

T-helper 1 and 2 serum cytokine assay in chronic opioid addicts

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ABSTRACT. There are a few studies with conflicting results on the effects of opioids on the functioning of immune system. This study was performed to investigate the *in vitro* production of interferon- γ and interleukin-10 after antigenic stimulation of cells using whole blood from opioid addicts. Blood samples were taken from 20 chronically opioid-addicted persons, who voluntarily enrolled for detoxification (10 opium and 10 heroin addicts). Blood samples were also taken from 10 healthy individuals with no history of drug abuse as the control. Cell culture was performed in a whole blood culture assay. Diluted blood samples were stimulated with phytohemagglutinin or with lipopolysaccharide and the supernatants were collected to measure cytokine production. The results demonstrated a significant decrease in interferon- γ production and an increase in interleukin-10 secretion in heroin addicts, relative to the control group (35.9 ± 26.3 versus 110.2 ± 60.3 pg/mL, $p < 0.01$ and 71.8 ± 28.4 versus 17.1 ± 13.5 pg/mL, $p < 0.01$, respectively), however the changes in these values in opium addicts were not significant compared to healthy individuals. The results could suggest that opioid addiction leads to a shift in the Th1/Th2 cytokine balance of peripheral CD4+ cells towards the Th2 response, and opioid addicts demonstrate reduced mitogenic responsiveness of lymphocytes relative to healthy individuals.

Keywords: cytokine, interferon gamma, interleukin-10, opioid addicts

Conflicting results are reported concerning the effect of opioid drugs on immune function. Several authors have reported that opioid addicts have an increased susceptibility to various infectious diseases [1, 2], and morphine or heroin may sensitize users to virus-bound infection due to the impaired immune function in these individuals [3, 4]. Similarly, down-regulation of phagocytic cell function and disturbances in cytokine production have been reported [1, 5]. It has also been noted that morphine could inhibit interferon- γ (IFN- γ), tumor necrosis factor alpha (TNF- α) and superoxide radical production *in vitro* by mitogen-stimulated, peripheral blood mononuclear cells in an opioid-mediated way [6-8]. Morphine inhibits natural killer (NK) cell responses when used experimentally in animals and in human volunteers [9, 10]. Conversely, some experimental data suggest that the opioid effects on the immune system are not necessarily deleterious; in fact, endogenous opioids administered *in vitro* to human peripheral blood mononuclear cells augment NK activity in a naloxone-sensitive manner [11]. Similarly conflicting results shows that a low dose of morphine has no effect on the production of interleukin (IL)-6 and TNF- α by human peripheral mononuclear cells [6].

In cell-mediated immunity, CD4+ T helper cells are usually classified into two subsets based on the types of cytokines they produce. T helper 1 (Th1) cells produce cytokines such as IFN- γ and IL-2 are mainly involved in macrophage activation. In contrast, T helper 2 (Th2) cells synthesize IL-4 and IL-10, and are mostly responsible for regulating humoral immune responses and progression of infection [12]. The delicate balance between Th1 and Th2 cytokines is thought to influence the outcome of disease. Production of IFN- γ by Th1 cells is usually a predictive index of good response in cell-mediated immunity, and secretion of IL-10 by Th2 cells contributes to impaired cellular immunity [12]; therefore in our study we investigated the *in vitro* production of IFN- γ and IL-10 after stimulation of the cells, with phytohemagglutinin (PHA) or with lipopolysaccharide (LPS), in whole blood from opioid addicts compared to healthy individuals.

SUBJECTS AND METHODS

Subjects

After ethical approval from the local Ethics Committee, 20 opioid addicts with no history of alcoholism and/or addic-

tion to other substances, who were admitted to the Detoxification Department of Local Health Center (in Kerman, Iran), were enrolled into this study and provided written consent to participate. The control group also consented to take part in this study. All subjects were men, aged 21 to 40 years. None had had any recent infections or a positive HIV or HBsAg test. They were free of drugs affecting the immune system and had no history of recent vaccination. Four to five milliliters of venous blood were drawn from each subject into a sterile, endotoxin-free, polystyrene tube containing 100 units sodium heparin. Each blood sample was processed within one hour after collection. After all the experiments were complete, the opioid addicts were divided into opium and heroin addiction groups. An age matched group of 10 healthy individuals, between 20 and 40 years old, served as the control group.

