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Alteration in specific opioid-receptor labeling on peripheral blood leukocytes of bile duct-ligated rat

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Abstract

Cholestasis is associated with increased tonus and activity of opioidergic system. Opioid peptides have also immunomodulatory effects through stimulation of specific opioid receptors on the immune cells, or in an indirect fashion via the central nervous system. The combination of immunofluorescent technique and flow cytometry has proven to be sensitive method for the detection of leukocyte opioid receptors. This study was designed to examine the effect of cholestasis on the opioid-receptor labeling on the leukocytes from bile duct-ligated rats. Seven days after surgery, leukocytes were isolated from the peripheral blood of bile duct-ligated or sham-operated rats. The cells were incubated with naltrexone-fluorescein, in the absence or presence of unlabeled naltrexone, as a competitor and analysed by flow cytometry. Monocytes and granulocytes from bile duct-ligated rats showed an increase in the percentage of opioid-receptor labeling (29.6 ± 2.08 for cholestatic versus 23 ± 1.9 for sham, $p < 0.001$; 50.6 ± 3.18 for cholestatic versus 39.6 ± 1.7 for sham, $p < 0.05$; respectively). Furthermore, there was a decrease in the expression of opioid receptors on leukocytes due to cholestasis. In conclusion, changes in specific opioid-receptor labeling and percent of labeled leukocytes indicate that endogenous opioid-receptor interaction may be altered in peripheral blood leukocytes in acute cholestasis.

Keywords: Peripheral blood leukocytes; Opioid receptor; Direct immunofluorescence; Rat; Cholestasis

1. Introduction

Endogenous opioid peptides are known to circulate in low levels in the plasma of mammals including humans and rats [1,2]. Many researchers have described a marked elevation of endogenous opioid levels in plasma of patients with cholestatic liver diseases and animal models of cholestasis [2–6]. Based on accumulated evidence, it has been suggested that endogenous opioids are implicated in the pathophysiology of cholestasis [2,7,8]. Observations compatible with this hypothesis include precipitation of an opioid withdrawal-like syndrome in patients with chronic cholestatic liver disease by administration of an opioid antagonist [3,4] and a global down-regulation of central mu-opioid receptors in the cholestatic rats [9].

Sibinga and Goldstein have demonstrated the presence of opioid receptors on cells of the immune system [10]. Opiates and opioid peptides induce an enhancement or a suppression of immune function, such as macrophage phagocytosis [11] and monocyte chemotaxis [12] depending on concentration

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and/or class of opioid used, as well as the type and/or activation/differentiated status of effector cells monitored. In addition, many researchers demonstrated that cholestasis is associated with abnormality of immune functions. The examples are disturbed neutrophil adhesion [13], chemotaxis and phagocytosis [14], in cholestatic rats, and also abnormal T-lymphocyte responses in primary biliary cirrhosis [15].

Although the characterization of brain opioid receptors has generally involved radioligand and competition binding assays with various selective opioid compounds, it has been difficult to identify classical opioid receptors on leukocytes by using radioligand binding assay [16]. This is probably because of very low opioid-receptor expression on immune cells. In addition only a small proportion of cells within heterogenous leukocyte population express opioid receptors [17]. Over two decades ago, a new method for studying opioid-receptor expression was described by Kolb et al. [18,19]. They synthesized three fluorescent-conjugated opioid ligands including naltrexone, naloxone, and oxymorphone, and demonstrated their high affinity at rat brain synaptosomal plasma membrane and biological activity at the guinea pig ileum. Then, many researchers have studied opioid receptors on isolated cells, using these probes [20,21]. As an increased plasma endogenous opioid activity has been demonstrated in cholestasis, it has been proposed that relative opioid-receptor density on the circulating leukocytes might also change. A previous study reported a change in the proportion of leukocytes with opioid receptors in the cholestatic rats. Specific opioid-receptor labeling (i.e. the relative density of opioid receptors) on leukocytes of cholestatic animals, however, was not evaluated [22].

The present study was carried out to evaluate whether specific opioid-receptor labeling on peripheral immune cells was changed in a rat model of acute cholestasis.

