

Alterations of Immune Functions in Heroin Addicts and Heroin Withdrawal Subjects¹

PIYARAT GOVITRAPONG, TUNDA SUTTITUM, NAIPHINICH KOTCHABHAKDI and THONGCHAI UNEKLABH

Neuro-Behavioural Biology Center (P.G., T.S., N.K.), Institute of Science and Technology for Research and Development, Mahidol University at Salaya, Nakornpathom 73170, Thailand and Thanyarak Hospital (T.U.), Department of Medical Services, Ministry of Public Health, Thunayaburi, Pathumthani 12130, Thailand

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ABSTRACT

Conflicting results, both decreased and increased, have been reported concerning the function of T-lymphocytes in heroin addicts. We investigated the alterations of T-lymphocyte proliferative responses and immunophenotypic markers on lymphoid cells in heroin addicts and during different periods of heroin withdrawal in addicted subjects. This study has demonstrated a decrease in the response of T-lymphocytes to 1.2, 2.5, 5 and 10 $\mu\text{g/ml}$ of phytohemagglutinin stimuli in heroin addicts and 1- to 5-day heroin withdrawal subjects compared with controls. Similarly, in an *in vitro* study, 10^{-4} , 10^{-6} and 10^{-8} M concentrations of morphine were shown to suppress 0.6 and 2.5 $\mu\text{g/ml}$ of PHA-stimulated T-lymphocyte obtained from naive subjects. This inhibitory effect of morphine on PHA stimulation was completely abolished by 100 μM naloxone. The immunological parameters of total T-lymphocytes (CD3), T-

helper cells (CD4), cytotoxic T-cells (CD8), B-cells and natural killer cells that are the immunophenotypic markers studied by flow cytometric analysis were altered in heroin addicts, 15- to 21-day and 6- to 24-month heroin withdrawal subjects, when compared with controls. These results suggest that heroin addicts and short period (15 to 21 days and 6 to 24 months) of heroin withdrawal have decreases in their immune system functioning and that the heroin withdrawal subjects seem to gradually reverse their immunological parameters to normal levels when withdrawal was sustained ≥ 2 years. This is the first report examining immune function in heroin withdrawal subjects using the "cold turkey" method. The results are beneficial for further study of the mechanism responsible for the opioid-induced changes in immune function.

Because of the AIDS epidemic, interest in studying the effects of drugs of abuse, especially opiates, on the immune system has increased greatly. This issue is now of paramount importance because of the association of AIDS with intravenous drug abuse. Heroin abusers are a high-risk group for the development of AIDS and HIV infection. Intravenous drug abusers account for 7.3% of the total AIDS cases in Thailand (Wongkhomthong *et al.*, 1995). Indeed, the HIV-seropositivity rate among intravenous drug abusers can be even greater, ranging from 5% in certain areas to 75% in others (Curran *et al.*, 1984), and this group is generally regarded as posing the most substantial risk of furthering spread of the disease. Even in the absence of AIDS, increasing evidence indicates that chronic use of opioid drugs can affect the functioning of the immune system. Heroin addicts have an increased susceptibility to a variety of infectious diseases (Louria *et al.*, 1967), and alterations in a wide vari-

ety of immune parameters also have been reported among them (for reviews, see Rouveix, 1992; Sibinga and Goldstein, 1988; Carr *et al.*, 1996).

A variety of changes in the immune system have been observed, indicative of both decreased and increased functioning in heroin addicts. The absolute number and percentage of total and active T lymphocytes in the peripheral blood of opiate addicts and T-cell rosette formation were significantly depressed (McDonough *et al.*, 1980). In contrast, an increase in the absolute number of T-cells in the blood of heroin addicts who were not malnourished was reported in another study (Heathcote *et al.*, 1981). Similar conflicting results have been reported concerning the functional activity of T lymphocytes from heroin addicts. Brown *et al.* (1974) found impaired *in vitro* responsiveness of lymphocytes to each of the three mitogens (PHA, concanavalin A, pokeweed mitogen) in heroin addicts relative to control values. Similarly, a suppressed PHA response in methadone patients was reported (Quagliata *et al.*, 1977); but Reddy *et al.* (1987) found normal T-proliferative responses to both concanavalin

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ABBREVIATIONS: PHA, phytohemagglutinin; NK, natural killer; HIV, human immunodeficiency virus; AIDS, acquired immune deficiency syndrome; HPA, hypothalamus-pituitary-adrenal; IL-1, interleukin-1; RPMI, Roswell Park Memorial Institute; DAMGO, [D-Ala^2 , MePhe 4 , Gly-ol 5]enkephalin; HBsAg, hepatitis B antigens.

A and tetanus toxoid antigen in another group of healthy addicts.

