B $p < 0.05$; groups A vs. C, D and E NS; anti-SRBC IgG: group A vs. B $p < 0.01$; group A vs. C $p < 0.05$; groups A vs. D and E $p < 0.0002$; groups C vs. D and E $p < 0.05$; anti-SRBC IgM: group A vs. C $p < 0.02$; groups A vs. B, D and E NS; group B vs. C $p < 0.02$. Asterisks (*), point out $p < 0.05$ as a level of statistical significance. + ve = positive

reduction tendency was seen in groups treated with methadone (8.4 ± 0.55) and fentanyl (8.4 ± 0.55) and statistically significant decrease in IgG titres was observed in mice treated with all opioids compared to positive control group of mice. Administration of naloxone to morphine treated animals significantly increased the antibody titres. The results are expressed as indices of the base of two (log 2) in Figure 3. IgM titres were calculated as the differences between the Ab titer in the whole sera and in the sera treated with 2-ME. The analogous results were obtained from sera of recipients of SRBC-fed Mf (data not shown).

Enhancement of zymosan-induced extracellular respiratory burst in peritoneal macrophages of mice treated with OPs

The kinetic reaction of production of reactive oxygen intermediates (ROIs), measured as lucigenin-dependent chemiluminescence, showed a significant increase in ROIs formation by zymosan-stimulated Oil-Mf isolated from donors treated with morphine (peak 4256) and fentanyl and methadone (peak about 3750) in comparison to the values obtained in control animals (peak 2750) (Fig. 4A). Similar results were obtained from OPs treated, PCL sensitized donors of R-Mf (Fig. 4B), where morphine and methadone were also the most stimulatory.

The influence of OPs on the induction phase of contact hypersensitivity (CHS) to PCL

The influence of OPs on the induction phase of CHS was studied in mice treated for 5 days with OPs, which on the third day of opioid administration were skin sensitized with 5% PCL. Five days after sensitization, mice were challenged with PCL on ears and early phase of CHS reaction was measured 2 h after challenge and delayed CHS reaction after 24 h. Figure 5 shows that the 2 h early response, dependent on B1 lymphocytes and complement, as well as the late 24 h response, dependent on Th1 lymphocytes and macrophages, were inhibited in morphine-treated mice but significantly increased in mice treated with methadone. Morphine and naloxone used together were more inhibitory than morphine alone. Fentanyl also moderately increased the late reaction. Results are shown in Figure 5.

The influence of OPs on the effector phase of CHS to PCL in mice

Mice had been skin sensitized with PCL and three days later the treatment with opioids began. Five days after the sensitization the CHS reaction was measured. OPs treatment of mice in the effector phase of sensitization to PCL gave the outcomes reverse to those observed when OPs were used during the induction phase. Methadone treatment increased the early

