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ANTIBODIES TO MORPHINE IN WORKERS EXPOSED TO OPIATES AT A NARCOTICS MANUFACTURING FACILITY AND EVIDENCE FOR SIMILAR ANTIBODIES IN HEROIN ABUSERS

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Summary

According to the International Narcotics Control Board, over 45,000 kg of morphine and 54,000 kg of codeine were ethically manufactured in 1986 at three facilities in the United States. Little information exists about possible adverse health effects associated with workplace exposure to opiate compounds in this industry. Because there are no specific federal standards for workplace exposure to narcotic dusts, exposure-control defaults to the nuisance dust standard (10 mg/m^3, as an 8 hr time-weighted average). Narcotics manufacturing workers were evaluated for anti-morphine IgG before and 10 mo. after the implementation of an improved respiratory protection program (RPP). Significantly elevated IgG levels were measured before the improved RPP ($P<0.005$). After the improved RPP, a significant reduction was observed ($P<0.001$), suggesting that specific antibody levels could be used as biomarkers of exposure. Inhibition studies showed that the antibodies were specifically directed against morphine with some cross reactivity with morphine derivatives. Preliminary results are also shown which indicate that similar anti-morphine antibodies are present in the sera of intravenous heroin abusers. Elevated levels ($P<0.05$) of anti-morphine antibodies were detected in sera from heroin abusers, providing evidence that similar antibodies may be produced from non-occupational exposure to opiates. These finding have potentially far-reaching implications for addiction research and drug testing.

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Health hazards associated with the manufacture of opiate-containing pharmaceuticals have been known for centuries, being first reported by Ramazzini in 1715 (1). The histamine-releasing properties of opiates are now well documented (2); and although there have been sporadic reports of asthmatic symptoms in opiate abusers (3,10,11,12) and opiates have been shown to have a variety of effects on the immune system (4,5), no one has yet described the de facto immunogenic potential of opiates in humans. Animals which are immunized to morphine-protein conjugates readily produce IgG class antibodies to morphine (6,7,8). These polyclonal anti-morphine antibodies, or monoclonal antibodies produced by hybridomas (8) are the source of antibodies for analytic immunoassays for morphine and other opiates.

IgE class specific anti-morphine antibodies from parental morphine exposure have not been described in the animal literature (9). Previous reports of immunologic and pharmacologic effects in animals (4) and reports of apparent asthmatic reactions in humans abusing opiates (3,10,11,12,41) or occupationally exposed (13,42), provided sufficient indication that opiates could possibly induce immunologic as well as pharmacologic hypersensitivity reactions in exposed humans under proper conditions. 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 that included dyspnea, wheezing, headaches, malaise, tiredness and skin reactions temporally related to opiate and/or solvent(s) exposure. The workers were also exposured to a variety of solvents (trade secret concerns dictate that the specific identity of solvents used in the process not be divulged), some of which were irritant; however, evaluation of industrial hygiene data, biological monitoring results and material safety data sheets failed to identify solvents which could account for systemic and respiratory complaints. Therefore, our research focused on the potential effects of inhaled opiate dusts.

Materials and Methods

Subjects

The average age of the study participants was 45 $\pm$ 11.2 years (mean $\pm$ standard deviation, SD). Ninety-five percent were male with a mean employment duration at the factory of 11.2 $\pm$ 9.4 years. Twenty-eight percent of the employees were current smokers with a mean smoking history of 12.5 $\pm$ 17 pack-years. Informed consent was obtained from all participants in the present HHE study, which was approved by the NIOSH Human Subjects Review Board (HSRB).

During the initial HHE site visit in February 1988, a serum sample was obtained from each of 35 workers. An updated respiratory protection program, designed to minimize dust exposures, was initiated shortly after the February 1988 survey. During a return visit to the factory in December 1988, serum samples were taken from 25 exposed process workers and from 6 other administrative and clerical workers employed at the same factory, who either had little or no known occupational exposure to opiates. No sera from unexposed individuals was available at the February visit. Samples were available from 21 workers who participated at both sera collection periods. Blood was withdrawn by antecubital venipuncture and allowed to clot at room temperature. The sera was separated by centrifugation at 1000 x g for 10 minutes. The sera were stored at -20°C until the immunoassays were performed at the

authors' laboratories.

