Effects of Morphine on the Rat Lymphoid organs and Adrenal Glands: Results of Enzyme Histochemical and Histometric Investigations

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SUMMARY

Effects of morphine on the rat lymphoid tissues and adrenal glands were investigated by means of histological, histometrical and enzyme histochemical methods. The experimental animals were treated with 5 mg/kg morphine hydrochloride at daily intervals for 30 days. Morphine treatment caused severe lymphoid cell depletion from spleen, thymus and lymph nodes whereas, ileal Peyer’s patches were affected moderately. Both peripheral blood lymphocyte percentage and ANAE-positive lymphocyte frequency in the lymphocyte fraction decreased. The changes in lymphoid organs provided morphological evidence for morphine induced immune suppression. Along with these, observation of the thickening of the adrenal cortex and medulla might show that morphine induced immuno suppression may occur via increased glucocorticoid secretion.

KEY-WORDS : morphine - rat - lymphoid organs - adrenal glands.

Introduction

Opioids and opioid agents are known to have profound immune suppressive effects [31]. An opioid agent, morphine is a drug of abuse with an ability to down-regulate immune responsiveness that could have potentially serious consequences in both heroin addicts and in the clinical environment. Results of the functional assays have shown that acute morphine administration inhibits peripheral blood lymphocyte activity and causes a definite decline in the peripheral blood lymphocyte counts in the rat [11] and human splenic natural killer (NK) cell activity [31]. LUZA [22] has shown that morphine might cause significant loss of lymphocyte viability in vitro. The decrease was 34 % for peripheral blood lymphocytes, 14 % for splenic lymphocytes and 18 % for thymocytes.

BRYANT et al [3] have implanted 75 mg morphine pellets into mice and have observed marked atrophy and reduced cellularity of the spleen and thymus, and an attenuated lymphocyte proliferative response to T-and B-cell mitogens, concavalin-A (con-A) and bacterial lipopolysaccharide (LPS), respectively.
EISENSTEIN et al [8] have shown that morphine inhibits splenic macrophage functions and also decreases their number. Similarly, GOMEZ-FLORES et al [15] determined significant reduction in nitric oxide production of macrophages in morphine treated rats, although there was no change in their numbers. Hilburger et al [19] have also reported that relative numbers of macrophages and B-lymphocytes decreased, whereas T-cell counts increased in the spleens of morphine treated mice. However, any definite changes were not observed in CD4+/CD8+ lymphocyte ratio in the animals. They also found that morphine suppresses antibody production directed against to sheep red blood cell (SRBC) as a result of impaired macrophage functions in the mice.

The mechanism related with the morphine-induced immune suppression is still obscure, although two pathways have been suggested [13]. The direct activity is possibly via the opiate receptors on the lymphocyte surface but the nature of such receptors is still in question [13]. HERNANDEZ et al. [17] have reported that central opioid pathways are involved in the immunosuppressive effects of morphine and these pathways may be distinct from those participating in opioid induced analgesia and adrenal activation.

As an alternative route, an indirect mechanism has also been assumed to act in two possible ways, one of them through activation of hypothalamo-hypophysial-adrenal arc (HHAA) which is resulting in significant increase of potential immune suppressive agents, adrenocorticoids. The second way of the indirect action occurs via activation of sympathetic nervous system in which catecholamine-releasing increases [13]. Related with these, the researchers [13] have suggested that morphine suppresses the NK cell activity in the the mouse via elevating serum corticosterone levels. They found that corticosterone significantly depressed the NK cell activity in vitro, whereas morphine was unable to alter directly the NK cell activity. Corticosteroids are well known T-lymphocyte suppressors and corticosteroid resistance is regarded as a sign of T-Cell immuno competency and maturation [32].

During the maturation, thymus originated T-lymphocytes also gain some specific enzymes playing important roles in their functions [1]. Alpha-naphthyl acetate esterase (ANAE) has been largely used as a marker to identify the mature, immunocompetent T-lymphocytes in various animal species and humans. The frequency of ANAE positive peripheral blood lymphocytes have shown to reflect the frequency of in the peripheral blood mature T-lymphocytes accurately in the mouse [24], in the cow [20], in the dog [33], in the chicken [23], in the human [18], and in the rat [12]. KNOWLES and HOLCK [21] applied the technique to the frozen tissue sections. The results showed that the technique is also suitable for determining T-lymphocytes in the tissue sections.