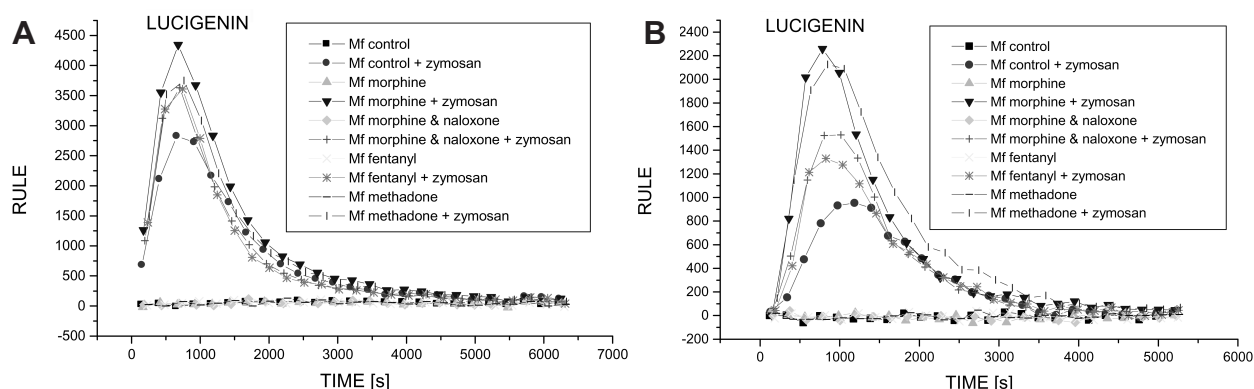


Fig. 4. Production of reactive oxygen intermediates (ROIs) by oil-induced (Oil-Mf) or resident (R-Mf) peritoneal Mf from OPs treated mice. Ten μ M lucigenin and zymosan (10 particles per cell) were added to 5×10^5 Oil-Mf (**A**) or R-Mf (**B**) in RPMI 1640 nutrient medium and photon emission was measured over 75–100 min (abscissa). For details see Materials and Methods. The results of one out of three experiments are demonstrated. Chemiluminescence (ordinate) is expressed in arbitrary units (RULE). $\blacksquare$ – untreated Mf; $\bullet$ – untreated Mf + zymosan; $\blacktriangle$ – morphine Mf; $\blacktriangledown$ – morphine Mf + zymosan; $\blacklozenge$ – morphine and naloxone treated Mf; $+$ – morphine and naloxone treated Mf + zymosan; $*$ – fentanyl treated Mf; $\times$ – fentanyl treated Mf + zymosan; $-$ – methadone treated Mf; $|$ – methadone treated Mf + zymosan

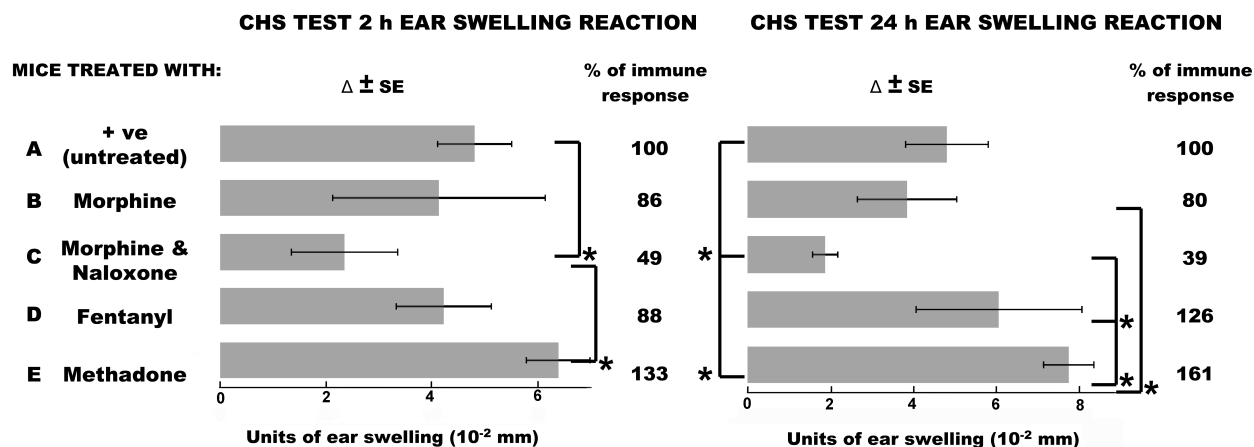


Fig. 5. Effects of OPs on the induction phase of contact sensitivity reaction in mice actively immunized with picryl chloride. Experimental groups of mice were treated with opioids for 5 days (groups **B–E**). On the 2nd day of opioids administration, both the tested and control (non-treated) groups were immunized on the shaved abdomen skin with 5% PCL. Five days after immunization (i.e., 3 days after discontinuation of opioid treatment) mice (groups **A–E**) were challenged on ears with 0.4% PCL solution in acetone/olive oil (for details see Materials and Methods). Contact hypersensitivity response (CHS) was tested 2 h (early CHS) and 24 h (delayed CHS) after challenge and results are expressed in $\Delta \pm SE$ of units $\times 10^{-2}$ mm of ear swelling and as a % of response in comparison to positive control group (**A**). Results obtained in the negative control group (approx. 2 units), resulting from unspecific chemical irritation from hapten, were subtracted from experimental values. Statistics: 2 h CHS response: group **A** vs. **C** $p < 0.05$; group **C** vs. **E** $p < 0.01$ groups **A** vs. **B**, **D** and **E** NS; 24 h CHS response: groups **A** vs. **C** and **E** $p < 0.02$; group **B** vs. **E** $p < 0.05$; group **C** vs. **D** and **E** $p < 0.001$; groups **A** vs. **B** and **D** NS. Asterisks (*), point out $p < 0.05$ as a level of statistical significance. + ve = positive

phase of sensitization to PCL but significantly inhibited the late reaction in comparison to mice from the positive control group (group **A**). The late phase of reaction in morphine recipients was significantly higher than in animals treated with methadone as well as than in the positive control group (Fig. 6).