Mitogen and antigen

Culture stimulators were the mitogen, phytohemagglutinin (PHA, 5 $\mu\text{g}/\text{mL}$) (L9017, Sigma, Sweden) and lipopolysaccharide (LPS, 1 $\mu\text{g}/\text{mL}$) (*Escherichia coli* 0111; B4, Boivin method, Difco Laboratories, Detroit, MI, USA). Mitogen and LPS were used at optimal stimulatory concentration as described elsewhere [13, 14].

Stimulation of whole blood

A heparinized, whole blood sample from each subject was diluted to 1:5 with RPMI-1640 (Gibco Life Technologies, Paisly, UK). The diluted blood was cultured in a one ml volume in endotoxin-free, polystyrene, round-bottomed sterile tubes with caps and in duplicate. The cells from the diluted whole blood were either cultured alone as control or stimulated with LPS (1 $\mu\text{g}/\text{mL}$) or with PHA (5 $\mu\text{g}/\text{mL}$). The tubes were incubated in a humidified air atmosphere at 37°C with 5% CO₂ for 72 hours. After incubation, all antigen-treated, PHA-treated and control tubes were centrifuged at 300 \times g and the supernatants were removed and kept frozen at -20°C until assayed for cytokine concentrations. These fluids were coded and laboratory personnel were blinded throughout the experimental procedures.

Cytokine measurements

The frozen, culture supernatant fluids were thawed at room temperature and cytokines, IL-10 and interferon- γ levels were measured by Enzyme-Linked Immunosorbant Assay (ELISA) using commercial assay kits supplied by Bio-source Europe S.A. (Nivelles, Belgium) according to the manufacturer's instructions. The absorbance of each well

was read at 492 nm and cytokine concentrations in the samples were calculated with a standard curve generated from recombinant cytokines. Cytokine values were expressed as picograms/milliliter (pg/mL).

Statistical analysis

The results are presented as the mean \pm standard deviation (S.D.). Student's *t*-test was used. Two-group comparison was performed using the Wilcoxon test (paired) or the Mann-Whitney *U*-test (unpaired). Statistical analysis of the data was carried out using SPSS, version 13. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Levels of immune response parameters were evaluated before the start of detoxification. The base-line characteristics of all subjects; opium addicts (O; $n = 10$), heroin addicts (H; $n = 10$) and healthy control (N; $n = 10$) groups for the cytokine production study are shown in *table 1*. The ages of the subjects ranged from 21 to 58 years. The dosage range of opium that was used by opium addicts was 500 to 3 000 mg/day. The dosage range of heroin that was used by heroin addicts was 500 to 2 000 mg/day. The duration of the addiction in the opium and heroin groups was 8.7 ± 8.03 and 6.7 ± 5.2 years, respectively.

Data for IFN- γ and IL-10 production in cell cultures of whole blood after stimulation with PHA or with LPS is shown in *table 2*. The mean production of IFN- γ in response to LPS or to PHA by cells from opium addicts was lower than corresponding values in the control group (mean \pm SD: 67.6 ± 24.5 versus 110.2 ± 60.3 pg/mL in response to LPS). However, this was not significant ($p > 0.05$) (*table 2, figure 1*). In heroin addicts, the mean production of IFN- γ in response to LPS or to PHA was significantly lower than corresponding values in the control group (35.9 ± 26.3 versus 110.2 ± 60.3 pg/mL in response to LPS, $p < 0.01$), (*table 2, figure 2*).

