Short Communication

The Alterations of Immunological Reactivity in Heroin Addicts and Their Normalization in Patients Maintained on Methadone

(heroin / methadone / peripheral blood leukocytes / proliferation / cytokine production)

A. ZAJÍCOVÁ[1, 2], H. WILCZEK[3], V. HOLÁŇ[1, 2]

[1]Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic

[2]Faculty of Science, Charles University, Prague, Czech Republic

[3]3rd Medical Clinic, 1st Faculty of Medicine, Charles University, Prague, Czech Republic

Abstract. Drug addiction influences many physiological functions including reactions of the immune system. The higher occurence of infectious and other diseases in drug addicts has been explained by the depression of immunity due to the harmful effects of the drug. To test this assumption, we tested the proliferative responsiveness and cytokine production of PBL from a group of heroin addicts (N = 19), patients maintained on methadone (N = 15) and healthy controls (N=15). The results show that Con A-induced proliferation of PBL from heroin addicts was even enhanced in comparison with PBL from the control group. Similarly, production of IL-2, IL-10 and IFNγ was higher in the group of heroin addicts than in healthy controls. The enhanced proliferation of PBL or the increased production of cytokines observed in heroin addicts was partially or completely normalized in the group of patients maintained on methadone. A significantly higher production of IL-6 was found in both unstimulated and stimulated PBL from heroin addicts and patients maintained on methadone, when compared with PBL from healthy controls. The results thus showed enhanced proliferative activity and increased production of various cytokines in heroin addicts and partial or complete adjustment of these alterations in patients maintained on methadone.

Received December 8, 2003. Accepted December 12, 2003.

This work was supported by grants NF/6824-3 and NJ/6632-3 from the Ministry of Health of the Czech Republic, and projects LN00A026 and MSM 113100003 from the Ministry of Education of the Czech Republic

Corresponding author: Alena Zajícová, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Flemingovo nám. 2, 166 37 Prague 6, Czech Republic. Tel: 420 220 183 460; Fax: 420 224 310 955; e-mail: zajicova@img.cas.cz

Abbreviations: Con A – concanavalin A, FCS – foetal calf serum, IFN – interferon, IL – interleukin, PBL – peripheral blood leukocytes, PBS – phosphate-buffered saline.

Opiates represent a group of drugs that influence different physiological functions in the body, including reactions of the immune system. The most common opiate, at present with several million addicts worldwide, is heroin (dimethylmorphine). Heroin can influence the immune system either directly by acting on the opioid receptor on the lymphocytes and macrophages (Stefano, 1996; Nelson et al., 2000) or indirectly through its effect on the nervous system (Peterson et al., 1998).

It has been extensively demonstrated that various immunological parameters, such as proliferation of T and B cells, cytotoxicity of T lymphocytes and activity of NK cells, are depressed in heroin (or morphine)-treated experimental animals or in heroin addicts (Bryant et al., 1987; Lysle et al., 1993; Eisenstein and Hilburger, 1998; Pacifici et al., 2000). As a consequence, an increased incidence of bacterial and viral infections in heroin addicts was reported (Louria et al., 1967) and confirmed by the enhanced susceptibility to oral bacterial infections in morphine-treated mice (McFarlane et al., 2000).

For the treatment of heroin dependence, a methadone substitutional pharmacotherapy (methadone maintenance treatement) has been introduced (McLachlan et al., 1993). Methadone is a synthetic opiate (methadonium chloratum), which is recently also used as an analgetic and antitussive (Bossong and Scheerer, 1989; Payte, 1991) and which has no adverse effects (withdrawal syndrome, tolerance) as in the case of heroin. A normalization of the majority of physiological functions has been observed after three months of methadone treatment, and after one year of the treatment, a significant stabilization of the functions and hypothalamic-pituitary axis was reported (Kreek and Koob, 1998).

Since there are only limited and highly variable reports concerning the impacts of heroin addiction on the functions of the immune system, we compared the proliferative activity and cytokine production of peripheral blood leukocytes (PBL) from the group of heroin addicts, patients maintained for more than three years on methadone and healthy controls. The results show that production of various cytokines is rather enhanced in heroin addicts and that these changes are partially or completely normalized in patients maintained on methadone.

Material and Methods

Patients

A group of 19 heroin addicts (15 male, 4 female, average age of 26.9 years) was tested. In addition, a group of 15 patients (6 male, 9 female, average age of 29 years) maintained for more than three years on methadone as a part of the Czech Methadone Programme was analysed. The group of 15 healthy blood donors (3 male, 12 female, average age of 28.3 years) served as controls.