Transfer of PCL sensitized lymphocytes to OPs-treated recipients

The influence of the OPs treatment of recipients of PCL specific lymphocytes on the passive transfer is shown in Figure 7. Recipients had been treated with

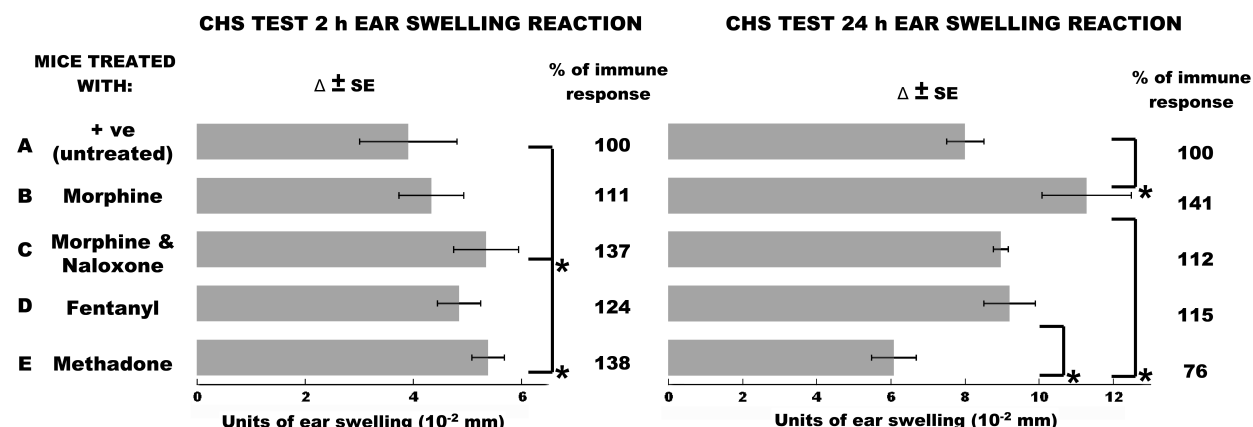


Fig. 6. Influence of opioids on the effector phase of contact sensitivity reaction in mice actively immunized with picryl chloride. Mice (from groups A–E) were immunized on the shaved abdomen skin with 5% PCL. On the third day after immunization the treatment of mice with different opioids (groups B–E) began and lasted for 5 days. Five days after immunization all tested mice (groups A–E) and the negative control group were challenged on ears with 0.4% PCL solution in acetone/olive oil (for details see Materials and Methods). Contact hypersensitivity response (CHS) was tested 2 h (early CHS) and 24 h (delayed CHS) after challenge. Results are expressed in $\Delta \pm SE$ of units $\times 10^{-2}$ mm of ear swelling and as a % of response in comparison to positive group (A). The size of ear swelling caused by unspecific chemical irritation by the hapten, observed in negative control group, was subtracted from experimental groups values. Statistics: 2 h CHS response: group A vs. C $p < 0.05$; group A vs. E $p < 0.02$, groups A vs. B and D NS; 24 h CHS response: group A vs. B $p < 0.02$; group D vs. E $p < 0.05$; group B vs. E $p < 0.002$; A vs. C, D and E NS. Asterisks (*), point out $p < 0.05$ as a level of statistical significance

