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Dr. Raymond E. Biacini , Gerry M. Henningsen & Stephen L. Klincewicz

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Immunologic Analyses of Peripheral Leukocytes from Workers at an Ethical Narcotics Manufacturing Facility

RAYMOND E. BIAGINI
GERRY M. HENNINGSEN
Centers for Disease Control and Prevention
Division of Biomedical and Behavioral Science
Atlanta, Georgia
STEPHEN L. KLINCEWICZ
Division of Surveillance Hazards Evaluation and Field Studies
Hazards Evaluation and Technical Assistance Branch
National Institute for Occupational Safety and Health
Cincinnati, Ohio

ABSTRACT. Little information exists about possible adverse health effects associated with workplace exposure to opiate compounds. We have previously reported opiate-specific IgG antibodies, positive epicutaneous tests, and pulmonary function decrements in workers exposed occupationally to opiates. In the present work, we extended these findings to investigate the effect of occupational opiate exposure on lymphocyte subpopulations and mitogen-induced lymphoblastogenesis. Thirty-three opiate-exposed workers and 8 nonexposed control workers were evaluated for lymphocyte subpopulation absolute numbers and percentages, by evaluating cell surface antigen expression with flow cytometry. A complete blood count with differential, common clinical chemistry parameters, and serum immunoglobulin levels were also evaluated. Opiate-exposed workers showed significantly ($p < .05$) increased absolute numbers and percentages of HLA-DR$^+$ cells (MHC class II histocompatibility antigen), significantly ($p < .01$) decreased percentages of T helper-inducer (CD4$^+$) cells, and significantly ($p < .05$) decreased numbers of basophils, compared with nonexposed opiate workers from the same factory. A trend toward reduction in the T helper-inducer (CD4$^+$)/T cytotoxic-suppressor (CD8$^+$) lymphocyte ratio was also evident. There was also a significant decrease in lymphocyte activity stimulated by pokeweed mitogen ($p < .05$) in opiate-exposed workers. These data indicate that occupational opiate exposure may change the number and types of circulating peripheral blood leukocytes, or alternatively, alter the expression of receptors on the surface of these cells. In addition, occupational opiate exposure appears to decrease the sensitivity of B-cells to pokeweed mitogen stimulation. The significance of these findings as to the pathogenesis of occupational opiate-induced asthma are unclear at the present time, but the cellular changes reported in the present work suggest that lymphocyte alterations may be important.

THE PRESENT STUDY was part of a National Institute for Occupational Safety and Health (NIOSH) Health Hazard Evaluation (HHE) investigation of workers employed in a factory who extract morphine and other related alkaloids from opium gum or related opium poppy (Papaver somniferum) concentrates. The workers complained of adverse health effects, including dyspnea, wheezing, headache, malaise, tiredness, and skin

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reactions related temporally to opiate exposure. Health hazards associated with the manufacture of opiate-containing pharmaceuticals have been known for centuries, being first reported by Ramazzini in 1715.[1] We have previously reported on significantly elevated specific antimorphine IgG levels,[3] lower epicutaneous threshold concentrations (ETC) for positive skin tests, and changes in pulmonary function from occupational exposures to opiate compounds.[4]

The effect of opiates on the cells of the immune system were reported as early as 1909.[2] Evidence was first reported in 1979, suggesting that human blood T-lymphocytes possess specific (naloxone-reversible) receptors for morphine.[28] Subsequent investigations[11] have confirmed this finding and also have shown that opiate exposure may alter the expression of the E-rosette receptor marker (CD2), T-helper/inducer (CD4) and T-suppressor/cytotoxic (CD8) markers. Opiate-induced alterations in the microdisplacement of receptors (molecular configurational changes of cell surface markers during their integration into the lipid-bilayer/glycocalix of the cell surface membrane) has been proposed as a potential mechanism for these findings.[11]

In the present work, we report on lymphocyte cell surface antigen changes, alterations in lymphoblastogenesis response to mitogens, and changes in differential white blood cell count parameters in workers exposed occupationally to opiate compounds.

Materials and Method

Process description, exposures, and environmental measurements. The manufacturing process at the plant included conversion of raw morphine base to various morphine salts (hydrochlorides, phosphates, tartrates) or derivatives of morphine, such as codeine and dihydrocodeine, oxycodone, hydrocodone, and similar compounds. Short-term and full-shift personal breathing zone samples for alkaloid dusts were collected and analyzed for codeine and morphine, using high-pressure liquid chromatography (HPLC). The limit of detection (LOD) for morphine was 5.0 mg/sample. The reader is referred to our previous descriptions published elsewhere[3,4] for detailed descriptions of process, exposures, and analytical methods.