The aim of this study was to determine overall effect of morphine on immune system. These were investigated on the rat lymphoid organs by means of histological, histometric and enzyme histochemical techniques. In order to investigate the indirect pathway related with the effects of morphine in adrenal cortex mentioned above, adrenal histological structure was investigated histometrically.

### Materials and Method

#### A) ANIMALS AND EXPERIMENTAL DESIGN

Four young (30-32 days of age and weighing 120-125g) Wistar rats (equal numbers from both sexes) were randomly divided into 2 groups each having 20 animals (10 of them were male and the remaining 10 were female). The animals were housed individually in the cages located in a pathogen free, temperature- and humidity-controlled colony room which is maintained under a 12-hours day-night illuminating cycle with free access to food and water. Prior to the experiment, the animals were prepared for manipulations during a 1-week habituation period in the Experimental Medicine Research and Application Center of Medical Faculty, Selçuk University.

The animals in the first group served as controls and injected subcutaneously (SC) with 1ml/kg physiological saline (0.9 % NaCl). Morphine hydrochloride, at a dose of 5 mg/kg in 1ml/kg volume of physiological saline was administrated subcutaneously to the animals in the second group, at daily intervals for 30 days.

#### B) HISTOLOGICAL AND ENZYME HISTOCHEMICAL METHODS

At the end of the administration period, blood samples from coxygeal veins of the animals were taken into heparinized (100 IU heparine/ml blood) tubes. From each sample, four blood films were prepared, air dried and two of them were stained with May Grunwald-Giemsma staining [7], whereas the other two were used for ANAE demonstration [12]. The animals were sacrificed by cervical dislocation and spleens, thymuses, ileal samples with Peyer’s patches, adrenal glands were removed and all the samples were divided into three pieces. One piece of the each sample was placed into buffered (pH 7.4) formal-saline. Following the fixation, the tissue pieces were processed for three chrome staining [2] by means of routine histological techniques. The second group of the pieces were fixed in alcholic formalin and processed for plasma cell staining [30]. The third group of the pieces were placed into formal-sucrose and kept for 24 hours at +4 °C. The samples were kept for another 24 hours in Holt’s syrope, frozen sections (10-12 µm) were taken at cryostat (Slee,London) and ANAE was demonstrated under strictly controlled incubation conditions according to the method of KNOWLES and HOLCK [21].

Lymphocytes with 2-5 large reddish-brown granules, giving dot-like positivity were regarded as ANAE-positive in both blood films and tissue sections. Monocytes and macrophages displayed a strong and diffuse-fine granular ANAE positivity.

#### C) HISTOMETRICAL ANALYSES

Splenic plasma cells in unit area (1.44 X 10⁴ µm² tissue area) were determined by counting in 10 randomly selected in subcapsular white pulp regions using an ocular square micrometer and results were expressed as cell count/unit area.
FIGURE 1.— Spleen sections of the control (A, ANAE demonstration, X128) and morphine treated (B, ANAE demonstration, X128) animals. Macrophages arrow heads mostly located around the follicles and interfollicular areas in the control section, ANAE + lymphocytes (arrows) heavily populate mantle zones. Severe lymphocyte depletion from germinal centers (GC) and mantle zones (mz) of the follicles are definite in the section of morphine treated animals (B).

FIGURE 2.— Thymic sections of the control (A, Trichrome, X300) and morphine treated (B, Trichrome, X240) animals. Highly cellular cortex (C) and less cellular medulla (M) are seen. In the section of the morphine treated animal (B) severe thymocyte depletion from both cortex and medulla is definite.

FIGURE 3.— Sections of the Ileal Peyer’s patches of the control (A, Trichrome,X128) and morphine treated (B, Trichrome, X128) animals. Lymphoid cell depletion of lymphoid cells and partial connective tissue increase are prominent in the section of the morphine treated animal (B).
Histometrical measurements on the spleens, adrenals and thymuses were done with the aid of an ocular linear micrometer. For this purpose, 10 tissue sections (7 µm) were taken at 21 µm intervals, from each animal and the results were expressed as mean values. Leucocyte formulae were determinated by counting at least 200 leucocytes in each May Grunwald-Giemsa stained blood film. ANAE-positive lymphocyte ratio of each animal was determined by counting 200 lymphocytes in the ANAE demonstrated blood films.