Immunophenotypic markers on lymphoid cells in human addicts have been studied using flow cytometric analysis. There was a profound decrease in the T-helper/cytotoxic T-cell (CD4/CD8) ratio in heroin addicts (Donahoe *et al.*, 1987). Shine *et al.* (1987) found a normal pattern of T-cell subsets and a normal CD4/CD8 ratio in another group of healthy intravenous drug abusers and methadone patients. Most of the data have suggested that opiates are involved in the cell-mediated immune responses in heroin addicts. However, data of immune responses in heroin-withdrawal subjects during various withdrawal periods are not available at this moment. Thus, the evidence for the immunomodulatory, and even the immunocompromising potential of opioids is compelling. Still, our understanding of the effects of opioids on the immune system is incomplete. Controversial results, in part, may be due to the specific mechanisms responsible for morphine-induced changes in the immune system being undefined. In addition, most of the heroin addicts are polydrug users. It is necessary to better understand the way in which heroin modulates immune responses, as well as the relationship between these effects, and to investigate whether this results in increased susceptibility to infections. We focused our attention on studies of the heroin-addicted and heroin withdrawal subjects by considering the lymphocyte proliferative responses, the expression of total T lymphocytes (CD3), T-helper cells (CD4), cytotoxic T-cell (CD8) antigenic markers of T-cells, B cells and NK cells. We conducted studies in humans because humans are significantly different from even the closest primates, and certainly profoundly different from rodents, with respect to certain specific immune indices and the pharmacokinetics of many drugs, as well as neuroendocrine functions. This is the first study of the immune function in heroin withdrawal subjects who used the "cold turkey" method, with no supplement of methadone or other drugs in the withdrawal period.

Methods

Subject selection. Subjects consisted of parenteral heroin abusers and heroin withdrawal subjects from Thanyarak Hospital, which is one of the biggest hospitals in Thailand, with ~100 to 200 cases of outpatient drug abusers daily. All subjects were men, aged 20 to 40 years, and none had recent infections, active inflammatory disease or a positive HIV or HBsAg test. They were free of drugs affecting the immune system and had no history of neuropsychiatric disorders. Subjects participating in this study gave written informed consent. All of the parenteral heroin abusers were selected from those who had a history of intravenous injection of heroin for ≥ 1 year, with a daily heroin dosage of not less than 600 mg. They absolutely did not abuse other drugs. The heroin withdrawal subjects used the "cold turkey" method for withdrawal from heroin, after which they experienced "craving" with some withdrawal symptoms, such as rhinorrhea, lacrimation, piloerection, restlessness, irritability, insomnia, abdominal cramps, muscle "bone" aches and so on. They were free of drugs for suppressing the withdrawal symptoms.

Serum and urine analyses. Blood and urine from volunteers in all groups of this study were obtained between 9:00 to 11:30 a.m. Blood was collected into polyethylene tubes containing 1000 U/ml heparin. Routine medical and immunological histories were obtained from all subjects. Routine urine analysis was performed, emphasizing the measurement of type and quantity of substances abused. Urine morphine was reported positive by a cutoff limit at >5.50

$\mu\text{g/ml}$. Sera from all subjects were tested for HIV antibodies using a particle-agglutination test for screening of antibodies to HIV (Serodia-HIV, Tokyo, Japan) at Thanyarak hospital and double-checked by using MICRO RED kit (BioRad, Hercules, CA) for screening of HIV-1/HIV-2 antibodies in our laboratory. Blood chemistry studies to assess hepatic function were determined in all subjects.

Lymphocyte cultures. Freshly drawn venous blood (10 ml) from subjects was diluted 1:1 with Hanks' balanced salt solution. The cell suspension (20 ml) was layered on 4 ml of Ficoll-paque solution (Pharmacia, Uppsala, Sweden) (specific gravity, 1.083) for separation of lymphocytes according to the method of Sacerdote *et al.* (1991). After centrifugation at $600 \times g$ for 20 min, the layer containing lymphocytes was transferred to another centrifuge tube, washed twice in 10 ml of Hanks' balanced salt solution and then centrifuged at $1200 \times g$ for 10 min. The cells were resuspended again with RPMI media containing 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin, 100 mg/ml streptomycin, 1% L-glutamine (Sigma Chemical, St. Louis, MO) and 1 mM HEPES (Sigma). Then, the suspension was centrifuged at $1200 \times g$ for 10 min. The pellets were lymphocytes that were brought to culture for studying proliferation.

Lymphocyte cells were counted with the viable dye, 0.1% trypan blue (Sigma) and adjusted to a final concentration of 1×10^6 cells/ml in RPMI media. Triplicate cultures containing 2×10^5 cells in 200 μl of RPMI media/well were seeded onto 96-microwell plates and treated with a mitogen, PHA (Seromed, Berlin, Germany), in concentrations of 1.2, 2.5, 5 and 10 $\mu\text{g/ml}$. Lymphocyte cultures were incubated for 48 hr at 37°C in 5% CO_2 and aired in a humidified incubator. After incubation, cells were pulsed for an additional 18 hr with 1 μCi of [^3H]thymidine (2.0 Ci/mmol) (New England Nuclear, Boston, MA). Cell proliferation was determined by the incorporation of [^3H]thymidine into cellular DNA. A semiautomated cell harvesting apparatus (Nunc, Naperville, CT) was then used to lyse the cells with distilled water and precipitate the labeled DNA on glass filter paper (Whatman, Clifton, NJ). The filter pads were dried, 1 ml of scintillation fluid was added and then the radioactive material trapped on filter paper was counted as cpm by a Beckman Instruments (Columbia, MD) LS5000CE Scintillation Spectrometer. The counting efficiency for tritium was $\sim 45\%$.