Study participants (general). The workers' major complaints, which they felt were associated with occupational opiate exposure, included dyspnea, wheezing, headache, malaise, tiredness, and skin reactions. The majority of evaluated employees were process workers; however, some worked in the laboratory and other areas that involved sporadic, lower, and infrequent opiate exposure. Process workers were exposed mainly during hand-scoop unloading of centrifuges, which sometimes proceeded without respiratory protection. During a visit to the factory in December 1988, blood samples were obtained from 33 exposed process workers and from 8 other administrative and clerical workers employed at the same factory who either had little or no known occupational exposure to opiates (NJ Referent). Thirty-one of the 33 exposed workers were male (91%), and 7 of the 8 referent workers were male (88%). There was no statistically significant difference in the mean ages of the opiate-exposed and NJ referent groups. Informed consent was obtained from all participants in the present HHE study, which was approved by the NIOSH Human Subjects Review Board (HSRB).

Asthmatic status. Ten individuals reported receiving a diagnosis of new-onset adult asthma from their physician after beginning work with narcotics. Those who reported new-onset asthma were not significantly different from those not reporting new-onset asthma with respect to age, smoking pack-years, proportion of current smokers, or years of employment at the facility. Asthmatics reported a higher percentage of episodes of wheezing and increased episodes of wheezing the month preceding the study.

Sample collection. Blood was withdrawn by antecubital venipuncture into tubes containing (a) acid, citrate, and dextrose (ACD) for flow cytometry studies, and (b) ethylenediaminetetraacetic acid (EDTA) for hemocytometry and lymphoblastogenesis assays; in some cases, the blood was allowed to clot at room temperature. The sera was separated by centrifugation at 1 000 *g* for 10 min for immunoglobulin and other serum chemistry analyses.

Flow cytometry. Flow cytometry was performed within 12 h of blood collection. Samples were processed and stained with a Simultest immune monoclonal antibody screening kit (Becton-Dickinson; San Jose, CA), using a protocol for whole blood lysis of erythrocytes that accorded with the manufacturer's instructions. Samples were analyzed on a FACS 440 flow cytometer equipped with a Consort 30 data analysis system (Becton-Dickinson). Laser alignment, fluorescence standardization, and electronic compensation for spectral overlap of the fluorochromes was performed with latex beads (Calibrite, Becton-Dickinson). The 488-nm line from an argon laser was used to excite the fluorescein isothiocyanate (FITC) and phycoerythrin (PE) dyes. Green fluorescence due to FITC conjugated monoclonal antibody-tagged cells was collected with a 535/15 nm band-pass filter. Red fluorescence due to PE-tagged cells was collected, using a 560 nm dichroic mirror and a 575/15-nm band-pass filter. Forward- versus side-light-scatter histograms were collected, and a lymphocyte gate was set to select lymphocytes from the whole blood preparation. Contaminating monocytes were excluded from analysis by use of dual CD45-FITC (pan-leukocyte) and CD14-PE (Leu-M3, monocyte) staining (Becton-Dickinson), a process that allowed their identification in plots of red versus green fluorescence. Five thousand mononuclear cells were analyzed for each subject's sample. Total lymphocyte-subset counts were calculated by multiplying the percentage positive by the total number of lymphocytes, as reported by a commercial clinical laboratory (MetPath, Inc., Teterboro, NJ). Cells were stained with monoclonal antibodies (Becton-Dickinson) to enumerate total T cells (CD3[Leu 4]$^+$/HLA-DR$^-$),

HLA-DR$^+$ (CD3[Leu 4]$^-$/HLA-DR$^+$), T helper-inducer (CD4[Leu 3a]$^+$/CD8[Leu-2a]$^-$), T cytotoxic-suppressor (CD4[Leu 3a]$^-$/CD8[Leu-2a]$^+$), and natural killer cells (CD57[Leu-7], NK cells).

Clinical chemistry and hemocytometry. Selected clinical chemistry and hemocytometry parameters were performed by a commercial laboratory (MetPath). Complete blood counts with differentials (eosinophils, basophils, neutrophils, monocytes, lymphocytes, total white blood cells, hematocrit, and hemoglobin) were performed by laser light scatter and enzyme cytochemistry; serum albumin was analyzed by a colorimetric method; serum IgE was analyzed by immunoassay; serum IgA, IgG and IgM were analyzed by nephelometry.