2. Materials and methods

2.1. Animals

Adult male albino Wistar rats weighing 220–240 g (purchased from the Pasteur Institute of Iran) were used in this study. The animals were housed in temperature controlled room ($24 \pm 1\,^{\circ}C$) on a 12-h light: 12-h dark cycle for at least 1 week before experimentation. Animals had a free access to food and water. All procedures were carried out in accordance with recommendations of the Ethics Committee on Animal Experiments of the Medical School (Tehran University of Medical Sciences, Tehran, Iran). A midline laparatomy was performed under general anaesthesia, induced by an intraperitoneal injection of ketamine (50 mg/kg) and promazine (10 mg/kg). In the bile duct-ligated (BDL) group the bile duct was isolated and doubly ligated by using method of Cameron and Oakley [23]. In sham-operated group, the bile duct was identified and manipulated without ligation. Seven days after recovery from operation, when the BDL group had shown signs of overt cholestasis (jaundice, dark urine and steatorrhea), midline laparatomy and blood sampling from the inferior vena cava were performed under general anaesthesia (as described previously) and the serum bilirubins level were determined by using commercially available kits (Zist-Shimi, Tehran, Iran).

2.2. Chemicals

Chemicals used were obtained as follows: dextran sulfate sodium (MW: 500,000 kDa) ICN Biochemical (Costa Mesa, CA, USA); naltrexone hydrochloride, Sigma Chemical Co. (St. Louis, MO, USA); naltrexone-fluorescein isothiocyanate (naltrexone-FITC) (Molecular Probes, The Netherlands). Two last drugs were dissolved in Krebs' buffer containing NaCl, 115 mmol/L; KH_2PO_4, 2 mmol/L; $MgCl_2$, 2.4 mmol/L; $NaHCO_3$, 25 mmol/L; KCl, 8 mmol/L; and $CaCl_2$, 1.3 mmol/L.

2.3. Leukocyte isolation

Leukocyte isolation was performed in accordance with the method of Boyum [24]. Briefly, 6 mL of fresh heparinized blood were diluted with two volumes of isotonic saline solution, and divided in two tubes each containing 1.5 mL dextran 6% (w/v) in NaCl 0.9% (w/v). The mixture was incubated at room temperature for 45 min to allow aggregation and sedimentation of the erythrocytes. The leukocyte rich supernatant was collected and washed (centrifuged at $200 \times g$ for 15 min at $4\,^{\circ}C$) with phosphate-buffered saline (PBS, pH 7.4). The washing was repeated once more. The contaminating erythrocytes were lysed by ice-cold isotonic ammonium chloride (NH_4Cl) solution, as necessary. Then, cells were washed three times as above at $200 \times g$ for 5 min at $4\,^{\circ}C$ with Krebs' buffer (pH 7.4). The differential cell counts were determined by a hemocytometer. The viability of the cells was tested by trypan blue exclusion.

2.4. Direct fluorescence labeling of opioid receptors

By using the naltrexone-FITC as an opioid probe, labeling of opioid receptors was performed as described previously [20]. Briefly, in a final assay volume of 60 μL of Krebs' buffer, 5×10^5 cells were incubated with 20 μL of 2–8 μM naltrexone-FITC for 30 min at $25\,^{\circ}C$ and in the dark, for determination of optimal staining of opioid receptors. The opioid-receptor antagonist naltrexone in 20 μL volume also was titrated (0.4–8 mM) to obtain the optimal concentration necessary for measurement of nonspecific fluorescence. The control sample consisted of leukocytes incubated with 40 μL Krebs, buffer alone. The assay was performed in triplicate. Samples were chilled on ice, diluted with 0.5 mL Krebs' buffer and centrifuged at $200 \times g$ for 3 min at $4\,^{\circ}C$. The supernatant was aspirated and cells were washed twice more and resuspended in a final volume of 0.5 mL of Krebs' buffer for the flow cytometric analysis.

2.5. Flow cytometric analysis

Samples were analysed on a Becton-Dickinson FACStar Plus flow cytometer. In each sample approximately 10,000 leukocytes were collected. Data including forward scatter and side scatter and fluorescein fluorescence were measured and analysed using LYSIS 2 software (Becton-Dickinson, San Jose, CA, USA). Median peak values of relative fluorescence intensity distribution were used to compare the fluorescence among samples. In each of lymphocyte, monocyte, and granulocyte population, percent of labeling was obtained by subtraction of nonspecific histogram (minus control histogram) from total histogram (minus control histogram) of the 100% gated population. Also, the percent of specific labeling of the opioid receptor was calculated as: [(Total fluorescence − background fluorescence) − (Nonspecific fluorescence − background fluorescence)/(Total fluorescence − background fluorescence)] × 100 as described previously [25]. At all study days, the cell isolation process was initiated immediately after blood sampling. In addition procedures of direct immunofluorescence and flow cytometric analysis were performed soon after obtaining a pure leukocyte suspension.