In addition to the worker samples described above, we subsequently obtained 28 plasma samples from the National Institute on Drug Abuse (NIDA) for use in a double-blind study. Eighteen of the samples were obtained from 8 healthy male, HIV negative, individuals admitted to NIDA for treatment. These subjects identified heroin as their drug of choice, had been drug-free for a minimum of three days, and all had non-detectable levels of heroin or morphine (opiates) in their urine. Seven other samples were obtained from burn patients prior to surgery, who were considered drug-free except for perhaps sporadic use of morphine for analgesia. Three control samples were obtained from NIDA staff members. The plasma samples were treated with thrombin after recalcification to induce clotting, and the sera separated by centrifugation as above for subsequent analysis.

Preparation of Morphine-6-hemisuccinate-Human Serum Albumin (M-6-HS-HSA) Conjugates

Morphine-6-hemisuccinate (M-6-HS, Figure 1) was prepared by heating morphine (Morphine Alkaloid Powder U.S.P., obtained from the Company) with succinic anhydride in pyridine as previously described (6,8), to yield white crystals which melted at 242°C to 243°C. Desorption probe mass spectrometry identified the synthesized compound to be highly purified M-6-HS, as no extraneous mass ion peaks other than those due to this compound were observed. The M-6-HS was then conjugated to human serum albumin (HSA, Sigma Chemical, St. Louis, MO.) by a mixed anhydride method (6), dialyzed under reduced pressure (Micro-ProDicon model 320, Bio-Molecular Dynamics, Beaverton, OR.), and purified by gel filtration (Sephadex G-25M, Pharmacia, Upsala, Sweden). Protein concentration was measured with a commercial Coomassie Brillant Blue assay kit (Bio-Rad, Richmond, CA.). The amount of morphine conjugated to HSA was estimated by radioimmunoassay (Abuscreen Radioimmunoassay for Morphine, Roche Diagnostic Systems, Nutley, N.J.), using morphine standards supplied by the kit manufacturer that were diluted in human serum. The morphine content of the M-6-HS-HSA conjugate was about 7.5 moles morphine per mole of human serum albumin.

Industrial hygiene measurements of the work environment in February 1988 revealed air opiate concentrations as high as 10 mg/M^3 (data not shown). It has been shown with other occupational allergens (18) that circulating levels of free or bound hapten can significantly reduce the sensitivity of subsequent in vitro immunoassays. In order to control for this possibility, serum morphine levels (14) were determined in the sera of 17 of the 25 (December 1988 sampling period) workers who agreed to participate in this analysis, using the same morphine radioimmunoassay method as above (Roche Diagnostic Systems).

Specific Antibody Assays

Radioallergosorbent (RAST) testing for M-6-HS-HSA specific IgE antibodies was performed using M-6-HS-HSA coupled to methylcellulose discs by cyanogen bromide treatment, and the RAST analysis was performed as previously described (15). Specific IgG and IgG_4 antibodies to M-6-HS-HSA were measured by a modified indirect microtitre plate ELISA method previously described (17). Briefly, 50 µg M-6-HS-HSA diluted in 0.1 M $NaHCO_3$, pH 8.6, was placed in each well and incubated at room temperature for two hours. Each well was then washed three times with

phosphate buffered saline (0.02 M phosphate buffer, pH 7.4, containing 0.9% NaCl) and a similar wash repeated between all subsequent steps. Aliquots (200 µl) of each diluted serum sample (1:10 in 5% BSA-deionized water) were (tested in triplicate on several occasions to confirm reproducibility) added to the wells and allowed to incubate at room temperature for two hours. After washing as above, 100 µl of goat anti-human IgG alkaline phosphatase conjugate (Sigma Chemical Co.) or goat anti-human IgG_4 alkaline phosphatase conjugate (ICN Immunological, Lisle, IL.), diluted 1/100 in 1% BSA, was added to each well of the 96-well plates (Easy Wash,