Lymphoblastogenesis assays. Peripheral blood mononuclear cells were isolated for lymphocyte proliferation assays by means of a Ficoll-Hypaque density-gradient centrifugation. The cells were resuspended in complete assay medium (CM; RPMI 1640 + 100 units/ml penicillin with 100 µg/ml streptomycin and 2 mM L-glutamine, [GIBCO Laboratories; Grand Island, NY] + 10% human type AB serum [human AB serum (pooled) obtained from male donors, less than 35 y old with no history of transfusion, certified as negative for antibodies to hepatitis B virus and human immunodeficiency virus I]) at a concentration of 1×10^6 viable cells/ml (by trypan blue exclusion). One-hundred-µl aliquots of the cell suspensions were dispensed into appropriate wells of 96-well plates to yield 1×10^5 cells/well in individual wells (cellular additions were performed in triplicate for all dilutions). The following mitogens were diluted in CM and added at the final concentrations specified: phytohemagglutinin (PHA, Difco, Detroit, MI), final concentrations of 50 µg/ml, 5 µg/ml, and 0.5 µg/ml; Concanavalin A (ConA, Sigma Chemical Co.; St. Louis, MO), final concentrations of 20 µg/ml, 2 µg/ml, and 0.2 µg/ml; and pokeweed mitogen (PWM, Sigma), final concentrations of 1 µg/ml, 0.1 µg/ml, and 0.01 µg/ml. The first 3 wells in each row received media in place of mitogen and served as a nonstimulated background control. The plates were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Incubation times were 72 h (PHA) or 120 h (ConA and PWM). The wells were pulsed with 50 µl of ^{3}H-thymidine (ICN; Costa Mesa, CA; specific activity, 2-10 ci/mmol), prepared as a fresh stock of 20 µCi/ml diluted in CM. After 4-6 h, the cells were harvested on a cell harvester and placed into minivials with 2 ml scintillation cocktail. Disintegrations per minute (dpm) of triplicate samples were counted on a liquid scintillation counter.

Analysis of data. All null hypothesis tests were performed using nonparametric methods, as the results of tests for population distribution indicated the data were not distributed normally. Kruskal-Wallis's ANOVA (Number Cruncher Statistical System, Dr. Jerry Hintze, Kaysville, UT) followed by Wilcoxon signed rank tests were used to investigate group differences. A Type 1 error level of $p < .05$ was considered to be statistically significant.

Table 1.—Hematologic Parameters and Immunoglobins for Opiate-Exposed and Referent Group Ethical Narcotics Manufacturing Workers

Parameter	Number of workers and group: Opiate exposed ($n = 33$)	Referent group ($n = 8$)
Albumin (g/dl)	4.5 (0.1)*	4.7 (0.1)
White blood cell (thous./mm^3)	7.0 (0.4)	6.8 (0.5)
Polys (/mm^3)	3 891 (274)	4 159 (532)
Lymphs (/mm^3)	2 431 (182)	2 015 (145)
Eosinophils (/mm^3)	191 (23)	129 (24)
Basophils (/mm^3)	58 (5.7)	41 (5. 7)†
Monos (/mm^3)	377 (28)	442 (45)
Hematocrit (%)	44.3 (0.7)	46.2 (0.7)
Hemoglobin (g/dl)	14.0 (0.2)	14.9 (0.5)
IgE (units/ml)	97 (15)	59 (18)
IgA (gm/dl)	308 (23)	257 (33)
IgG (gm/dl)	1 401 (90)	1 279 (116)
IgM (gm/dl)	162 (19)	106 (19)

*Standard error (in parentheses).
†$p < .02$.