2.6. Statistics

Data have been expressed as means ± S.E.M. For the lymphocytes difference between means was calculated using unpaired Student *t*-test. The data for opioid-receptor labeling on monocytes and granulocytes were not normally distributed, as determined by Bartlett' *s*-test, so the nonparametric *t*-test Mann–Whitney was used for these data. A *p*-value less than 0.05 was considered as statistically significant.

3. Results

3.1. Induction of cholestasis

One day after bile duct ligation, the animals showed signs of cholestasis (jaundice, dark yellow urine), which persisted until the day of sampling. The plasma of BDL rats was dark yellow and serum total bilirubin levels were high in BDL rats (94 ± 12.9 μM in BDL versus 5.1 ± 0.5 μM in sham-operated rats, $p < 0.01$). Also, there was a significant increase in the proportion of both monocytes and granulocytes ($p < 0.001$), in association with a decrease in the proportion of lymphocytes ($p < 0.0001$) in BDL rats, compared with sham-operated animals (Table 1).

3.2. Subpopulations of leukocyte as identified by flow cytometry

The flow cytometric analysis of freshly isolated leukocytes (viability > 95%) showed three distinct populations of lymphocytes, monocytes, and granulocytes and this was confirmed by H & E staining of harvested cells from a cell sorter. The profile of these regions was same for both sham and BDL groups, although more granulocytes were observed in the dot plots obtained from the samples of cholestatic rats. This finding was consistent with increased granulocyte counts in this model (Table 1) [13]. Fig. 1 shows dot plot produced by flow cytometry of the sample obtained from a BDL rat (A) and also gating applied in the forward scatter versus side scatter of light (B). Forward scatter is a representative of cell size and side scatter representative of cell granularity. The "gate" was drew as following [26]: (a) the debris were discarded, (b) because of smaller size and no granularity, lymphocyte population was gated (R1) at nearly left lower quadrant (in the right side of debris), (c) the bigger cells, monocytes that those dots starts at nearly right side of lymphocytes appears in the right lower quadrant (R2), and (d) the granulocytes region was gated (R3) approximately in the right upper quadrant, because of their granularity and intermediate to large size, compared with lymphocytes and monocytes (Fig. 1).

Table 1
Differentials of isolated leukocytes from cholestatic and sham rats 7 days after the operation

Leukocyte subpopulations	Sham (%)	Cholestatic (%)
Lymphocytes	66.8 ± 3.1	40.1 ± 2.9***
Monocytes	7.7 ± 1.9	15.7 ± 2.2**
Granulocytes	25.5 ± 2.7	44.2 ± 2.3**

Data represent the means ± S.E.M. of six rats per group.
** $p < 0.001$ compared with related sham cell population.
*** $p < 0.0001$ compared with related sham cell population.

3.3. Optimal concentration of opioid ligands for opioid-receptor labeling procedure

Freshly isolated leukocytes from sham animals were incubated with 2–8 μM of naltrexone-FITC. Relative median peak fluorescein fluorescence values of total histogram of lymphocytes were 3.8 ± 1.2, 13.6 ± 1.7, and 8.3 ± 2 arbitrary units, at 2, 4, and 8 μM of naltrexone-FITC, respectively. The assay demonstrated 4 μM was the appropriate concentration of naltrexone-FITC for staining of the opioid receptors on the leukocytes. In addition, titration of the opioid-receptor antagonist naltrexone (0.4–8 mM), determined that 4 mM of unlabeled naltrexone to be an appropriate concentration for measurement of percentage specific labeling on the leukocytes (Fig. 2). Thus, 4 μM naltrexone-FITC and 4 mM unlabeled naltrexone were used for all opioid-receptor labeling experiments.