Separation of leukocytes

The leukocytes from peripheral blood were separated by the protocol of Pharmacia Biotech (Pharmacia Biotech, Uppsala, Sweden). Three or 4 ml of blood diluted 1 : 1 with phosphate-buffered saline (PBS) containing 2% of heat-inactivated foetal calf serum (FCS, Sigma, St. Louis, MO) were layered on the top of Ficoll-Paque (Pharmacia Biotech) and centrifuged for 20 min at 1200 *g* at room temperature. The separated leukocytes were collected and washed twice in PBS containing 2% of FCS. The leukocytes were diluted in RPMI 1640 medium (Sigma) containing 10% FCS, antibiotics (100 U/ml of penicillin, 100 μg/ml of streptomycin), 10 mM HEPES buffer and 5×10^{-5} M 2-mercaptoethanol (hereafter referred to as complete RPMI 1640 medium).

Proliferation assay

The leukocytes were diluted in complete RPMI 1640 medium and cultivated at a concentration 0.5×10^6 cells/ml in a volume of 200 μl of the culture medium in 96-well tissue culture plates (Nunclon, Roskilde, Denmark) alone or in the presence of concanavalin A (Con A) (1.5 μg/ml, Sigma) for 72 h in 37°C. Cell proliferation was determined by adding 0.5 μCi of ^{3}H–thymidine/well (Institute for Research, Production and Application of Radioisotopes, Řež, Czech Republic) for the last 6 h of the 72-h incubation period.

Cytokine production and detection

The leukocytes in complete RPMI 1640 medium were cultivated at a concentration 0.5×10^6 cells/ml in a volume of 700 μl of the culture medium alone or in the presence of Con A (1.5 μg/ml) in 24-well plates (Nunclon). The supernatants were collected after a 24-h (interleukin (IL)-2 determination), 48-h (interferon (IFN)γ) and 72-h (IL-4, IL-6, IL-10) incubation period.

The presence of cytokines in the supernatans was detected by ELISA using Eli-pair kits purchased from Diaclone Research (Diaclone Research, Besancon, France). High-binding 96-well plates (Costar Corning, New York, NY) were coated with capture anti-cytokine antibodies (100 μl/well) diluted with PBS and the plates were incubated overnight at 4°C. The plates were washed twice with PBS containing 0.5% of Tween 20 (Sigma) and blocked for 2 h with saturation buffer at room temperature. The plates were dried and left for 24 h. The cytokine standards of known concentration and the tested samples were dispensed (100 μl/well) with detection biotinylated antibodies (50 μl/well) and incubated 1–3 h (respective to the type of the cytokine) at room temperature. The plates were then washed three times with wash buffer, and streptavidin-horseradish peroxidase (SAv-HRP) diluted with buffer (PBS containing 0.1% of Tween 20 and 1% of bovine serum albumin) was added (100 μl) into each well. The plates were incubated 20 min at room temperature and washed three times. The TMB substrate (100 μl/well) was added into each well and the plates were left to develop 10–15 min in the dark. The reaction was stopped by adding 100 μl of 1 M H_2SO_4 and measured by an ELISA reader (Sunrise, Tecan Group Ltd., Maennedorf, Switzerland, read absorbance at 450 nm with a reference filter 630 nm).

Statistics

The results are expresed as mean ± standard error (S.E.). The statistical significance of differences between the means of individual groups was calculated using Student's t-test.

Results

Proliferative activity of PBL from heroin addicts and patients maintained on methadone

PBL from heroin addicts, patients maintained on methadone or healthy controls were cultivated unstimulated or were stimulated with Con A. As demonstrated in Fig. 1, Con A-stimulated PBL from heroin addicts had higher proliferative activity than PBL from healthy controls. Proliferation of PBL from patients maintained on methadone reached values between those for heroin addicts and healthy controls (Fig. 1).

Cytokine production by PBL from heroin addicts and patients maintained on methadone

The leukocytes from the tested donors were cultivated alone or in the presence of Con A and production of the cytokines IL-2, IFNγ, IL-4, IL-6 and IL-10 was determined by ELISA.