With regard to the production of IL-10, it was observed that much higher levels were produced by the cells in response to LPS or to PHA in heroin addicts compared with this value in the control group (71.8 ± 28.4 versus 17.1 ± 13.5 pg/mL in response to PHA, $p < 0.01$) (*table 2, figure 3*). In opium addicts, stimulation of the cells with LPS or with PHA resulted in an elevated level of IL-10 in the culture supernatants compared with the control group (28.6 ± 14.5 versus 17.1 ± 13.5 pg/mL in response to PHA, $p > 0.05$) (*table 2*).

Table 1
Baseline characteristics (mean \pm SD) of the subjects in the study

	Opium addicts (n = 10)	Heroin addicts (n = 10)	Healthy controls (n = 10)
Age (years)	31 \pm 10.3	28.9 \pm 5.7	26.7 \pm 3.2
Dosage of opioid addiction (mg/day)	500-3 000	500-2 000	-
Duration of opioid addiction (years)	8.7 \pm 8.03	6.7 \pm 5.2	-
HIV test	Negative	Negative	Negative
HBs Ag test	Negative	Negative	Negative

Table 2
Supernatant cytokine levels, interferon- γ (IFN- γ) and interleukin-10 (IL-10), of whole blood cells from opium addicts, heroin addicts and healthy control groups, stimulated *in vitro* with lipopolysaccharide (LPS)/phytohemagglutinin (PHA). Cytokine levels are given in pg/mL. Data are expressed as mean \pm SD

Groups	Cytokines	Cytokine production stimulated by LPS	Cytokine production stimulated by PHA
Opium addicts	IFN- γ	67.6 \pm 24.5	162.8 \pm 45.6
	IL-10	36.1 \pm 13.1	28.6 \pm 14.5
Heroin addicts	IFN- γ	35.9 \pm 26.3*	76.3 \pm 40.9*
	IL-10	88.3 \pm 30.4*	71.8 \pm 28.4*
Healthy controls	IFN- γ	110.2 \pm 60.3	213.3 \pm 61.6
	IL-10	26.1 \pm 12.8	17.1 \pm 13.5

* p < 0.01 compared with control group.

DISCUSSION

In experimental studies involving the effects of morphine (the most abundant opioid molecules in opium extracts) on the cells of the immune system, the literature points to the conclusion that morphine given *in vivo* modulates a variety of immune responses (including T- and B-cell function, macrophages, natural killer cell and polymorphonuclear leukocyte function) [15, 16]. This assumption is supported by numerous studies in humans which show that morphine or heroin (a morphine derivative) inhibits proliferation of T- and B-cells, suppresses T cell-mediated cytotoxicity and decreases the activity of NK cells *in vitro* [10, 17]. Experimental studies have demonstrated immunosuppressive effects of morphine and heroin. However, controversial results have been reported in clinical studies. We thus decided to evaluate the *in vitro* cytokine production of cells in whole blood from a group of opium addicts and a group of heroin addicts.

In our study, the group of opium addicts showed higher production of IL-10 and lower production of IFN- γ compared to the healthy control group (table 2, figure 1), but this difference was not significant (p > 0.05). Similar results have been reported by Peterson *et al.*, who found intact production of IFN- γ by concavanalin A- (con-A)

stimulated peripheral blood from patients maintained on methadone [18]. However, we found comparable production of IL-10 by cells of whole blood from opium addicts. In addition, a previous study by Brugo *et al.*, showed no evidence of *in vitro* suppression after con-A stimulation of peripheral blood from heroin addicts or patients maintained on methadone [19]. Another study by Shine *et al.* indicated a normal pattern of T-cell subsets in a group of healthy, intravenous drug abusers and patients maintained on methadone [20]. Similarly, a normal T-cell response to both con-A and tetanus toxoid antigen in another group of healthy addicts has been reported [21].