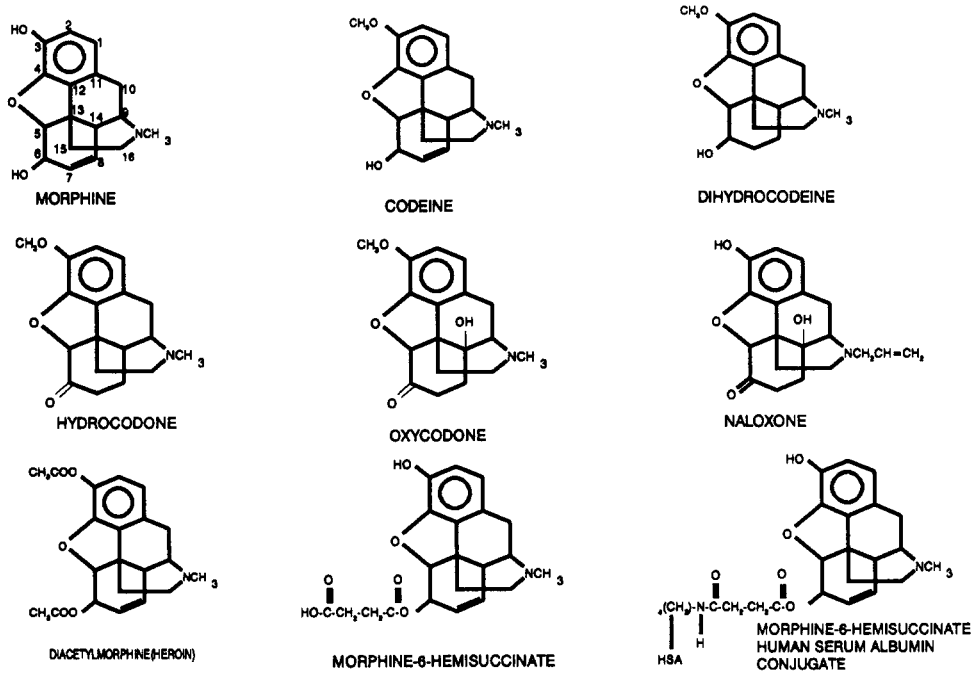

FIG. 1.
Structures of morphine and related alkaloids and conjugates.

Corning, NY) and incubated for 2 hrs at room temperature. The plates were washed and 100 µl of 0.6 mM p-nitrophenyl phosphate disodium (Sigma Chemical Co.) substrate solution, diluted in alkaline glycine buffer (0.05 M glycine and 0.5 mM magnesium chloride, pH 10.4), was added. After 30 min, the reactions were terminated with 50 µl of 2 N NaOH. Optical density at 410 nm was read on an automated ELISA plate reader (model MR700, Dynatech, Alexandria, VA.).

ELISA Inhibition

Varying concentrations of M-6-HS-HSA, morphine sulfate, codeine phosphate, dihydrocodeine bitartrate, oxycodone HCl, hydrocodone (all obtained from the Company) and naloxone HCl (Sigma Chemical Co.) solutions were incubated with a

positive serum known to have high levels of specific IgG antibodies to M-6-HS-HSA, for two hours at 37°C. Following this 2 hr preincubation, the sera were analyzed by ELISA as above. The amount of specific IgG binding (in triplicate, represented by optical density) contained in the inhibited serum was compared to that in the uninhibited serum. The percent inhibition was calculated as previously described (18).