Cell preparation for immunofluorescent staining in flow cytometric studies. Peripheral blood samples were collected into EDTA anticoagulant tubes and processed within 2 hr of collection. All samples were prepared using lysed whole blood and stained with the following panel of two-color antibody conjugates: LeucoGATE (CD45FITC/CD14PE) [CD45/CD14], isotype control (IgG₁FITC/IgG₁PE)[γ_1/γ_2], CD3FITC/CD16+56PE(Leu4/11C+19), CD3FITC/CD19PE(Leu-4/12), CD3FITC/CD8PE(CD3/CD8)[Leu-4/2] and CD3FITC/CD4PE (CD3/CD4) [Leu-4/3]. After a 15-min room temperature incubation period, the red blood cells were lysed with FACS-Lysing Solution (Becton Dickinson, San Jose, CA). Samples were then fixed in a 1.0% paraformaldehyde solution before flow cytometric analysis.

Flow cytometric analysis. Flow cytometric studies were performed on a FACScan Analyzer (Becton Dickinson, San Jose, CA). The FACScan used an air-cooled argon ion laser with emission at 488 nm. The FACScan was calibrated using an automatic software, AutoCOMP and CaliBRITE beads. SimulSET software was used for data acquisition and analysis, and LeucoGATE defined the lymphocyte gate, which included $\geq 95\%$ of the total lymphocyte population. Isotype controls defined autofluorescence and positioned the quadrant markers to calculate the percent positive cells for a given antibody.

Statistical analysis. Results were expressed as mean \pm S.E.M. Student's *t* tests were performed on continuous variables. For studies involving multiple comparisons, data were analyzed by Tukey-Kramer multiple comparison test.

Results

PHA-stimulated T lymphocyte proliferation in heroin-addicted and heroin withdrawal subjects. The base-line characteristics of all subjects; normal (N; $n = 17$), heroin addicts (H; $n = 19$) and heroin withdrawal subjects (HW; $n = 17$) in the T-lymphocyte proliferation study are shown in table 1. The ages of the subjects ranged from 22 to 28 years. The dosage range of heroin that was used by heroin addicts and heroin withdrawal subjects was 600 to 1200 mg/day. The durations of heroin abuse were 2.95 ± 0.53 and 1.36 ± 0.18 years in heroin addicts and heroin withdrawal groups, respectively. Urine morphine of $>5.50 \mu\text{g/ml}$ was detected in the heroin-addicted group. The duration of withdrawal periods ranged from 1 to 5 days. They all were negative in HIV and HBsAg tests.

T-lymphocyte function was determined by the incorporation of [^3H]thymidine into the intracellular DNA of the cells stimulated by PHA, a specific T-cell mitogen. The percentage of T-lymphocyte proliferation compared with those of B-lymphocyte was $>99\%$. T-lymphocytes obtained from normal, heroin-addicted and heroin withdrawal subjects were proliferated as a dose-dependent manner in the presence of various concentrations (1.2, 2.5, 5 and 10 $\mu\text{g/ml}$) of PHA (fig. 1). The data represent the percentage of the control (unstimulated cells of each group expressed as 100%). The proliferative responses of the cells from heroin addicts in the presence of 1.2, 2.5, 5 and 10 $\mu\text{g/ml}$ PHA in culture were suppressed significantly compared with normal subjects. In the heroin withdrawal (HW) group, significant immunosuppression ($P < .01$) was also found in cells stimulated by every concentration of PHA. However, the [^3H]thymidine incorporation into DNA of the cells obtained from heroin withdrawal subjects was significantly increased ($P < .01$) compared with those from the heroin-addicted (H) group. To determine the possibly direct effect of morphine on lymphocyte function, various concentrations of morphine were included in the T-lymphocyte cultured cells from normal subjects. Morphine concentrations ranging from 0.01 to 100 μM induced a significant reduction ($P < .05$ and $P < .01$) in lymphocyte [^3H]thymidine incorporation in cultures stimulated with PHA 0.6 (fig. 2A) and 2.5 (fig. 2B) $\mu\text{g/ml}$, respectively.

To determine whether the inhibition of T-lymphocyte proliferation by morphine was abolished by naloxone, an opioid receptor antagonist, cells were cultured with 0.01, 1 and 100 μM morphine in the presence of 100 μM naloxone. The morphine-induced inhibition on 2.5 $\mu\text{g/ml}$ PHA-stimulated T-

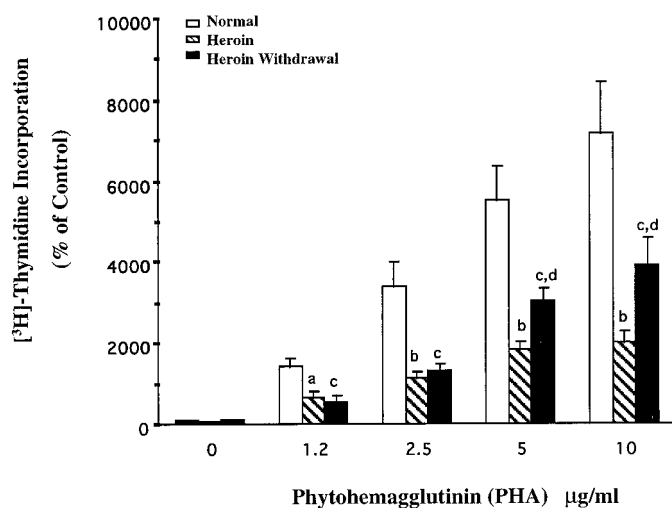


Fig. 1. Effect of 1.2, 2.5, 5 and 10 $\mu\text{g/ml}$ of PHA on proliferation of T-lymphocytes in normal ($n = 17$), heroin-addicted ($n = 19$) and heroin withdrawal subjects ($n = 17$). Cell proliferation of T-lymphocytes extracted from three different groups of subjects were assayed as described in Materials and Methods. T-lymphocyte proliferation responses were expressed as mean \pm S.E. of [^3H]thymidine incorporation into DNA compared with each unstimulated cell group (as percent control). ^a, Significant difference between heroin-addicted and normal subjects ($P < .05$). ^b, Significant difference between heroin-addicted and normal subjects ($P < .01$). ^c, Significant difference between heroin-withdrawal and normal subjects ($P < .01$). ^d, Significant difference between heroin-withdrawal and heroin-addicted subjects ($P < .01$).