Results

Analyses of hematologic parameters and immunoglobulin levels showed no significant differences ($p > .05$, Table 1) in the parameters measured, except for a statistically significant increase in number of basophils ($p < .05$) in opiate-exposed workers (58 ± 33 versus 41 ± 14 basophils/mm^3), compared with nonexposed referents. Flow cytometric analyses (Table 2) indicated that there were significantly increased ($p < .02$) percentages and numbers of cells bearing the CD3$^-$HLA-DR$^+$ phenotype (which are most probably B-cells) and significantly decreased ($p < .01$) percentages of cells bearing the CD4$^+$ (T helper-inducer) phenotype in opiate-exposed workers, compared with the referent group. No significant ($p > .05$) differences were found in percentages or numbers of CD8$^+$ (T cytotoxic-suppressor cells) or CD57$^+$ (NK cells) or in numbers of cells bearing CD4$^+$ (T helper-inducer), compared with the referent group (Table 2). T helper-inducer/T cytotoxic-suppressor (CD4$^+$/CD8$^+$) ratios tended to be lower in the exposed workers (1.8 [exposed] versus 3.0 [referent]), but this finding was not significant statistically ($p > .10$). Lymphocyte proliferation in opiate-exposed individuals was reduced significantly ($p < .03$), compared with nonopiate-exposed referents at all concentrations of PWM tested, but not with the other mitogens tested (PHA, ConA; Table 3).

Discussion

We believe this to be the first report of the effect of opiate exposure on peripheral leukocytes from workers exposed to opiates in the ethical narcotics industry. Much work has been done on peripheral leukocyte

Table 2.—Percentages and Absolute Numbers of Lymphocyte Surface Markers from Opiate-Exposed and Referent Group Ethical Narcotics Manufacturing Workers

Phenotype	Cell type	Number of workers and group: Opiate exposed (n = 30)	Number of workers and group: Referent group (n = 6)
$CD3^+/HLA\text{-}DR^-$	T-cell (%)	65.0 (2.1)†	75.0 (4.5)
$CD3^+/HLA\text{-}DR^-$	T-cell (no.)	1 570 (141)	1 619 (106)
$CD3^-/HLA\text{-}DR^+$	$HLA\text{-}DR^+$ (%)	14.9 (1.1)	10.9 (0.9)‡
$CD3^-/HLA\text{-}DR^+$	$HLA\text{-}DR^+$ (no.)	342 (34)	236 (23)‡
$CD4^+/CD8^-$	T-helper/inducer (%)	36.8 (2.5)	51.6 (4.3)§
$CD4^+/CD8^-$	T-helper/inducer (no.)	910 (105)	1 120 (114)
$CD4^-/CD8^+$	T-cytoxic/suppressor (%)	26.0 (1.6)	20.7 (3.0)
$CD4^-/CD8^+$	T-cytotoxic/suppressor (no.)	609 (63)	485 (69)
$CD4^+/CD8^+$ Ratio	N/A	1.8 (0.3)	3.0 (0.8)
$CD57^+$	Natural Killer (%)	7.6 (1.4)	8.1 (4.0)
$CD57^+$	Natural Killer (no.)	186 (52)	180 (89)

*D = cluster of differentiation nomenclature.
†Standard error (in parentheses).
‡$p < .02$.
§$p < .01$.

Table 3.—Results of Lymphoblastogenesis Assays on Opiate-Exposed and Referent Group Ethical Narcotics Manufacturing Workers

Mitogen	Concentration (μg/ml)	Opiate exposed (DPM × 1000)		Referent group (DPM × 1000)	
No. of workers		25		6	
ConA*	0	3 834	(1 768)†	2 051	(815)
ConA	0.2	18 761	(3 127)	29 362	(2 786)
ConA	2.0	14 756	(2 184)	20 607	(2 839)
ConaA	20	8 742	(1 189)	6 564	(1 373)
No. of workers		31		7	
PHA‡	0	874	(336)	292	(65)
PHA	0.5	19 702	(2 515)	26341	(2 675)
PHA	5.0	1 302	(1 794)	76 211	(51 330)
PHA	50	3 822	(1 571)	5 286	(1 164)
No. of workers		28		7	
PWM	0	1 983	(315)	1 887	(379)
PWM	0.01	3 150	(468)	6 696	(1 477)§
PWM	0.10	20 110	(1 972)	29 603	(3 278)#
PWM	1.0	22 912	(2 293)	36 365	(3 507)‡

*See text for details of mitogens used.
†Standard error (in parentheses).
‡$p < .01$.
§$p < .005$.
#$p < .03$.

changes in street opiate addicts; however, street opiate addicts have exposure to numerous adulterants, and they have unique lifestyles and a nutritional status that may compromise conclusions of the immunological effects of opiates on their cohort.