The data were statistically analyzed with Student’s t-test.

Results

A) SPLEEN

The splenic lymphoid follicles of the control animals had typical lymphoid organ histology for the rats splenic follicles which were consisted of a pale germinal centers (GC) and a mantle zone surrounding the GCs. ANAE-positive lymphocytes were rarely seen in the GCs, whereas most of the lymphocytes in the mantle zone displayed a strong dot-like ANAE positivity. Lymphatic cords constituted cellular...
sheets, paraarteriolar lymphoid sheaths (PALS) around the central arteries (Fig. 1A). The lymphocyte population of PALS were mostly positive for ANAE. Megakaryocytes were less in number and splenic red pulp was observed as narrow regions between the lymphatic follicles and cords. Hemosiderin pigment existed weakly in the spleens of controls. In morphine treated animals, spleen was severely affected. Both morphometric (Table I) and histological results have showed severe lymphocyte depletion from both GC and mantle zone of the spleen (Fig 1B). As a result of the lymphoid cell depletion, the splenic lymphoid tissue has regressed and the capsule of the organ has contracted and thickened significantly (P < 0.01). Both GC-diameter decreased and mantle zone thickened significantly (P < 0.05) in the morphine treated animals compared to those of the controls. Mean splenic plasma cell count of the morphine given animals was also lower than those of the controls (Table I). Haemosiderine was frequently observed and widely distributed in the subcapsular areas of the spleens from morphine treated animals. Splenic megakaryocyte counts have relatively increased in the treatment group (Table I).

B) THYMUS

Lymphoid tissue of the thymus organized as a dense cellular cortex and less cellular medulla (Fig. 2A). Hassal’s corpuscles were rarely seen in the thymic medulla. Morphine treated animals displayed severe thymocyte depletion in both cortical and medullary areas of the thymus. Partial fibrosis was also observed in the thymic medullae of the treatment group (Fig. 2B). Both thymic cortices and medullae have regressed significantly (P < 0.05, Table I).

C) ILEAL PEYER’S PATCHES

Ileal Peyer’s patches (IPP) in the control group were covered by a lymphoepithelium densely populated with lymphoid cells. Lymphoid tissue of the IPP’s formed by aggregated lymphoid follicles having deeply located, small GCs (Fig. 3A). Ileal Peyer’s patches of the experimental group (Fig 3B) have been affected slightly and partial lymphoid cell depletion was observed.

D) MESENTERIC LYMPH NODES

Mesenteric lymph nodes of the control group had larger cortical areas which were occupied by lymphoid follicles, paracortical zones formed by lymphatic cords and medullary areas containing large lymphatic sinuses. All sinuses were heavily filled with lymphocytes (Fig. 4A). The lymph nodes (Fig. 4B) have been affected slightly and partial lymphoid cell depletion was observed in the experimental group.

E) ADRENAL GLANDS

Adrenal glands of the control animals displayed typical morphology with a larger cortical area and a centrally located medulla region (Fig. 5A). Overall thickness of adrenal cortex has increased in the treatment group when compared to

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<tr>
<th>Groups</th>
<th>ANAE positive Lymphocyte (%)</th>
<th>Leucocyte types (%)</th>
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<tr>
<td>Controls n = 20</td>
<td>62.90 ± 8.8</td>
<td>60.62 ± 6.8</td>
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<tr>
<td></td>
<td>35.61 ± 4.9</td>
<td>30.2 ± 0.8</td>
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<td>0.72 ± 1.0</td>
<td>0.03 ± 0.02</td>
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<tr>
<td>Morphine treated n = 20</td>
<td>46.11 ± 8.8</td>
<td>54.7 ± 7.7</td>
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<td></td>
<td>39.03 ± 7.3</td>
<td>3.59 ± 1.2</td>
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<td></td>
<td>0.5 ± 0.1</td>
<td>0.16 ± 0.1</td>
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<td>Significance</td>
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* : P < 0.05

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TABLE II. — Mean leucocyte, ANAE positive lymphocyte percentages of the control and morphine administrated animals and results of statistical analyses.
those of the controls (Fig. 5B). Statistical analysis have showed that fascicular layer of the adrenal cortex thickened significantly (P < 0.05) compared to those of the controls. Medullar thickness has also increased significantly (P < 0.05) in the morphine treated animals. Histometric results obtained from the lymphoid tissues and adrenal glands are given in Table I.