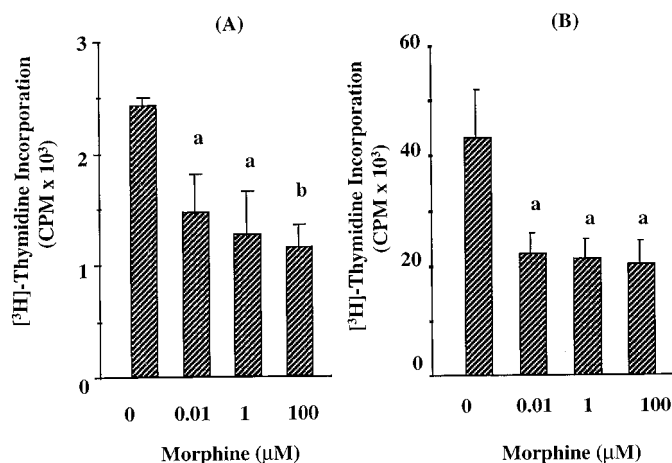


Fig. 2. Concentration-response effect of morphine on 0.6 (A) and 2.5 (B) $\mu\text{g/ml}$ PHA stimulated T-lymphocyte proliferation in normal subjects. Cell proliferation was assayed using 1×10^6 cells/ml of lymphocyte cells in 96-microwell plate containing varying concentrations of morphine (0.01–100 μM) and PHA as indicated in a final volume of 200 μl . After incubation (48 hr, 37°C 5% CO_2), all cells were pulsed for an additional 18 hr with 1 μCi [^3H]thymidine, and the radioactivity associated with the cells was determined. Data presented as mean \pm S.E. of triplicate determinations from seven normal subjects. ^a, Significant difference from without morphine group ($P < .05$). ^b, Significant difference from without morphine group ($P < .01$).

lymphocyte proliferation was totally reversed by 100 μM naloxone (fig. 3). A significant difference ($P < .05$) in T-lymphocyte proliferations was shown when compared between each concentration of morphine group, with and without naloxone, except those from the without-morphine-added group (fig. 3).

Comparison of lymphocyte subsets among normal, heroin-addicted and heroin withdrawal subjects. To further evaluate the immunosuppressive responses in heroin

TABLE 1

Base-line characteristics (mean \pm S.E.) of normal, heroin addicts and heroin withdrawal subjects in T-lymphocyte proliferation study.

	Normal	Heroin addicts	Heroin withdrawal
Sex	Male	Male	Male
Number of subjects	17	19	17
Age (yr)	28.24 ± 1.24	26.21 ± 0.94	21.29 ± 0.18
Urine morphine ($\mu\text{g/ml}$)	Negative	>5.50	Negative
Dosage of heroin abuse (mg/day)		600–1200	600–1200
Duration of heroin abuse (yr)		2.95 ± 0.53	1.36 ± 0.18
HIV test	Negative	Negative	Negative
HBsAg test	Negative	Negative	Negative
Withdrawal period			1–5 days

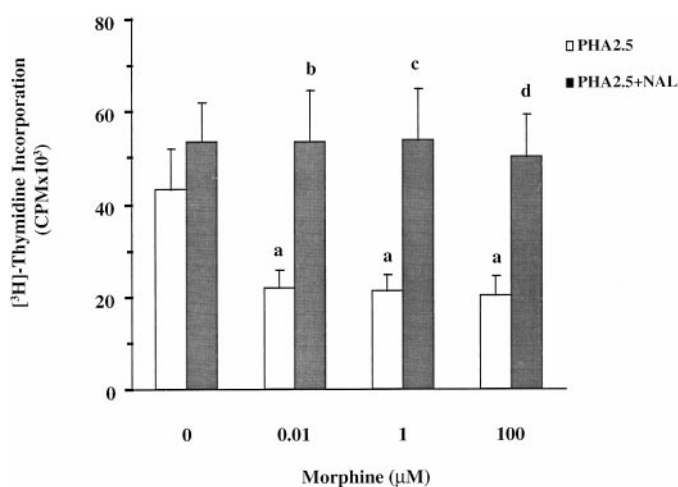


Fig. 3. Concentration-response effect of morphine on 2.5 µg/ml PHA-stimulated T-lymphocyte proliferation in normal subjects is shown in a black bar with naloxone and in a white bar without naloxone. Cell proliferation was assayed using 1×10^6 cells/ml of lymphocyte cells in 96 microwell plate containing naloxone (100 µM) 45 min before adding morphine (0.01–100 µM). After incubation (48 hr, 37°C, 5% CO₂), all cells were pulsed for an additional 18 hr with 1 µCi of [³H]thymidine, and the radioactivity associated with the cells was determined. Data presented as mean ± S.E. of triplicate determinations from 10 normal subjects. ^a, Significant difference from without morphine group ($P < .05$). ^b, Significant difference from 0.01 µM morphine without naloxone group ($P < .05$). ^c, Significant difference from 1 µM morphine without naloxone group ($P < .05$). ^d, Significant difference from 100 µM morphine without naloxone group ($P < .05$).