Analysis of Data

All null hypothesis tests were performed using non-parametric methods, as the results of tests for population distribution indicated the data were not normally distributed. 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 in specific IgG and IgE antibodies to M-6-HS-HSA (February 1988 exposed, December 1988 exposed, and December 1988 non-exposed factory cohort. Sera from a non-exposed factory cohort was not available at the February sera acquisition period. All ELISA results were normalized by calculating an absorbance ratio (19) (absorbance of experimental sera/absorbance of volunteer negative control sera [N=6]). The negative control sera were obtained from individuals in the Cincinnati, OH, area with no known chronic or abusive opiate exposure except for possible sporadic therapeutic exposure to low levels of codeine-containing analgesics and antitussives. A Wilcoxon test for paired observations was used to investigate differences in ELISA absorbance ratios for the individual workers who provided sera at each of the two testing periods. A Type 1 error level of ≤ 0.05 was considered to be statistically significant. Positive serum samples were defined as those with an absorbance ratio of $\geq$ 2.5 (19). For inhibition experiments, inhibition by a test substance of $\geq$ 50 percent of noninhibited values was considered a positive effect (18).

For analyses of the NIDA sera, all samples were analyzed individually. Kruskal -Wallis non-parametric ANOVA followed by Mann-Whitney U tests for non-paired data were used to investigate differences between groups. Control samples from NIDA and normal individuals from the Cincinnati, OH, area were combined (N=6) to increase power, as the two control groups were statistically indistinguishable.

Results

Morphine Specific Antibodies

Twenty of the 35 workers' sera tested in February, 1988, had positive evidence of morphine antibodies; in December, 1988, only 2 of 25 workers had positive evidence of antibodies. One of 6 of the "non-exposed" workers' serum collected in December gave positive results of morphine antibodies. Mean ($\pm$SE) absorbance ratios for the three groups of workers were: February 1988 exposed = 4.48 $\pm$ 0.74; December 1988 exposed = 1.19 $\pm$ 0.24; and December 1988 non-exposed = 1.61 $\pm$ 0.99. These results are given as open circle data points immediately to the right of the individual workers' data (see Figure 2, Panel A). In panel B of Figure 2, results from workers who provided sera at both the February and December, 1988, testing periods are given. Data are presented as mean $\pm$ SE absorbance ratios (5.4 $\pm$ 0.95 in February, 1988 and 1.19 $\pm$ 0.95 in December, 1988). All data points are the mean of triplicate values. Null hypothesis statistical tests were performed by Kruskal-Wallis analysis (Panel A) or Wilcoxon's test for paired observations (Panel B). No worker sera samples gave positive evidence of M-6-HS-HSA specific IgE or IgG_4 antibodies at either sera acquisition periods.

ELISA results for the NIDA plasma samples are shown in Fig. 3. Two of 8 of the burn patient samples and 7/18 of the heroin abuser samples were positive ($\geq$2.5 times mean control absorbance). Mean absorbance values for the heroin abusers were significantly greater than control values ($P<0.05$). Sera from burn patients, while having positive evidence of antibodies in 2 individuals, was statistically indistinguishable from control sera.