We found that immune cells from heroin addicts presented a significantly enhanced production of IL-10 and lower production of IFN- γ (p < 0.01) after PHA or LPS stimulation in comparison with immune cells from a healthy control group (table 2, figure 2). This finding is supported by the result from Govitrapong *et al.* who demonstrated a decrease in the response of T-lymphocytes to a wide range of concentrations of PHA in heroin addicts [22]. This result is supported by experimental studies by Fecho *et al.* who showed that administration of heroin in rats **produced a dose-dependent suppression of, proliferation of T cells, production of interferon- γ , lipopolysaccharide-stimulated proliferation of B cells, in the spleen** [23]

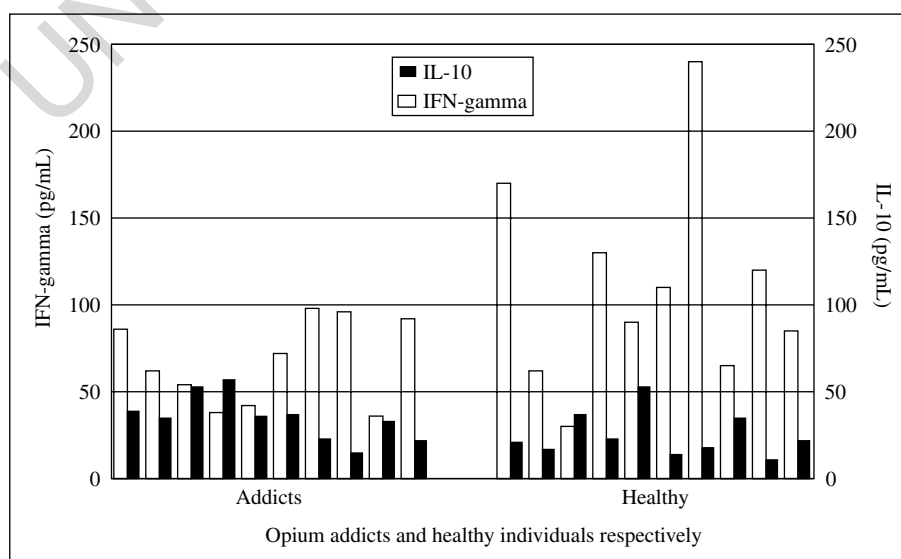


Figure 1

In vitro cytokine (interferon-gamma and interleukin-10) production of lipopolysaccharide-stimulated whole blood cells from opium addicts and healthy control groups, respectively.

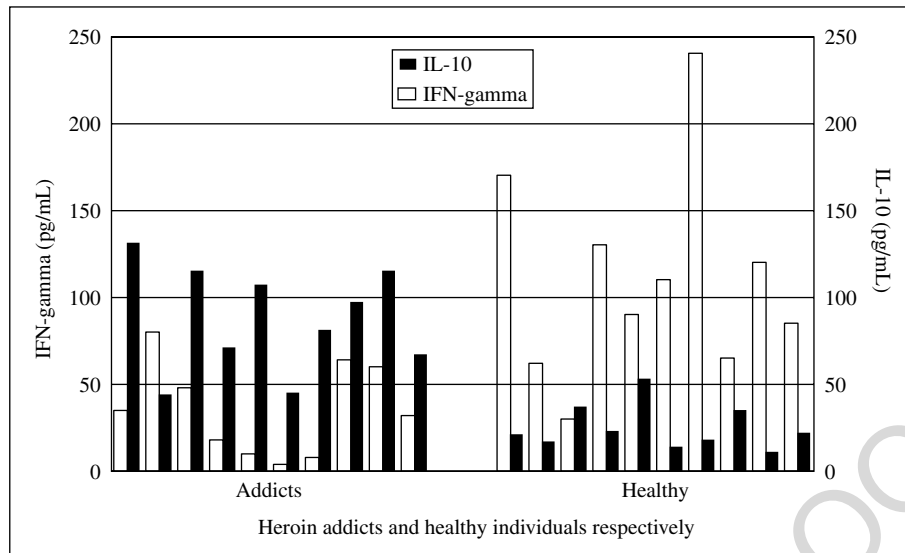


Figure 2

In vitro cytokine (interferon-gamma and interleukin-10) production of lipopolysaccharide-stimulated whole blood cells from heroin addicts and healthy control groups, respectively.