abusers and heroin withdrawal subjects during various periods of time, flow cytometric analysis was performed and subjects were selected accordingly. The base-line characteristics of all subjects: normal (N; $n = 17$), heroin addicts (H; $n = 15$), 15- to 21-day heroin withdrawal (HW1; $n = 15$), 6- to 24-month heroin withdrawal (HW2; $n = 16$) and 3- to 5-year heroin withdrawal (HW3; $n = 15$), are shown in table 2. The average age was between 21 to 31 years, with the same daily doses of heroin use (range, 600-1200 mg/day). The average durations of heroin abuse were 2.95 ± 0.53 , 1.90 ± 0.78 , 6.10 ± 1.22 and 6.00 ± 1.05 years in heroin addicts and in 15- to 21-day (HW1), 6- to 24-month (HW2) and 3- to 5-year (HW3) withdrawal groups, respectively. They all were free from HIV and HBs infections. The mean ± S.E.M. values of percent and absolute numbers of lymphocyte and total T-lymphocyte (CD3) obtained from various groups of subjects are shown in figure 4, A–D. There was no significant difference in the percentages of lymphocytes and total T-lymphocytes among different groups compared with controls. However, the absolute numbers of lymphocytes (fig. 4B) and total

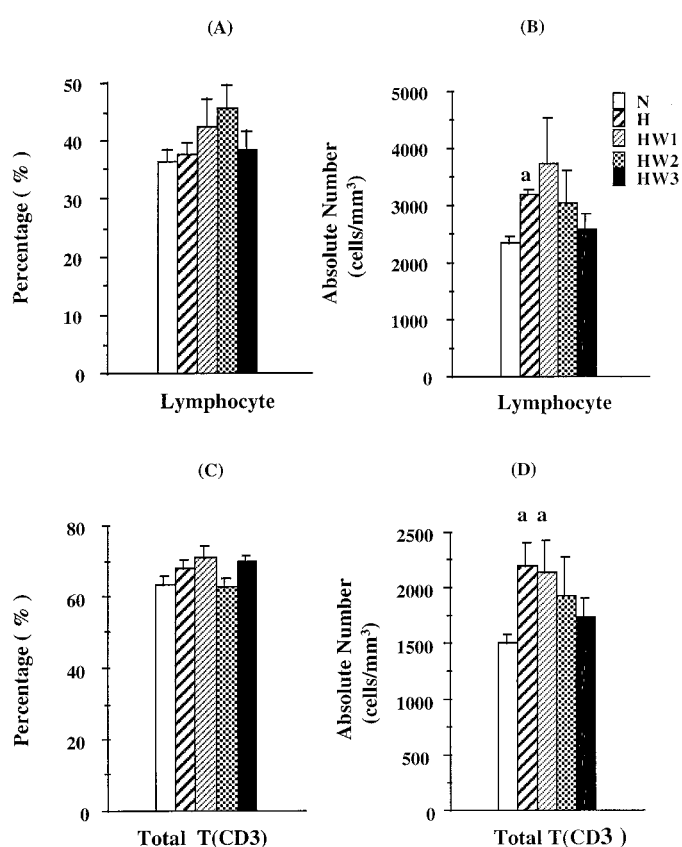


Fig. 4. Comparison of percentage of lymphocytes (A), absolute lymphocyte count (B), percentage of total T-lymphocytes [CD3] (C) and absolute CD3 count (D) among normal (N; $n = 17$), heroin addicts (H; $n = 15$), 15- to 21-day heroin withdrawal (HW1; $n = 15$), 6- to 24-month heroin withdrawal (HW2; $n = 16$) and 3- to 5-year heroin withdrawal (HW3; $n = 15$) subjects. The data were obtained by using a highly sensitive FACScan flow cytometer. Each group presented as mean ± S.E. ^a, Significant difference from normal group ($P < .05$).

T-lymphocytes (CD3) (fig. 4D) were both significantly increased in heroin addicts, gradually declining to normal levels as heroin withdrawal progressed. The percentages of T-helper cells (CD4) obtained from heroin addicts and short period heroin withdrawal groups were significantly decreased, reverting to normal levels when heroin withdrawal had continued for a longer period of time (6 months to 5 years) (fig. 5A). In contrast, the percentages of cytotoxic T-cell (CD8) obtained from heroin addicts and short period heroin withdrawal groups were significantly increased ($P < .001$), reverting to normal levels when heroin withdrawal had continued for a longer period of time (fig. 5C). The absolute

TABLE 2

Base-line characteristics (mean ± S.E.) of normal, heroin addicts and heroin withdrawal subjects in flow cytometric analysis

	Normal	Heroin addicts	Heroin withdrawal subjects		
			HW1	HW2	HW3
Number of subjects	17	15	15	16	15
Sex	Male	Male	Male	Male	Male
Age (yr)	28.53 ± 1.16	27.40 ± 1.78	21.2 ± 0.20	28.00 ± 1.93	29.80 ± 2.01
Urine morphine (µg/ml)	Negative	>5.50	Negative	Negative	Negative
Dosage of heroin abuse (mg/day)		600–1200	600–1200	600–1200	600–1200
Duration of heroin abuse (yr)		2.95 ± 0.53	1.90 ± 0.78	6.10 ± 1.22	6.00 ± 1.05
HIV test	Negative	Negative	Negative	Negative	Negative
HBsAg test	Negative	Negative	Negative	Negative	Negative
Withdrawal period			15–21 days	6–24 mo	3–5 yr