Fig. 3 shows fluorescence intensity distributions of a representative cell (granulocyte) population from a BDL sample, labeled with naltrexone-FITC in control or autofluorscence (A), total (B), and nonspecific (C) histograms. Note a leftward

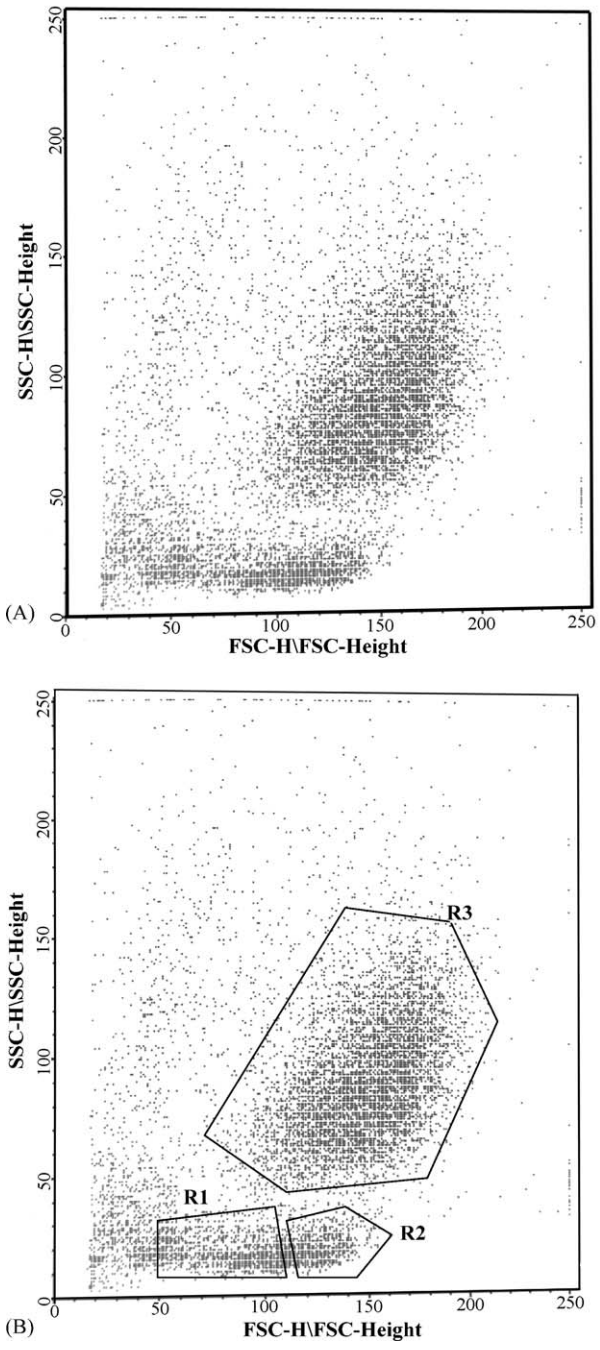

Fig. 1. Dot plot produced by flow cytometry of 10,000 leukocytes collected from sample of a cholestatic rat 7 days after the bile duct ligation (A). Using the light scatter data three regions (R1, R2, and R3) selected and gated (B). So, agranulocytes distribute horizontally and granular cells as vertically, in the plot. Thus, the relatively smaller lymphocytes appear in left lower part (R1), the bigger monocytes appear in right lower (R2), and the granulocytes appear approximately in right upper part of the plot (R3). Profile of these regions in dot plot, are highly consistent between experiments and between sham and cholestatic groups.

shift of fluorescein histogram in the presence of naltrexone. This demonstrates inhibition of binding of naltrexone-FITC by opioid antagonist naltrexone, in nonspecific histogram (Fig. 3).