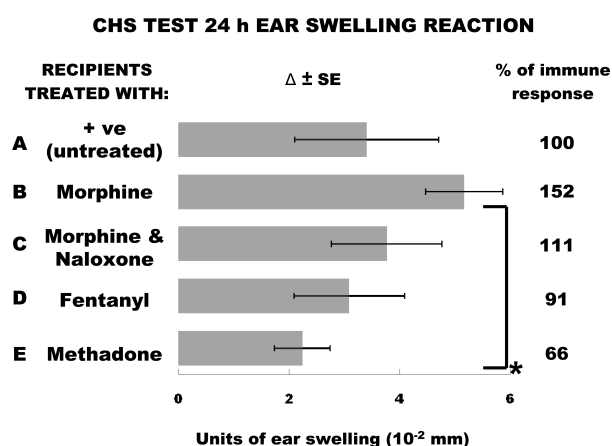


Fig. 7. Transfer of PCL-specific lymphoid cells to recipients treated with OPs. PCL-specific lymphocytes were obtained from spleens and axillary and inguinal lymph nodes from mice immunized on the shaved abdomen skin with 5% PCL five days before. Lymphocytes (7×10^7) were transferred into naive recipients (group A) or mice treated for 5 days with OPs (groups B–E). At the day of transfer recipients were challenged on ears with 0.4% PCL (for details see Materials and Methods and description to Fig. 5). Contact hypersensitivity response (CHS) was tested 24 h after challenge and net results are expressed in $\Delta \pm SE$ of units $\times 10^{-2}$ mm of ear swelling and as a % of response in comparison to positive control group (A). Each group of mice consisted of 5 animals. This experiment was repeated twice and both times identical results were obtained. Statistics: Groups B vs. E $p < 0.005$; groups A vs. B, C, D and E NS. Asterisks (*), point out $p < 0.05$ as a level of statistical significance. + ve = positive

OPs for 5 days, and afterwards they received 7×10^7 PCL immune cells *iv* and were tested for CHS response. Results showed the increased activity of the PCL hapten-sensitized lymphocytes in morphine-treated recipients in comparison to the recipients treated with methadone (Fig. 7), although there were no statistical differences in CHS responses in groups of opioid-treated mice in comparison to the positive control group.

Transfer of *in vivo* OPs-treated, *in vitro* TNP-labelled Oil-Mf into naive recipients

The procedure of experiment is shown in Figure 1D. Oil-Mf from OPs treated donors were harvested, labelled with TNP (TNP-Mf) and transferred into naive recipients. The CHS reaction to PCL was tested a week later, 2 h (early component) and 24 h (late component) after challenge. OPs stimulation had influence on antigen presenting activity of Mf in CHS to PCL. Results are depicted in Figure 8 and show an increase in the early phase of CHS mediated by TNP-Mf treated with all tested OPs in comparison to the control group. The late phase of CHS was enhanced only in case of morphine-treated TNP-Mf, fentanyl had no influence and methadone slightly diminished CHS reaction mediated by TNP-Mf in recipients.

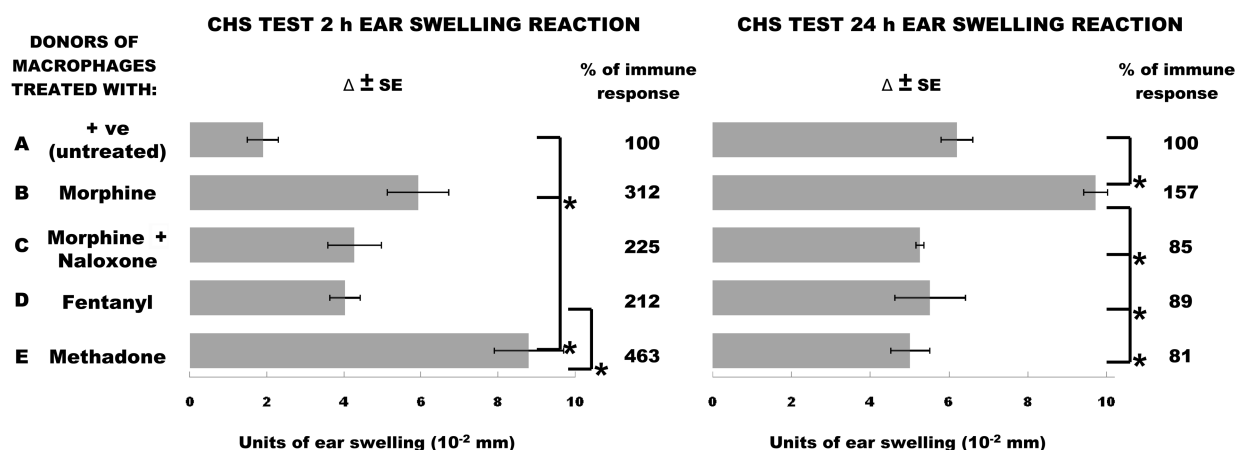


Fig. 8. Transfer of *in vivo* opioid-treated, *in vitro* TNP-labelled Oil-Mf into naive recipients. The procedure of experiment is shown on Fig. 1D. Oil-Mf from OPs-treated donors were harvested, labelled with TNP (TNP-Mf) and transferred into naive recipients. The CHS reaction to PCL was tested a week later 2 h (early component) and 24 h (late component) after challenge. For further explanation see above. Statistics: 2 h CHS response: group A vs. B $p < 0.05$; group A vs. D $p < 0.001$, group C vs. D $p < 0.01$, groups A vs. C and B vs. D NS; 24 h CHS response: group A vs. B $p < 0.01$; group B vs. C $p < 0.001$; group B vs. D $p < 0.005$; groups A vs. C, D and D vs. C NS. Asterisks (*), point out $p < 0.05$ as a level of statistical significance. + ve = positive