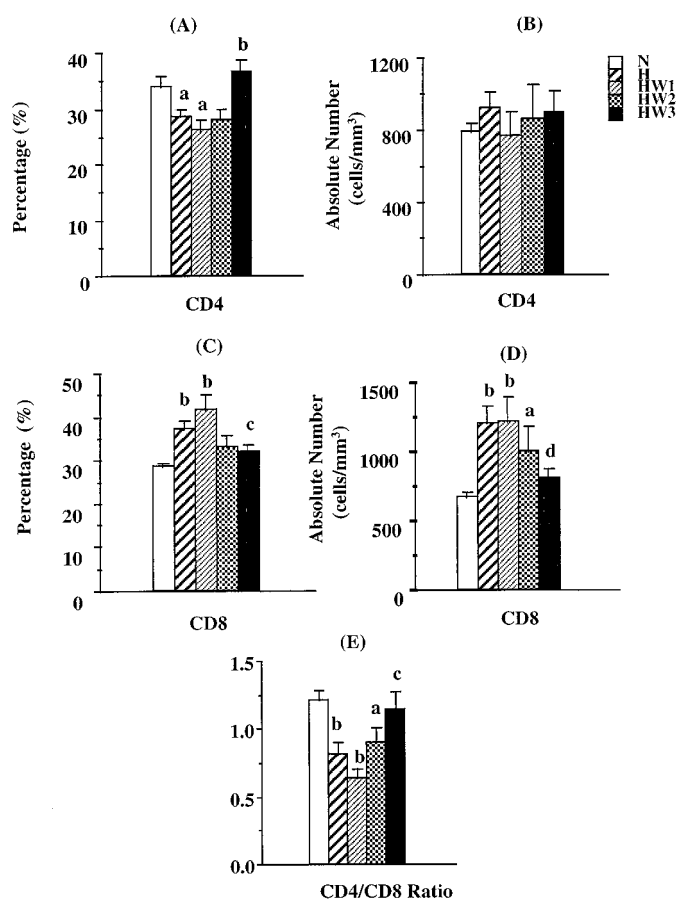


Fig. 5. Comparison of percentage of T-helper [CD4](A), absolute CD4 count (B), percentage of T-suppressor [CD8] (C), absolute CD8 count (D) and the T-helper/T-suppressor [CD4/CD8] ratio (E) among normal (N; $n = 17$), heroin addicts (H; $n = 15$), 15- to 21-day heroin withdrawal (HW1; $n = 15$), 6- to 24-month heroin withdrawal (HW2; $n = 16$) and 3- to 5-year heroin withdrawal (HW3; $n = 15$) subjects. The data were obtained by using a highly sensitive FACScan flow cytometer. Each group presented as mean \pm S.E. ^a, Significant difference from normal group ($P < .05$). ^b, Significant difference from normal group ($P < .01$). ^c, Highly significant difference from heroin-addicted group ($P < .001$). ^d, Significant difference from heroin-addicted group ($P < .01$).

numbers of CD8 in different groups of subjects were altered in the same manner as the percents of CD8 (fig. 5D), whereas the absolute numbers of CD4 in all cases did not show any significant difference among groups (fig. 5B). The ratios of T-helper/cytotoxic T-cell (CD4/CD8) were shown to be significantly decreased in heroin addicts ($P < .001$), in 15- to 21-day heroin withdrawal ($P < .001$) and in 6- to 24-month heroin withdrawal ($P < .05$) compared with normal subjects, whereas this ratio reversed to normal values in 3- to 5-year heroin withdrawal subjects (fig. 5E). Both the percentage (fig. 6A) and absolute numbers of B-cells (fig. 6B) obtained from heroin addicts and 15- to 21-day heroin withdrawal subjects were significantly increased ($P < .01$) compared with those obtained from normal subjects, whereas the numbers obtained from subjects during longer periods of heroin withdrawal were comparable to those of normal subjects. The percentage of NK cells (fig. 6C) obtained from heroin addicts and the 15- to 21-day heroin withdrawal group were significantly decreased ($P < .05$ and $P < .01$, respectively) compared with normal subjects, whereas the absolute numbers of NK cells (fig. 6D) from all groups of subjects were not signif-