The production of IL-2 (Fig. 2A) and IFNγ (Fig. 2B) was higher in the group of heroin addicts than in the control group or in the patients maintained on methadone. On the contrary, the level of IL-4 was low-

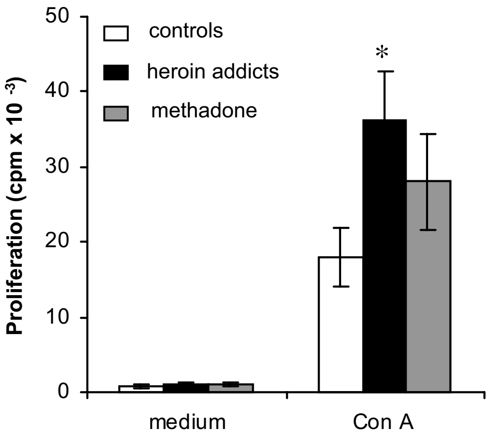

Fig. 1. Proliferation of PBL from the group of heroin addicts (N = 19), patients maintained on methadone (N = 15) and healthy controls (N = 15). Leukocytes were cultivated only in medium or in the presence of Con A (1.5 μg/ml). The proliferation was determined by incorporation of ^{3}H-thymidine.

est in the group of heroin addicts (Fig. 2C). The production of IL-10 was highest in heroin addicts and there was also a significant spontaneous production of IL-10 by PBL from heroin addicts (Fig. 2D). No significant difference in the production of IL-6 was found between heroin addicts and patients maintained on methadone after Con A stimulation (Fig. 2E). There was also a significantly higher spontaneous production of IL-6 in PBL from the groups of heroin addicts and patients maintained on methadone than in the group of control PBL donors (Fig. 2E).

Discussion

The increased incidence of various infections (for example: hepatitis B or C virus) and other diseases in heroin addicts has led to the idea that heroin administration could suppress the protective immune reactions (Louria et al. 1967, Govitrapong et al., 1998). This assumption was supported by numerous observations that heroin (or morphine) inhibits proliferation of T and B lymphocytes, suppresses T cell-mediated cytotoxicity and decreases the activity of NK cells *in vitro* (Bayer et al., 1992; Lysle et al., 1993; Pacifici et al., 2000). McFarlane et al. (2000) showed that morphine treatment reduced resistance to oral *Salmonella typhimurium* infection in mice.

Recent studies in experimental models suggested that some parameters of the immune system are rather augmented after heroin (or morphine) administration. It has been demonstrated in mice that production of some cytokines is increased within few minutes after morphine administration (Pacifici et al., 2000) and this increase is more pronounced among the proinflammatory cytokines (Peng et al., 2000). The findings of augmented production of proinflammatory cytokines are supported by the observation of an increased resistance to tumour growth in mice treated with heroin (Zagon and McLoughlin, 1981) or by stronger allotransplantation reactions described in heroin-treated mice (Holáň et al., 2003). In addition, Nelson and Lysle (2001) showed that systemic morphine administration prior to elicitation of the *in vivo* contact hypersensitivity response produced an extreme increase in inflammation at the site of antigen response.

Since experimental models demonstrated immunomodulatory effects of heroin (or morphine) on cytokine production and limited clinical studies yielded variable results, we tested the proliferative responsiveness and cytokine production of PBL from a group of heroin addicts and from patients maintained for more than three years on methadone.

We found that PBL from heroin addicts had a significantly enhanced proliferative response after Con A stimulation in comparison with PBL from control donors. This finding is different from the observations in experimental models, where acute or short-term treatments with morphine resulted in a depression of T-cell proliferation (Bryant et al., 1987; Bayer et al., 1992; Fecho et al., 1996). Our results are rather supported by the observation of Brugo et al. (1983), who did not find any evidence for suppression of proliferative reactions after Con A stimulation of PBL from heroin addicts and patients maintained on methadone.

A comparable production of IL-4 was found among the groups of controls, heroin addicts and patients maintained on methadone. On the contrary, the production of IL-2, IFNγ and IL-10 was highest in heroin addicts and this increased production was normalized in patients maintained on methadone. Similar effects of methadone on the immune system were observed by Peterson et al. (1998), who found intact production of IFNγ by Con A-stimulated PBL from patients maintained on methadone. However, we found enhanced and comparable production of IL-6 by PBL from heroin addicts and patients maintained on methadone. An augmented production of IL-6 after treatment with morphine was recently described in various experimental models (Roy et al., 1998; Zubelewicz et al., 1999), in heroin addicts (Peng et al., 1999) and in patients treated with morphine as a postoperative pain management (Kim and Hahm, 2001; Beilin et al., 2003). It has also been shown that the enhanced production of IL-6 after morphine administration is dependent on the activity of the hypothalamic-pituitary-adrenal axis and that the increased production of IL-6 can be blocked by adrenalectomy (Houghtling et al., 2000). On the other hand, adrenalectomy did not abolish the suppressive effects of morphine on the activity of the immune system (Houghtling et al., 2000). It appears that the stimulatory effects of opiates on IL-6 production are mediated through the effects of the drugs on the neuroendocrine system rather than by a direct action of the drug on the cells of the immune system.