F) PERIPHERAL BLOOD

Both percentage of the peripheral blood lymphocytes and the proportion of ANAE positive lymphocytes declined significantly (P < 0.05). Significant (P < 0.05) increases were also observed in the eosinophytic cell ratio of the morphine treatment group (Table II).

Discussion

The results of previous functional assays have revealed that morphine suppresses immune responses by inhibiting lymphocyte, macrophage and NK cell activities. FLORES et al [11] have found that morphine inhibits proliferative responses of lymphocytes to con A. Proliferative responses to bacterial LPS is also declined by morphine [3]. GARZA and PRAKESH [14] have observed moderately reduced mitogen induced IL-2 production and splenic CD4 and CD8 lymphocyte percentages in HIV-1 transactivator of transcription (TAT)- transgenic mouse model. They also found significant reduction in splenic NK cells and peritoneal cytotoxic T-lymphocyte (CTL) activities.

Significant declines in NK cell activity [13, 11, 31], chemotactic activity, cellular velocity and adherence capacity of macrophages have been profoundly depressed in morphine treated animals [29]. Primary antibody responses to SRBC [8] and delayed type hypersensitivity reactions [16] have also been impaired by morphine treatment.

Results of histological and histometrical investigations on the lymphoid organs of the morphine treated animals give some morphological evidence for morphine induced immune suppression. Morphometric results have indicated that morphine caused profound regressions in lymphoid cell populations in the rat spleens and thymuses (Table I). Severe lymphocyte depletion from both GC and mantle zone of the splenic lymphoid follicles was found in morphine treated animals. Splenic capsule has contracted and thickened significantly (P < 0.001, Table I) as result of the lymphoid cell depletion in the treatment group. Both GC-diameter and mantle zone-width decreased in the morphine treated animals compared to those of the controls. Mean splenic plasma cell count of morphine given animals was also lower than those of the controls (Table I). These results give some morphological evidence for morphine induced suppression of primary antibody response [19]. Morphine has also been reported to induce splenic atrophy [8, 25]. BUSSIÈRE et al [5] have found decreased spleen weight in morphine treated animals, although the authors pointed out that immune suppression did not correlate with the splenic atrophy or mortality. Whether immuno suppression is attributable to either decreased macrophage number or decreased functional capacity of individual macrophages is unclear although EISENSTEIN et al [8] have suggested that morphine induced suppression of primary antibody response is due to a deficit in the splenic macrophage function.

BRYANT et al [3] have implanted morphine pellets in mice and have observed splenic and thymic, and lymphocyte proliferative responses to T- and B-cell mitogens (con A and bacterial LPS, respectively). The authors have also found marked reductions in spleen (35 %) and thymus (56 %) weights following 48 hours in 75 mg morphine pelleted mouse relative to the weights in placebo-pelleted controls. Multiple pellet implantation caused similar effects, such as splenic and thymic atrophy, with reduced mitogen-induced lymphocyte proliferative responses. In accordance with the results above, we observed prominent thymocyte depletions from both thymic cortices and medullae in the morphine treated animals. Partial fibrosis was also definite in the thymic medullae of the treatment group. Thymic atrophy in the young animals may result in serious deficiency in the cellular immunity. Both peripheral blood lymphocyte percentage and the proportion of ANAE positive lymphocytes declined significantly (P < 0.05) in the treatment group. Histological observations of this study have showed that both lymphoid nodes and ileal Peyer’s patches have also been affected and slight lymphoid cell depletion was observed in the experimental group.

In this study, morphine administration caused significant declines (P<0.05) in both peripheral blood lymphocyte percentages and ANAE positive T lymphocyte frequency in the lymphocyte population (Table II). Similarly, FLORES et al [11] have observed 30 % decrease in the peripheral blood lymphocyte population which was not selective for any specific population of T-lymphocyte. LUZA [22] has assumed that morphine might cause significant loss of lymphocyte viability in vitro. The decrease they determined was 34% for peripheral blood lymphocytes, 14 % for splenic lymphocytes and 18 % for thymocytes.