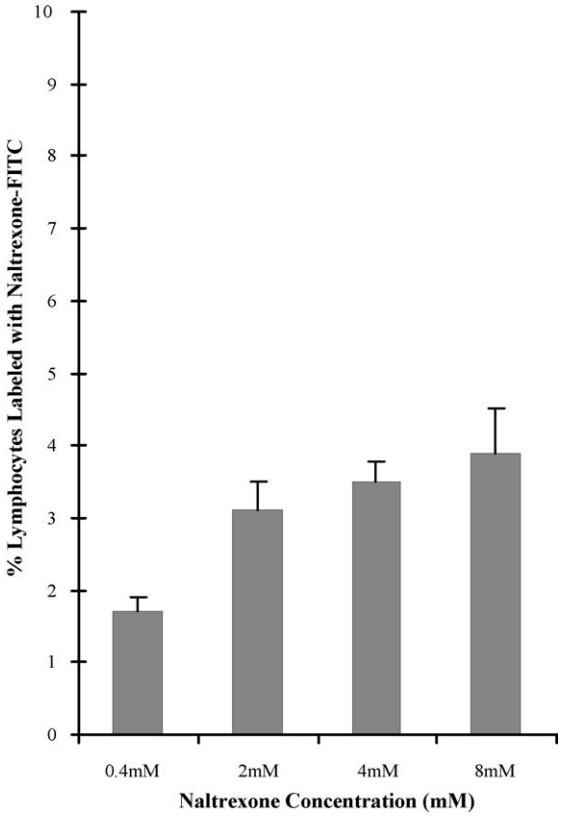

Fig. 2. Titration of unlabeled naltrexone for optimal labeling of opioid receptors. Freshly isolated leukocytes, from sham rats, were incubated with 4 μM naltrexone-FITC and 0.4–8 mM naltrexone. This scheme demonstrates increasing naltrexone concentration, increases percent of labeling, on the lymphocytes for opioid receptor. Data are the mean ± S.E.M. from five experiments, each performed in triplicate.

3.4. Effect of cholestasis on specific opioid-receptor labeling on the peripheral blood leukocytes

It was found that greater percentages of specific opioid-receptor labeling, defined as FITC fluorescence inhibited by naltrexone, were demonstrated by the monocytes (29 ± 2.08 for BDL versus 23 ± 2 for sham, $p < 0.001$) and granulocytes (50 ± 3.2 for BDL versus 39.6 ± 1.7 for sham, $p < 0.05$) from BDL rats. But, change of lymphocytes from BDL rats, was not statistically significant (15.4 ± 1 for BDL versus 18 ± 1.08 for sham, $p = 0.1$) (Fig. 4).

Leukocytes from BDL rats showed smaller percent of labeling, compared with sham rats. The difference was statistically significant for lymphocytes (Table 2).

4. Discussion

The results of this study showed that the specific opioid-receptor labeling of leukocytes was increased in the cholestatic rats in association with a relative decrease in the percent of labeled leukocytes.