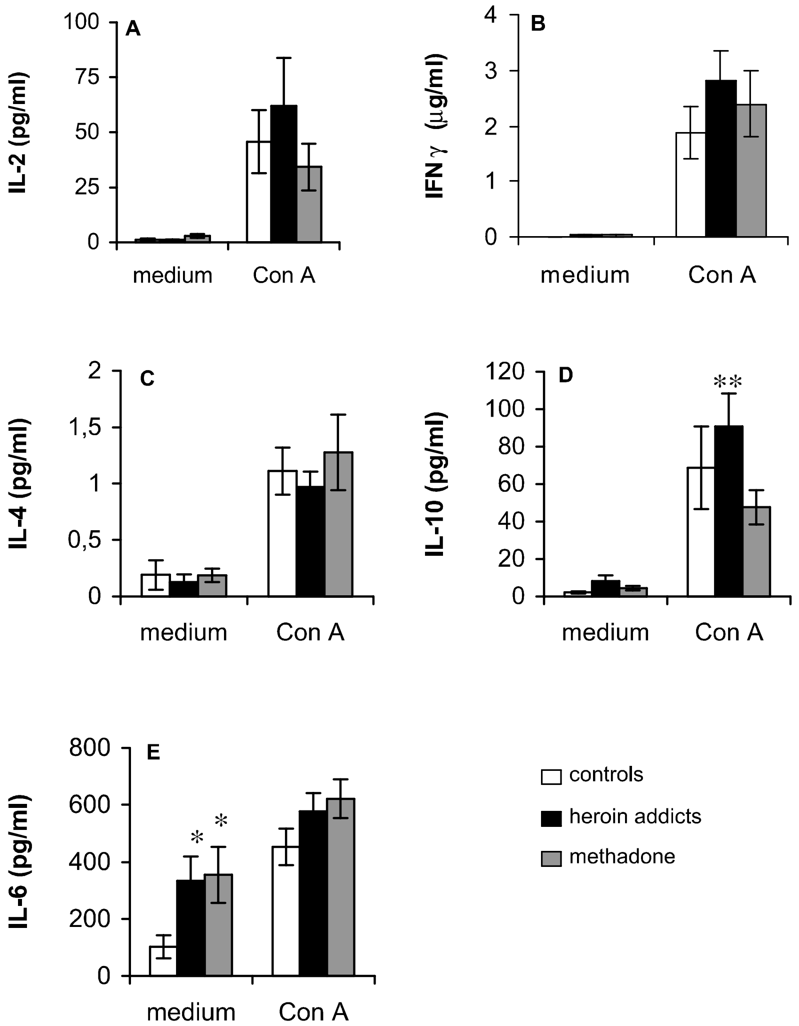

Fig. 2. Production of the cytokines IL-2 (A), IFNγ (B), IL-4 (C), IL-10 (D) and IL-6 (E) by PBL from heroin addicts (N = 19), patients maintained on methadone (N = 15) and healthy controls (N = 15). PBL were cultivated unstimulated or in the presence of Con A (1.5 µg/ml). The production of cytokines was measured by ELISA in the supernatants collected after a 24-h (IL-2 detection), 48-h (IFNγ) or 72-h (IL-4, IL-6 and IL-10) incubation period. *Statistically significant ($P < 0.05$) difference from the control group, **significantly ($P < 0.05$) different from the group of patients maintained on methadone.

The group of heroin addicts had apparently higher production of IL-10 than was observed in healthy controls or in patients maintained on methadone. Even spontaneous production of IL-10 by unstimulated PBL was higher in the group of heroin addicts. IL-10 is known to have inhibition effects on the immune system and it can be considered that the increased production of IL-10 might be at least partially responsible for the decrease of immunity observed in some models after heroin administration. We showed that the changes of immunity seen in the group of heroin addicts are partially normalized in patients maintained on methadone. Similar results were also reported by Novick et al. (1989), who studied the activity of NK cells and the composition of cell populations. These authors described a normalization of immune functions in patients maintained on methadone when compared with heroin addicts.

In summary, the results showed that administration of heroin influences the functions of the immune system and that individual parameters of the immune response, in dependence on the dose of the drug, timing and the way of administration, may be increased or decreased. We suggest that in dependence on the role of these parameters in the immune response, the immune system can demonstrate augmented or decreased resistance to the infectious agent or a variable reponsiveness to the antigen.

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