Data concerning the effect of street opiate exposure on numbers and percentages of lymphocytes and subsets are conflicting. Decreases in absolute numbers and percentages of T-lymphocytes[5,6] have been reported. In contrast to this, again in street addicts, other investigators have found increases in absolute numbers of T-cells with no change in percentage expression of this marker.[7] Increases in absolute numbers of T helper-inducer and T cytotoxic-suppressor cells have also been reported in peripheral blood from heroin addicts.[8] Other investigators have found inverted T helper-inducer/T

cytotoxic-suppressor ratios in addicts who were hospitalized with a variety of serious infectious diseases[9]; however, the effect of infection on the observed T helper-inducer/T cytotoxic-suppressor ratio reduction could not be ruled out. In still another study, no abnormalities in T-cell subsets were observed in healthy, HIV-negative parenteral drug abusers and methadone patients.[10] It has been proposed that the duration of opiate exposure may be important in opiate-induced lymphocyte toxicity, as decreased T helper-inducer/T cytotoxic-suppressor ratios have been reported to be present in addicts who had used heroin for more than 10 y.[11] Asthmatic reactions have also been reported in heroin abusers,[12–15] and in these individuals immunomodulating effects have been described, e.g., lymphopenia with concurrent increased number of null cells and increased lymphocyte proliferation by phytohemagglutinin (PHA).[5]

Animal studies have shown that morphine exposure profoundly increases host susceptibility to bacterial and fungal infections[16,17] and tumor challenge,[18] inhibits the primary antibody response to sheep red blood cells,[19] and alters natural killer cell activity[20–22] and interferon production.[23,24]

The workers in the present study had potential inhalation exposure only to pharmaceutical-grade opiates. In these workers, we have shown statistically significant increases in percentage of T helper-inducer cells, with no effect on absolute numbers of T helper-inducer cells; significant increases in cells expressing the HLA-DR phenotype; and a significant decrease in lymphocyte transformation stimulated by pokeweed mitogen. Increases in percentages of T helper-inducer cells have been reported previously[8] in heroin addicts. However, to the best of our knowledge, this is the first report of increases in cells expressing the HLA-DR phenotype and decreased lymphoblastogenesis from PWM in opiate-exposed individuals.

A series of recent reports have emphasized that asthma is an inflammatory disease of the airways.[25–27] The inflammatory cells include changes in numbers of eosinophils, basophils, T-lymphocytes, and cytokines released from these cells. Our data from the workers of the present study support changes in basophils and T-cell subsets that may be associated with opiate exposure and may be related to the asthmatic responses from opiate exposure reported in these same workers.[3,4]

A single immunologic endpoint can rarely be pathognomonic; however, a pattern of clinical or laboratory changes may be indicative of either exposure or early disease. Whereas the flow cytometric results are emphasized in this paper, they are best interpreted in view of other indicated immune and hematologic test results reported elsewhere (significantly elevated specific antimorphine IgG levels, lower epicutaneous threshold concentrations [ETC] for positive skin tests, and changes in pulmonary function).[3,4] It should also be kept in mind that the subset changes reported in the present work could be the result of changes in the regulation of cell traffic in the peripheral blood rather than (or in addition to) changes in the distribution of all lymphocytes. The relationship of changes in the number and/or percentage of lymphocyte subsets to the pathophysiological interpretation of other findings (basophilia, suppressed pokeweed mitogenesis, specific anti-opiate IgG, the lack of anti-opiate IgE, positive epicutaneous prick tests, and pulmonary function changes associated with workplace opiate exposure) are yet unclear.

Given that the application of immunotoxicologic techniques to human populations exposed to xenobiotics is relatively new, there are difficulties in the interpretation of statistically positive results and their potential health significance. Group contrasts performed by classical statistical analysis of data from small-sample-sized groups (as is the case in the present work) can suffer from a number of shortcomings. This is further confounded by the relatively large inherent variance in some of the clinical immunological endpoints seen here and by other investigators using essentially equivalent methodologies.[29,30] Interpretation of the clinical significance of this type of data has to be couched within elements of the study design, i.e., was there credible evidence of exposure (preferably by biomonitoring) and measurable clinical endpoints (such as pulmonary function changes, etc. associated with exposure). Additionally, data from animal studies and from other studies of similarly exposed humans have to be taken into account during interpretation. Finally, interpretation of such findings is complicated because there is little quantitative data regarding the degree to which an immune parameter (especially a cellular immune parameter, except in well studied diseases such as AIDS) needs to be modified from xenobiotic exposure to become clinically diagnostic.[31]

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Requests for reprints should be sent to Dr. Raymond E. Biagini, NIOSH, Applied Biology Branch, Immunochemistry Research Section, 4676 Columbia Parkway, Cincinnati, OH 45226.

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