Percentage of the subpopulations of blood leukocytes in OPs-treated recipients in cytofluorimetric assay

Expression of surface markers characteristic for certain populations of blood leukocytes and observed in the mice treated with OPs was estimated in flow cytometry and is shown in Table 1 as percentages of total cells analyzed. Our results indicate that OPs change the propor-

tion of blood leukocytes in comparison to the values found in non-treated control mice. Morphine and methadone significantly reduced monocyte CD14⁺ population and fentanyl and methadone moderately decreased the levels of T lymphocytes (CD3⁺), of both CD4⁺ and CD8⁺ phenotypes. Reduction in the number of CD4⁺CD8⁺ Treg cells in all OPs treated groups was also found. Finally, NK cells level (NK1.1) was reduced in mice treated with fentanyl and methadone (Tab. 1).

Tab. 1. The percentage of selected leukocyte populations in the blood of mice treated with OPs. Blood from opioid-treated mice was collected in citric buffer anticoagulant and leukocytes were directly stained with fluorochrome-conjugated mAbs. The percentage of distinct populations of leukocytes was measured cytofluorimetrically. B cells were expressed as CD19 positive cells, T lymphocytes were CD3 and CD4 or CD8 positive, NK cells were stained with NK1.1 mAbs and monocytes were estimated as CD14 positive cells. CD4⁺CD8⁺ cells represented regulatory T cell population. The table shows the results of one of two experiments done

Cell surface marker	Control	Morphine	Morphine and naloxone	Fentanyl	Methadone
CD3	41.8	40.7	49.2	34.7	37.3
CD4	31.3	30.0	36.1	25.6	28.5
CD8	11.0	11.4	13.6	9.9	9.9
ratio CD4/CD8	2.8	2.6	2.7	2.6	2.9
CD4 ⁺ CD8 ⁺	0.4	0.2	0.4	0.2	0.2
CD19	6.2	6.6	8.4	7.0	7.0
NK1.1	3.8	3.6	2.8	2.0	2.4
CD14	10.2	5.5	6.6	11.6	5.8

Tab. 2. Expression of cell surface markers on peritoneal Mf obtained from mice treated with OPs. Oil-induced Mf from OPs-treated mice were stained with fluorochrome-conjugated mAbs. The percentage of cells positive for chosen markers was measured cytofluorimetrically. Mononuclear cells were selected as the total number or Mac3 positive Mf with co-staining of CD14, CD80, CD86 and DR (H2k) markers. The table shows the results of one of two experiments done. The basic autofluorescence of non-stained cells was subtracted from the results obtained from stained cells measurements

Cell surface marker	Opioid Cells	Control (untreated)	Morphine	Morphine and naloxone	Fentanyl	Methadone
CD14	total	25.8	17.2	19.1	9.3	11.8
	Mac3+	25.3	14.3	15.4	8.8	11.4
CD80	total	53.9	37.7	33.0	34.6	34.4
	Mac3+	25.3	16.5	16.5	15.2	15.2
CD86	total	50.3	37.2	32.3	41.4	38.7
	Mac3+	26.0	16.9	17.0	16.1	14.0
MHC II (DR)	total	57.2	45.4	37.8	43.9	52.6
	Mac3+	27.6	17.3	17.4	15.9	14.8
net value of % of positive cells in staining						

Cell surface markers expression on peritoneal Oil-Mf obtained from OPs treated donors in cytofluorimetric assay

The expression of cell surface markers characteristic for antigen presenting activity (CD80, CD86, MHC class II (DR)), as well as LPS receptor (CD14) was reduced in OPs treated Mac3⁺ Mf. Results are shown in Table 2.

The influence of OPs on the cytokine secretion by Oil-Mf

When not stimulated with LPS, Oil-Mf harvested from donors treated with OPs showed basal secretion of TNF- α , IL-6 and IL-10 cytokines, similar to this of naive (control) donors of Mf, although the production of TNF- α by OPs-treated Mf was reduced. LPS stimulated Mf from donors treated with morphine and methadone produced significantly higher amounts of proinflammatory cytokines (TNF- α , IL-6) as well as IL-10 while fentanyl did not change the production of the tested cytokines in comparison to control Mf. The results of ELISA assays of cytokines are shown in Table 3.