Please, could you check this sentence? Thanks. This has also been demonstrated by Roy and his colleagues, who investigated the effect of morphine treatment on Th1/Th2 cytokine production in an experimental study of an LPS-mediated sepsis model, which in animals treated with both LPS and morphine showed a significant shift towards a Th2 cytokine profile [24].

The nature of the immunomodulatory activity of the opioids has been the subject of a great deal of research over the last ten years. There is increasing evidence that effects of opioids on the immune response are mediated at several levels. Fecho and his colleagues have demonstrated that sympathoadrenal activity is involved in the suppressive effects of acute morphine treatment on the proliferative response of splenic T and B cells to Con A, LPS or ionomycin/phorbol myristate acetate [25]. Wang *et al.* who investigated the role of the mu-opioid receptor in chronic, morphine-mediated immunosuppression in mice,

showed that morphine-induced immunosuppression may be mediated directly by the mu-opioid receptor and they provide evidence that the inhibition of IFN- γ synthesis and activation of macrophage-cytokine synthesis is corticosterone-independent, and only partially dependent on sympathetic activation [26]. McCarthy and colleagues provide evidence of the impact of opioid treatment on antibody responses, and suggest that the molecular basis for this effect is likely due, at least in part, to the modulation of both cytokine and cytokine receptor expression [27]. Other authors have reported that opioid peptides, bound to various types of opioid receptors, detected on various cell types including blood mononuclear elements, which differentiate as macrophages in tissues, elicited a stimulatory effect [28]. Vallejo *et al.* believe that opiates behave like cytokines, modulating the immune response by interaction with their receptors in the central nervous system and in the periphery. So peripheral immunosup-

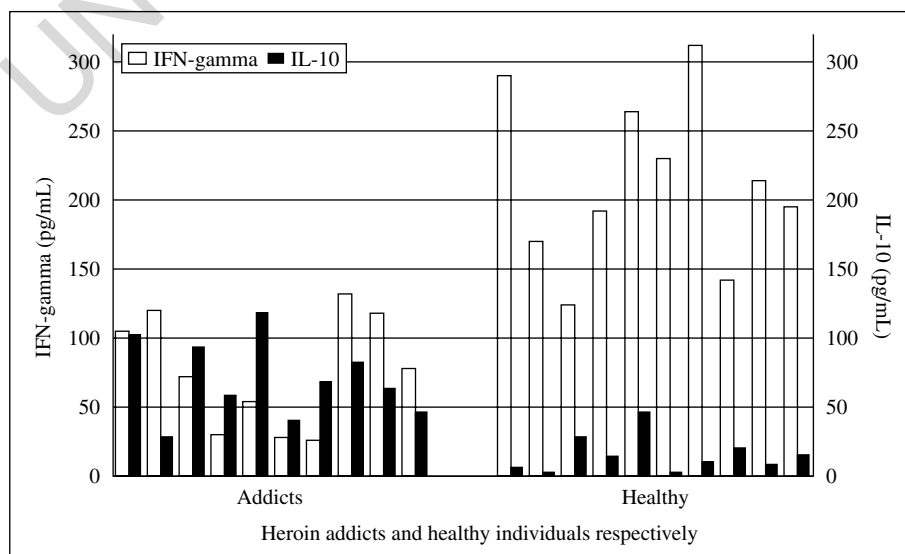


Figure 3

In vitro cytokine (interferon-gamma and interleukin-10) production of phytohemagglutinin-stimulated whole blood cells from heroin addicts and healthy control groups, respectively.

pression is mediated, at least in part, by opioid receptors located in the central nervous system, and intrathecally-administered opioids do not exert the same immunosuppressive effects. This may have important clinical implications for those patients receiving long-term opioid therapy for malignant and nonmalignant pain [29].

In conclusion, the results from our study indicate that opioid addiction could cause an *in vitro* shift in the Th1/Th2 cytokine balance of peripheral CD4+ cells towards a Th2 response, and that opioid addicts demonstrate reduced mitogenic responsiveness of lymphocytes compared to healthy individuals.

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