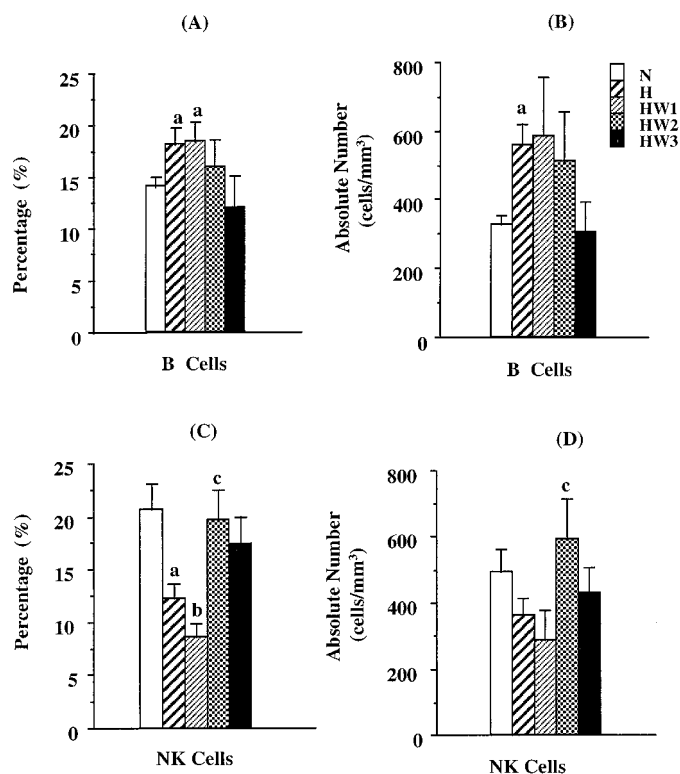


Fig. 6. Comparison of percentage of B-cell (A), NK cell (B), absolute B-cell count (C) and absolute NK-cell count (D) among normal (N; $n = 17$), heroin addicts (H; $n = 15$), 15- to 21-day heroin withdrawal (HW1; $n = 15$), 6- to 24-month heroin withdrawal (HW2; $n = 16$) and 3- to 5-year heroin withdrawal (HW3; $n = 15$) subjects. The data were obtained by using a highly sensitive FACScan flow cytometer. Each group presented as mean \pm S.E. ^a, Significant difference from normal group ($P < .05$). ^b, Significant difference from normal group ($P < .01$). ^c, Significant difference from heroin-addicted group ($P < .05$).

icantly different. However, the percentage (fig. 6C) and absolute number of NK cells (fig. 6D) in the 6- to 24-month heroin withdrawal group were increased significantly ($P < .05$) compared with heroin-addicted subjects.

Discussion

Alteration of immune function in heroin addicts. Opiates have been shown to produce effects on immune function *in vivo* (Weber and Pert, 1989), and clinical observations that opiate addicts have increased susceptibility to infections (Louria *et al.*, 1967) were subsequently shown to be related to deficits in immune function (Brown *et al.*, 1974). Our study has demonstrated a decrease in the response of T lymphocytes to a wide range of concentrations of mitogenic (PHA) stimuli in heroin-addicted subjects. This data supports the result from Roy *et al.* (1995), who demonstrated that chronic morphine treatment *in vivo* inhibited PHA-IL1-activated thymocyte proliferation. It has been proposed that the effects of opioids on lymphocyte proliferation may operate *via* a direct interaction with opioid receptors (Sibinga and Goldstein, 1988). To test the above hypothesis, the effect of morphine *in vitro* has therefore been studied. Morphine attenuated the mitogen-induced lymphocyte proliferation in a dose-dependent manner. Roy *et al.* (1991) showed a suppressive effect of chronic morphine treatment on macrophage colony formation in bone marrow. In addition, morphine inhibited

proliferation of PHA-IL-1 activation of naive mice thymocytes in a dose-dependent manner could be demonstrated *in vitro* (Roy *et al.*, 1995). Our data also indicate that this inhibitory effect was abolished by naloxone. However, our unpublished observation showed that neither morphine nor naloxone could significantly alter the background (without PHA) proliferation of lymphocyte. Roy *et al.* (1996) and Wick *et al.* (1996) demonstrated that morphine-mediated inhibition of Bac1.2 F5 macrophage in culture was partially reversible by naloxone, and their previous experiment showed that activation of thymocyte with PHA-IL1 *in vitro* resulted in a dramatic increase in (³H)-morphine specific binding (Roy *et al.*, 1992). Collectively, we suggest that PHA might induce proliferation of different population of lymphocytes containing different affinities of opioid receptors that are more or less sensitive to morphine.

Immunophenotypic markers on lymphoid cells have been determined using flow cytometric analysis in heroin addicts. There was a profound decrease in the proportion of CD4/CD8 and the percentage of CD4, with an increase in both the percentage and absolute numbers of CD8 and the absolute numbers of cell expressing CD3. The data obtained here are parallel with the proliferative responses. However, Shine *et al.* (1987) found a normal pattern of T-cell subsets in a group of healthy intravenous drug abusers and methadone patients, whereas Sei *et al.* (1991) and Novick *et al.* (1989) found that parenteral heroin abusers had higher absolute numbers of CD4 cells in the peripheral blood.

NK cell activity mediated predominantly by large granular lymphocytes is also regulated by opioid compounds. *In vitro*, β -endorphin and met-enkephalin augmented NK activity in a dose-dependent and naloxone-reversible fashion (Matthews *et al.*, 1983; Kay *et al.*, 1984). Morphine was slightly suppressive (Matthews *et al.*, 1983), and DAMGO suppressed NK activity (Weber *et al.*, 1989). Shavit *et al.* (1986) have previously shown that opiates interacting with opiate receptors in the brain are implicated in the suppression of NK activity. Those authors suggested novel central nervous system mechanisms through which the immune response might be regulated. In the present study, the percentage of NK cells was suppressed in heroin addicts. This was supported by a previous study showing that parenteral heroin addicts had significantly reduced NK activity while the absolute numbers of NK cells in peripheral blood were unchanged (Novick *et al.*, 1989). The similar findings had previously been reported by Reddy *et al.* (1987) and DePaoli *et al.* (1986), who observed reduced NK cell activity in HIV-seronegative parenteral drug addicts and a further reduction in HIV-seropositive patients.