Tab. 3. Cytokine production of oil-induced Mf isolated from naive mice or animals treated with OPs and *in vitro* stimulated or unstimulated with LPS. Oil-Mf in concentration of 5×10^5 /ml in RPMI medium supplemented with 5% FCS and stimulated or not with LPS were cultured at 37°C in 5% CO₂ atmosphere. For TNF- α and IL-6 measurements supernatants were collected after 24 h culture and for IL-10 after 48 h. The concentrations of cytokines were measured in capture ELISA test (see Materials and Methods) and are expressed in pg/ml. Table shows the results of one representative experiment out of three as the mean $\pm$ SD

Cytokines: Groups:	IL-6 (pg/ml)	IL-10 (pg/ml)	TNF- α (pg/ml)
Control	< 5	< 8	35 $\pm$ 3
Control + LPS	726 $\pm$ 54	21 $\pm$ 2	1309 $\pm$ 380
Morphine	< 5	< 8	14 $\pm$ 4
Morphine + LPS	1835 $\pm$ 253	60 $\pm$ 5	2415 $\pm$ 780
Morphine + naloxone	< 5	< 8	38 $\pm$ 4
Morphine + naloxone + LPS	354 $\pm$ 27	32 $\pm$ 4	807 $\pm$ 184
Fentanyl	< 5	< 8	15 $\pm$ 4
Fentanyl + LPS	503 $\pm$ 74	16 $\pm$ 3	1130 $\pm$ 380
Methadone	< 5	< 8	13 $\pm$ 7
Methadone + LPS	2130 $\pm$ 380	62 $\pm$ 7	1800 $\pm$ 350

Discussion

The results of the present study suggest a strong suppressive effect of opioid therapy on humoral immune response in mice illustrated by a significant inhibition of the creation of hemolytic plaques by splenocytes (in PFA) and of the production of antibodies against SRBC. Similar findings have been previously demonstrated by Lefkowitz and Chiang [14]. Our results indicate directly that monocytes/macrophages are responsible for this effect, which was confirmed by the comparison of the results obtained by the direct immunization of mice with SRBC and *ip* injection of Mf fed with SRBC (Fig. 2A and 2B). Macrophages are involved in the phagocytosis and processing of corpuscular antigen followed by its presentation and activation of B cells to produce specific antibodies. This activity can be assessed as formation of hemolytic plaques by splenocytes in PFA, and the secretion of serum antibodies at the stage of mature plasma cells. Present results indicate that the administration of OPs (morphine, methadone, fentanyl) has a statistically significant inhibitory effect on the effector phase of humoral immune response induced by both SRBC injection into OPs-treated mice and by transfer of SRBC-fed T-Mf, the latter emphasizing Mf activity in the processing and presentation of corpuscular antigen to initiate humoral response. This antigen-presenting activity can be down-regulated by OPs, acting through μ -opioid receptors on macrophages, and this effect is diversified between the drugs used for the therapy, depending on their particular pharmacokinetic properties. This reduction in humoral response may be due to either reduced phagocytosis by monocytes/macrophages under the influence of OPs [8, 36] or lower activity of antigen presentation.

The inhibitory effect of morphine on total anti-SRBC serum antibodies formation is presumably the result of decreased IgG production by plasma cells (Fig. 3). In case of fentanyl and methadone therapies, no differences in total serum anti-SRBC antibodies are observed, but the inhibition of humoral response maturation is suggested by an increase of IgM and decrease of IgG levels in comparison to the positive control. As the consequence of impaired immunoglobulin class switching process in opioid therapy, the lack of formation of immune memory B cells had been observed by Yahya et al. [36]. The present evidence points out the potential impact of modified cy-

tokine secretion profile on the inhibition of production of antibodies during treatment with OPs [34]. Moreover, this process also involves Mf as APCs, so may be affected by OPs therapy without direct opioid influence on B lymphocytes [34], as also shown in this report (see Fig. 2).