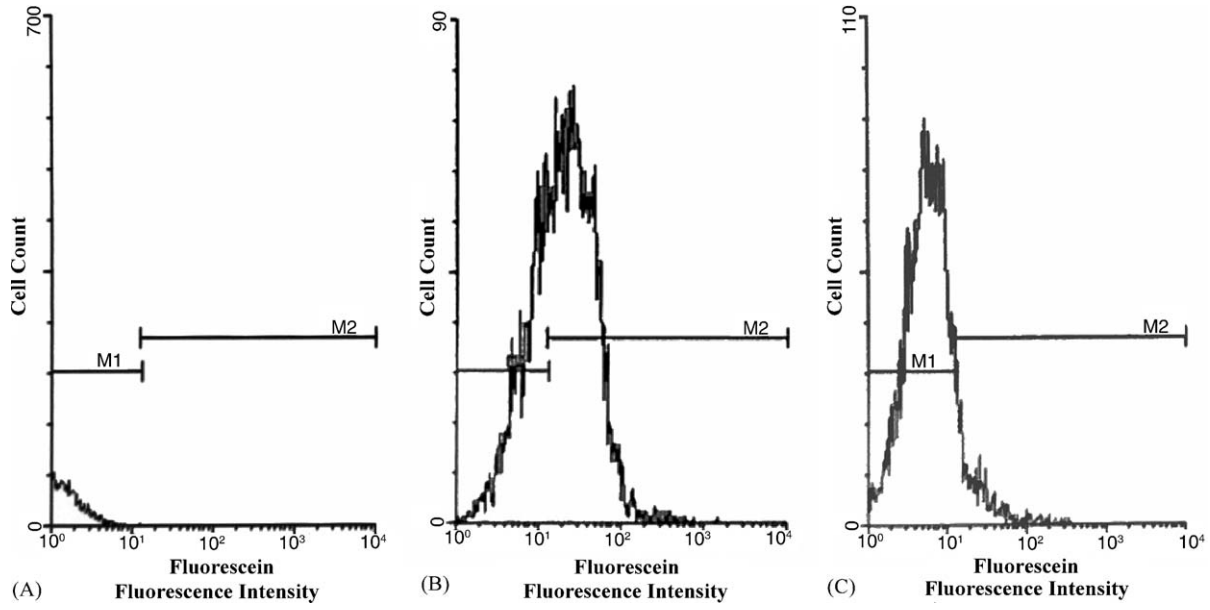

Fig. 3. Histograms of opioid-receptor labeling on the granulocyte subpopulation from a cholestatic rat. Fluorescence intensity distribution for cells labeled with naltrexone-FITC is showed as following: control sample including cells incubated only with buffer, autofluorescence (A); in the absence of naltrexone, total labeling (B); and in the presence of naltrexone, nonspecific labeling (C). These graphs shows a shift to left in the median peak FITC fluorescence because of inhibition of binding of naltrexone-FITC to opioid receptors by unlabeled naltrexone in nonspecific compared with total histogram.

The activity of opioid system is increased in cholestatic conditions. Elevated plasma levels of endogenous opioid peptides have been reported in patients with primary biliary cirrhosis [3,4] and also cholestatic animals [2,6]. Observations compatible with elevated plasma levels of opioids include precipitation of an opioid withdrawal-like syndrome in patients with cholestasis [3,4], as well as in the mouse model of acute cholestasis by administration of an opioid antagonist [8], a naloxone-reversible antinociception [5] and also, a global down-regulation of brain mu-opioid receptors [9] in bile duct-resected rats, and subsensitivity to opioid agonists in the isolated guinea pig ileum and mouse vas deferens obtained from the cholestatic animals [27].

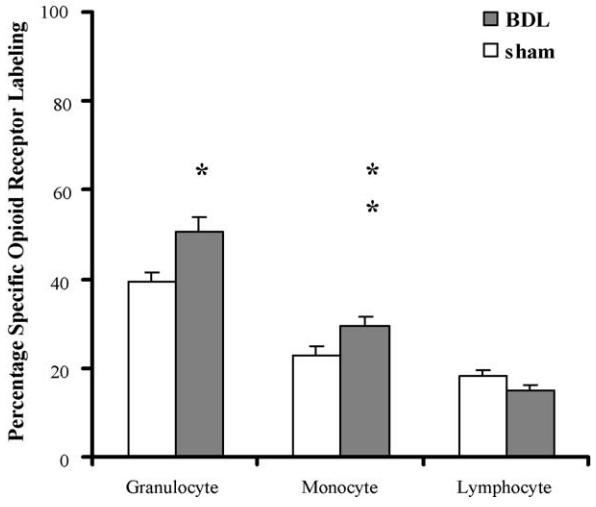

Fig. 4. Effect of cholestasis on specific opioid-receptor labeling on the peripheral blood leukocytes 7 days after bile duct ligation. As described in Section 2, freshly isolated leukocytes were incubated with 4 μM naltrexone-FITC in the absence or presence of 4 mM unlabeled naltrexone. The percentage specific opioid-receptor labeling was calculated as: [(Total fluorescence − background fluorescence) − (Nonspecific fluorescence − background fluorescence)/(Total fluorescence − background fluorescence)] × 100 [23]. Data are mean ± S.E.M. of specific opioid-receptor labeling from 6 to 9 experiments in sham or BDL group, each performed in triplicate. $^{*}p < 0.05$, and $^{**}p < 0.001$ compared with related sham cell population.

It has been demonstrated that endogenous opioid peptides including β-endorphin and dynorphin peptides modulate the functions of cells involved in host defense and immunity [10,17]. Endogenous opioids modulate immune functions by affecting central nervous system [28–30] and peripheral (immune cells) [11,12,17] opioid receptors [10]. In addition, immune cells express sites that show atypical opiate and opioid binding properties [16]. Accordingly, immunomodulatory effects of β-endorphin have been shown to depend on both naloxone-sensitive and naloxone-insensitive receptors, suggesting both brain-type and non-neuronal-type opioid receptors on immune cells [17].