Although the mechanism of the morphine-induced immune suppression is still unclear, a direct mechanism of action is suggested to operate through lymphocyte opiate receptors, but the nature of such receptors is still in question [13]. Nevertheless, HERNANDEZ et al. (17) have reported that a single systemic injection of morphine (10 mg/kg) to rats profoundly suppressed mitogen induced proliferation of blood lymphocytes by a receptor mediated mechanism. Their further results have revealed that central opioid pathways are involved in the immuno suppressive effects of morphine and these pathways may be distinct from the receptors participating in opioid induced analgesia and adrenal activation. Altough HERNANDEZ et al. [17] found an inhibited lymphocyte proliferation by 50 % without producing analgesia or a significant increase in plasma corticosterone in 1 µg/ml morphine injected animals. Although CARR et al. [6] have suggested that morphine induced analgesia and immuno suppression are mediated through a common opioid receptor type, BUSSIÈRE et al. [5] assumed that morphine may be acting through both opioid and non-classical opioid (e.g. a
mechanism that not blocked by naltrexone) mechanisms. SEI et al. [27] have proposed Ca $^{2+}$ dependent mechanism explaining the early events of immune suppression. The most affected cells were CD4 $^+$ T-lymphocytes. The authors claimed that morphine-induced inhibition of Ca $^{2+}$ mobilization in immune cells may be an early event of opiate-induced immuno suppression. In the latest study [10] it has been suggested that suppressive effects of morphine on the leukocyte functions result from the direct effects on functional abilities of leukocytes, rather than indirect effects.

Suppression of humoral responses by morphine might not primarily mediated by direct action of morphine on the immune system, glucocorticoids may be involved in the indirect mechanism by which morphine causes splenocyte dysfunction [26]. It has been suggested [13] that the alternative pathway is an indirect mechanism of action which is mediated by two possible ways, hypothalamic-pituitary-adrenal axis (HPA) activation resulting in high serum level of adrenal corticosteroids, or activation of sympathetic nervous system and concomitant catecholamine release. FREIER and FUCHS [13] have found significantly elevated serum corticosterone levels in the morphine treated mouse.

In accordance with the suggestions above, overall thickness of adrenal cortex has increased in the morphine treated animals in this study. Statistical analysis has showed that adrenal fascicular layer and medullar zone thickened significantly (P < 0.05) compared to those of the controls. Similarly, BRYANT et al. [4] have found adrenal hypertrophy in the morphine-pelleted animals (50 % increase in adrenal weight relative to placebo). The severity of both sphenic and thymic atrophy was reduced by about 50 % in adrenalectomized morphine-pelleted mice (17 % and 22 % reductions, respectively) compared to that in adrenalectomized mice implanted with placebo pellets. The authors [4] have suggested, that morphine induced immuno suppression is, at least in part, mediated by an increase in serum corticosterone levels after implantation of the morphine pellet.

The adrenal corticosteroids are well known T-lymphocyte suppressors and corticosteroid resistancy is regarded as a sign of T-Cell immunocompetency and maturation [32]. WEISSMANN [32] show that administration of hydrocortisone (HC) resulted in a striking degree of cortical thymocyte pycnosis by 24 hours with only minimal medullary thymocyte pycnosis. The author suggested that HC-resistance is acquired during maturation process which starts in the cortical zone of thymus and terminated in the medulla. The acquisition of HC-resistance (perhaps more properly the loss of sensitivity) to the lytic effects of HC is a late event in the maturation. The only «properly» matured thymocytes loose the glucocorticoid sensitivity, and thereby are assured survival in an environment which may contain relatively high concentrations of glucocorticoids on an episodic basis [28].

**Conclusion**

The results of this study have revealed that subcutaneous morphine administration at a dose of 10mg/kg for 30 days caused profound detrimental effects on rat lymphoid organs.

Thymus and spleen were the most affected organs. Lymphopenia and significant declines in ANAE positive T lymphocyte percentages were also observed in the morphine treated animals. Hypertrophy of both adrenal medullae and fascicular layer of adrenal cortex might give some morphological evidence for morphine-induced immuno suppression. Immuno suppression possibly occurs via an alternative pathway which is an indirect mechanism of action mediated by two possible ways, hypothalamic-pituitary-adrenal axis (HPA) activation resulting in high serum level of adrenal corticosteroids, or activation of sympathetic nervous system and concomitant catecholamine release.

**References**