On the other hand, we have demonstrated a significant activating effect of morphine and other OPs on the formation of oxygen radicals by Mf (Fig. 4A and 4B), which may enhance antigen processing. This effect may be important for compensation of previously described inhibition of phagocytosis [8, 36] and suggest strengthening of the natural cytotoxicity of Mf during opioid treatment. Our results are in opposition to the previous observations by Yahya [36], who showed that morphine, as the primary representative of OPs, inhibits the activity of Mf to generate oxygen radicals. This discrepancy may likely be due to the different experimental conditions used by the authors of previous studies. Additionally, other researchers showed the methionine-enkephalin-mediated, dose dependent enhancement of ROIs production by rat peritoneal macrophages [28]. This mechanism was suggested to be mediated *via* μ - and δ -opioid receptors [35], what is in line with our observations of the effects of treatment with naloxone. This antagonist of μ -opioid receptors inhibited the activation of ROIs production in case of *in vivo* morphine treated Oil-Mf and R-Mf (Fig. 4A and 4B).

The mechanism of contact hypersensitivity involves different cell populations. The early reaction (2 h after sensitization with hapten), mediated by B1 lymphocytes, mast cells and complement [29], is a less specific inflammatory process providing the proper microenvironment for the development of the late hapten-specific response. This reaction occurs 24 to 48 h after sensitization and engages appropriate effector cells (either Th1 CD4⁺ lymphocytes and Mf in PCL immunization – type IVa according to Pichler classification, or T CD8⁺ cytotoxic lymphocytes in OX or DNFB sensitization – type IVc [9, 21, 22]). Our detailed study on the impact of opioids on the specific cellular response in CHS to PCL suggests that OPs may influence different stages of the allergy to this hapten. Earlier reports demonstrating the activating effects of morphine and its derivatives, diacetylmorphines (heroin) on the effector phase of CHS responses [10, 19] were confirmed in our model. We have shown that the classical pathway of CHS, mediated by effector Th1 CD4⁺ lymphocytes and Mf, by

OPs is affected differently at early and delayed stages. The analysis of results demonstrates no significant effect of treatment with morphine and fentanyl on the process of induction of sensitization to PCL (see Fig. 5). The same was observed in CHS reaction to oxazolone (OX) (data not shown). However, the use of methadone increases sensitization to haptens in the induction phase (the first contact with the allergen) (see Fig. 5). In mice sensitized to PCL, methadone administration leads to the inhibition of late effector allergic reaction in comparison to the early activation stage (Fig. 6). In case of morphine, late effector phase of delayed allergy to PCL is significantly higher when compared to the methadone treatment. In case of an existing allergy to oxazolone, morphine therapy in the late effector phase also significantly enhances the sensitization, whereas methadone and fentanyl do not potentiate it (data not shown). Our results suggest that morphine activates the effector phase of CHS to PCL and OX haptens, as it was shown previously to be the case for DNFB-CS [18], while methadone and fentanyl do not potentiate this late reaction. Regardless of the different effector cells induced by immunization with PCL and OX, the mechanism of action of particular opioid seems to be identical and dependent only on the kind of drug used. While morphine activates the effector phase of both types of responses (IVa and IVc), fentanyl has no significant impact on them and methadone activates the induction phase of sensitization and inhibits the late reaction of effector phase of CHS.

Passive transfer of sensitized PCL effector cells to the recipients treated with OPs demonstrated that morphine affects the external environment of cells in the host organism and increases symptoms of allergic sensitization to PCL (Fig. 7), which may suggest its influence on the activating conditions of inflammation (eg., infiltration). On the other hand, compared with morphine, methadone significantly inhibits the activation process of CHS in the passive transfer. The analogous results were obtained in case of transfer of TNP-labelled Mf from OPs treated donors into naive recipients (Fig. 8). The similarities are particularly evident when comparison is made between the late CHS responses mediated by mentioned above transferred TNP-labelled Mf, CHS induced in actively sensitized, OPs treated mice (Fig. 6), and CHS adoptively transferred by PCL sensitized lymphocytes into OPs treated donors (Fig. 7).

Although influence of OPs therapy on transferred Mf is mediated solely by their opioid receptors, in case of *in vivo* therapy the observed inhibitory effect of methadone indicates that, in addition to direct impact of opioids through μ -opioid receptors, other peripheral mediators such as substance P [18] and presumably the vascular effect may participate in the regulatory roles of OPs. It discloses the inhibitory effect on the sensitization to PCL during methadone administration compared to morphine therapy. In addition, the activation of δ -opioid receptors on murine T cells [25] may impede the proliferation mechanism, consequently leading to a change in the proportion of peripheral blood lymphocytes and to the inhibition of the effector phase of CHS (see Tab. 1). It should be noticed that in CHS only a minority (about 1%) of infiltrating lymphocytes are specifically sensitized, so the observed effects of transferred cells action are usually modified by recipient's immune competent cells.