Our results showed that the granulocyte and monocyte subpopulations of leukocytes from cholestatic rats had a significant increase in specific opioid-receptor labeling compared with same subpopulations from sham animals 7 days after bile duct-ligation or sham operation, respectively (Fig. 4). These two subpopulations together constitute the greater proportion of peripheral leukocytes of BDL, compared with control rats, as described previously (Table 1) [13]. The increase in the specific opioid-receptor labeling of leukocytes observed in this study, may be a result of increased specific fluorescent staining due to increased opioid-receptor expression on these cells. It has previously been documented

Table 2
Effect of cholestasis on the percent of opioid-receptor labeled leukocytes 7 days after bile duct ligation

	Fluorescein fluorescence intensity (arbitrary units)[a]					
	Lymphocytes		Monocytes		Granulocytes	
	Sham	Cholestatic	Sham	Cholestatic	Sham	Cholestatic
Autofluorescence (control fluorescence)	2.7 ± 0.15	2.4 ± 0.13	2.8 ± 0.15	2.5 ± 0.13	5 ± 0.35	4.8 ± 0.3
Naltrexone-FITC (total fluorescence)	18.5 ± 0.59	16.83 ± 0.5	25.4 ± 0.49	14.09 ± 0.54	28.3 ± 1.2	26.7 ± 1.7
Naltrexone-FITC/naltrexone (nonspecific fluorescence)	15.4 ± 0.46	14.6 ± 0.46	20.9 ± 0.48	11 ± 0.4	19.6 ± 0.68	19 ± 1.95
% of labeled cells	3.1 ± 0.3	2.2 ± 0.2*	4.6 ± 0.5	3.5 ± 0.4	8.7 ± 0.7	7.7 ± 0.7

[a] Data showed the means ± S.E.M. of relative median peak fluorescence intensity values of 6–9 experiments per group, each performed in triplicate.
* $p < 0.05$ compared with lymphocytes of sham.

that plasma levels of methionine-enkephalin were significantly increased in cholestatic rats 5 days after bile duct-resection [2]. Collectively, we can conclude that opioid receptors on peripheral leukocytes of BDL group probably undergo agonist-induced up-regulation, as reflected by increased specific opioid-receptor labeling. Pharmacologically, it was expected that increased exposure of leukocytes to a high levels of exogenous [31,32] or endogenous opioids (such as methionine-enkephalin in the plasma of cholestatic rats), would result in down-regulation of their opioid receptors. But we observed a different phenomenon, an increase in specific opioid-receptor labeling on the peripheral leukocytes of BDL, compared with normal (sham) rats. It is probable that these changes be due to a central compensatory mechanism for increasing levels of functionally responsive opioid receptors to improving immune dysfunction in cholestasis [13–15].

Nevertheless, there are some studies that have demonstrated an up-regulation of opioid receptors on the leukocytes after opioid agonist such as heroin [33] and morphine treatments [34–36]. In a clinical study, it has been observed that the granulocytes of heroin abusers have an increased opioid-receptor expression compared to healthy controls [33]. Also, findings of the present study demonstrated an increase in specific opioid-receptor labeling on leukocytes from BDL group were in agreement with the changes obtained in human lymphocytes exposed to morphine, which showed an increase in the expression of mu-opioid receptor [34], and also an up-regulation of kappa-opioid-receptor expression [35,36]. In these studies, the changes were reversed by pretreatment of the cells with nonselective opioid antagonist naloxone, reflecting those changes were opioid receptor-mediated.

In the present study we also observed a reduction in the percent of labeled leukocytes in BDL compared with sham group (Table 2). This represents a smaller expression of opioid receptors on the leukocytes due to bile duct-ligation. This finding differs from results obtained previously, which showed increased percent of labeled leukocytes in the cholestatic rats [22].

Several workers have emphasized the implication of increased circulating levels of endogenous opioids, in the pathophysiology of cholestasis [7,8,37–40]. Accordingly, we suggest that probably this phenomenon may also contribute to the observed changes in the specific labeling of opioid receptors on peripheral leukocytes from BDL rats. The contribution of endogenous opioids may take place through a direct effect(s) on the immune cells or an indirect effect(s) at central opioid receptors, or both. In addition, changes in the central opioid receptors documented in rats with acute cholestasis [9] also, may be involved. The elucidation of the exact underlying mechanisms of these findings needs, however, further studies in this field.

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