The present study in heroin addicts has shown an increase in both the number and the percentage of B cells by immunofluorescence staining. Johnson *et al.* (1982) reported that α -endorphin *in vivo* significantly depressed primary antibody production to sheep red blood cells. Both met-enkephalin and leu-enkephalin are modulate inhibitors, whereas β - and γ -endorphin are not effective. Morphine seems to have no effect on antibody production where animals are immunized with a T-independent antigen. However, *in vivo* administration of β -endorphin inhibited the primary antibody response to keyhole limpet hemocyanin but enhanced the secondary response (Munn *et al.*, 1989).

Immune status in heroin-withdrawal subjects. The immune function in heroin withdrawal subjects also was a

focus of the present study. In most previous studies of heroin addiction, the data were obtained from patients who were not only polydrug abusers but also in the withdrawal state; most patients were either treated with drugs to suppress withdrawal symptoms, in methadone maintenance therapy or both. The patients in our study not only were single-drug abusers but, in the withdrawal state, also were free of any drug, as heroin withdrawal was carried out "cold turkey." This would make our results extremely beneficial for the better understanding of heroin withdrawal mechanisms. In a previous study (Novick *et al.*, 1989) of long-term stabilized methadone-maintained patients, NK cell activity and absolute B- and T-cell subset numbers were not significantly different from those of normal control subjects. Kind (1988) performed a study in unselected groups of former narcotic addicts in methadone maintenance treatment for varying periods of time, including patients both with and without continuing polydrug and alcohol abuse. He found that 53% of subjects had normal NK cell cytotoxicity. However, methadone itself has been shown to have immunomodulatory effects, and these effects are dose dependent. At doses below 75 mg/kg, it was possible to reverse the immunodepressive effect of heroin (Novick *et al.*, 1989). In contrast, in an *in vitro* study, Kind (1988) showed that neither opioid antagonist (naloxone) nor opioid agonist (methadone) altered NK cytotoxicity until drug concentrations $>10^{-4}$ M above pharmacological levels were reached, at which point both compounds reduced NK activity in parallel responses. In the present study, the suppression of lymphocyte proliferation responses was partially reversed in the heroin withdrawal subjects. All parameters, assessed by flow cytometric analysis, were altered in heroin addicts but gradually returned to normal levels, with the exception of the short period of withdrawal (15 to 21 days), where they seemed to get worse. However, the total recovery in almost all immunological parameters obtained here would not occur before 3 to 5 years after drug withdrawal. Our studies supported the notion that "the time course of the development of tolerance following initial drug exposure and the persistence of tolerance following sudden removal of the drug was found to parallel the development and decline of immune reactions" (Cochin and Kornetsky, 1964). Our result clearly demonstrates that the immunological deficits evidenced during drug consumption are reversible slowly after heroin is withdrawn, without taking methadone and concomitantly in parallel with the decline of the withdrawal signs and other neurobehavioral effects.

A considerable amount of evidence suggests that opioids administered *in vivo* modify the immune system indirectly. The HPA axis has been implicated in the morphine-mediated suppression of primary humoral immune responses (Pruett *et al.*, 1992). Other recent studies also suggest that morphine-mediated effects on the immune system operates through central processes (Hernandez *et al.*, 1993; Fecho *et al.*, 1993). However, there was an indication that the HPA axis was not necessarily involved in all opioid-mediated immunosuppressive effects because injection of opioid compounds into specific sites of the brain that resulted in a suppressive effect on immune function (*e.g.*, lymphocyte proliferation on splenic NK activity) did not change the circulating levels of corticosterone (Band *et al.*, 1992; Hernandez *et al.*, 1993). Collectively, these results provide convincing evidence for the local and systemic activity of opioids on immu-

nocompetence and immune homeostasis. Attempts to definitively prove the existence of opioid receptors on cells of the immune system have been unsuccessful in various laboratories, including our own by using radioligand binding. However, recently, molecular techniques have provided data (Sedqi *et al.*, 1995; Chuang *et al.*, 1995; Wick *et al.*, 1996; Roy *et al.*, 1996) indicating the existence of opioid receptors on cells of the immune system.

In conclusion, the results from our study indicate a depression in PHA-stimulated T lymphocyte proliferation, both as an *in vitro* effect of morphine and in heroin addicts, including the modulation of surface markers observed on T cells. The alteration of the T lymphocyte proliferative responses and surface markers seems to gradually reverse to normal levels when heroin is withdrawn. The mechanisms responsible for the opioid-induced changes in immune function are still unclear. They may be mediated directly *via* opioid receptors present on lymphocytes (Wick *et al.*, 1996; Roy *et al.*, 1996) and/or indirectly *via* opioid receptors in the central nervous system. Molecular biological and biochemical characterizations suggest that immune cell differentially express classic opioid receptors. In addition, the presence of a novel class of opioid receptor in immune cells has been suggested, and it is believed that the antiproliferative effect is mediated *via* this receptor type (Roy and Loh, 1996). However, multifactorial elements might be involved in such alteration. Efforts should be continued and expanded to facilitate an interchange of new ideas that will accelerate research and lead to the more rapid development of new and more effective treatments for substance abuse.

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Send reprint requests to: Piyarat Govitrapong, Ph.D., Neuro-Behavioural Biology Center, Institute of Science and Technology for Research and Development, Mahidol University at Salaya, Nakornpathom 73170, Thailand. E-mail: grpkk@mahidol.ac.th