Regulatory action of OPs on cytokines generation by Mf stimulated with LPS initially is demonstrated as an elevation of proinflammatory cytokines production (including IL-6 and TNF- α). Then, after a day of releasing proinflammatory cytokines, the secretion of IL-10, which inhibits the CHS, is activated (see Tab. 3). Activation of IL-6 secretion under the influence of morphine therapy has been reported previously [33], although the levels of cytokines secreted by Mf induced by morphine are often divergent and dependent on experimental conditions [15]. It is well known that PAMPs (eg., LPS) stimulation is a strong activator of production and secretion of proinflammatory cytokines by Mf. *In vivo* OPs treatment seems to inhibit TNF- α secretion by Mf cultured *in vitro* without LPS (see Tab. 3). This may offer one explanation for anti-inflammatory action of OPs observed in the clinical therapy of pain. The analysis of OPs influence on the macrophage surface markers expression shows that all tested molecules characteristic for antigen presentation activity (CD14, CD80, CD86, MHC class II) are significantly inhibited by every kind of OPs treatment (morphine, fentanyl or methadone) (see Tab. 2), which is the next evidence of impaired function of Mf as APCs. The diversity of OPs influence on CHS can also be explained by the alteration of cell surface markers expression on the peripheral blood immunocytes of mice sensitized to PCL. The regulatory T CD4⁺CD8⁺ lymphocyte population is considered to be important in the negative regulation

of CHS [31] and a decreased number of these cells was shown in mice treated with OPs. Morphine and methadone treatment reduces the percentage of blood monocytes (CD14⁺ population). Thus, the decreased sensitivity to PCL in the induction and effector phases of CHS (type IVa mediated by Th1 lymphocytes and monocytes/macrophages), in addition to the inhibition of Mf antigen presenting and effector functions, could be justified by a fall in monocytes/macrophages numbers. Methadone and fentanyl administration leads to the reduction of the percentage of NK1.1 cells in the blood. These opioid therapies also tend to reduce the level of T lymphocytes (CD3⁺), including CD4⁺, CD8⁺ and double positive CD4⁺CD8⁺ subpopulations in the peripheral blood of mice sensitized to PCL, which in turn may lead to a reduction of specific cellular (CHS) and humoral immune responses [25]. Previous studies by other authors [33] confirm our observation of changes in the level of the mouse peripheral blood NK and T cells. No significant changes in the population of B cells were reported by Yahya [36] confirming that the inhibitory effect of morphine on the activation of humoral immunity [33] is mediated by other cells, such as Mf. The inhibitory effect of morphine therapy was also previously reported in case of chronic infections [16] and activation of classical cellular immunity [33]. The effect of fentanyl through μ -opioid receptors is considered blocking rather than activating [26], which is also noticeable in our results, showing its inhibitory effect on immune response.

The OPs action on the immune system is a complex phenomenon which cannot be explained by a single mechanism and concerns different types of monocytes expressing opioid receptors. Additionally, the differences between OPs themselves influence the final effect of drug administration. Among them, differences in solubility and pharmacokinetic activity of OPs should be taken into consideration. Our present experiments concentrated mainly on the influence of OPs on Mf. All tested OPs reduced the antigen presenting activity of Mf. The investigated OPs have an influence on Mf activity in humoral response which was observed as the decreased number of PFC and reduced antibody titrations, accompanied by the possible impairment of B cells maturation expressed as IgG level reduced in favor of IgM. Opioid therapy seems to enhance the proinflammatory activation of Mf cultured *in vitro* in the presence of PAMPs (zymo-

san or LPS), while without PAMPs stimulation Mf show anti-inflammatory properties. Modulation of CHS to PCL by opioid treatment is orchestrated through the impact on Mf and several other cell populations (T effector cells, Treg, monocytes, NK) so the observed effects are complex and divergent. The results obtained in this work prove direct opioid regulatory function mediated through opioid receptors expressed on macrophages, which serve as important antigen presenting cells in the induction phases of humoral and cellular responses, as well as as the effector cells of CHS response to PCL. To conclude, our results determined the significant contribution of the influence on Mf to the opioid modulation of immune response.

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