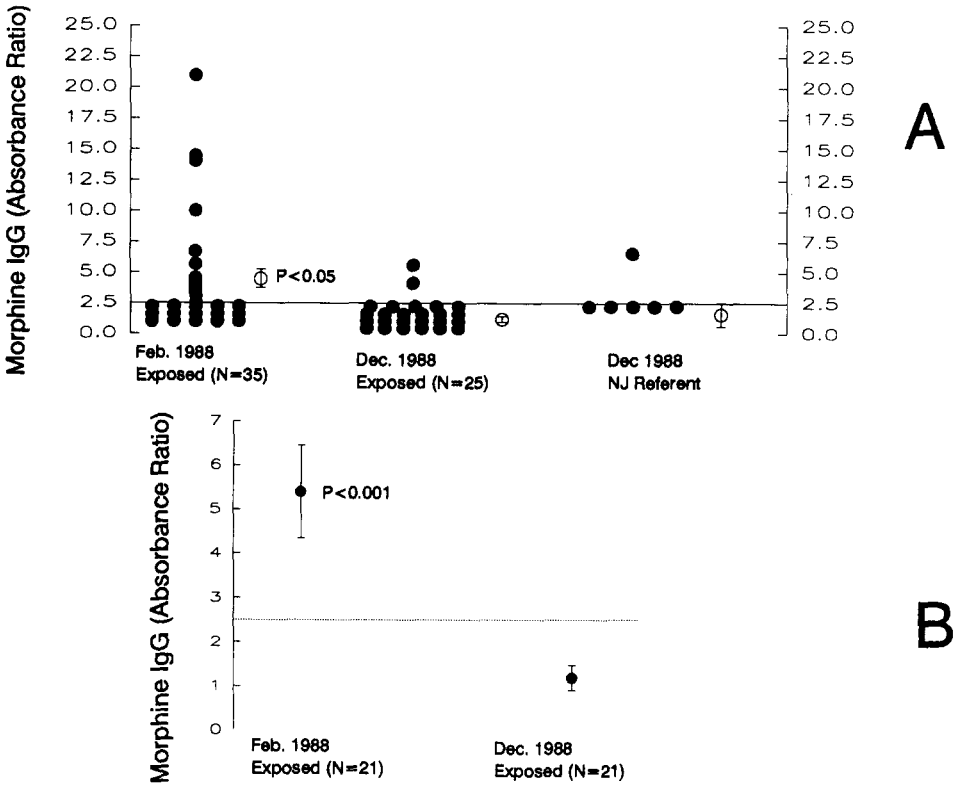

FIG. 2.
Worker ELISA results reported as binding ratios (see text). A ratio of $\geq$ 2.5 was considered a positive response (dotted line). Individual workers' data (filled circles) are plotted in Panel A (data points below the dotted line are not plotted to scale for clarity of graphical presentation). Mean values for workers who provided sera at both acquisition periods are given in Panel B.

ELISA Inhibition

The concentrations for 50% inhibition of non-pre-treated ELISA values (IC_{50}s) for the worker serum are shown in Table 1. These inhibition results agree quite well

with data from similar reports of the specificity of experimentally produced rabbit anti-M-6-HS-bovine serum albumin antisera (7) and suggest that opiate compounds with a carbonyl at position 6 (hydrocodone, oxycodone and naloxone) appear to be less effective inhibitors than those with hydroxyl moieties at this position (morphine, codeine, dihydrocodeine; see Figure 1). Reduction status of the 7-8 double bond seems to be unimportant, as both codeine (reduced 7-8 bond) and its oxidized 7-8 bond structural isomer, dihydrocodeine, appear to be equally effective inhibitors. Concentration-effect curves for the inhibition experiments are shown graphically in Figure 4.

Serum Opiates

Only three individuals of the 17 workers sampled in December 1988, who consented to serum opiate determinations, had trace levels of morphine or structural analogues in their sera (10-12 ng/ml, or 13-16 pmoles opiate based on morphine sulfate, MW 758 g). These levels were near the lower analytical limit of detection for morphine in sera by radioimmunoassay (approximately 10 ng/ml) (20). The worker whose serum was used in the inhibition studies did not consent to a serum opiate determination.

COMPETITOR	IC_{50} (nM)
M-6-HS-HSA	4 ± 3
Morphine sulfate	78 ± 36
Codeine phosphate	81 ± 17
Dihydyrocodeine bitartrate	88 ± 16
M-6-HS	290 ± 120
Hydrocodone HCl	800 ± 170
Naloxone HCl	7100 ± 2900
Oxycodone HCl	8000 ± 1700

TABLE 1. Concentration of Competitor for 50% Inhibition (IC_{50}) of Anti-Morphine ELISA by Related Morphine Derivatives.

Discussion

In the present report we describe human specific IgG antibodies to morphine in the sera of workers occupationally exposed to opiates in the course of their employment. We also demonstrated morphine-specific IgG in heroin abusers, extending the usefulness of these findings to subjects exposed non-occupationally to opiate compounds. No in vitro evidence for M-6-HS-HSA specific IgE or IgG_4 class antibodies were found in sera obtained from these groups of addicts or workers, although several of the workers complained of suspected opiate-related adverse health effects (tightness of the chest, skin rashes and others symptoms) that are compatible with allergic (IgE) or short-term-sensitizing IgG (IgG_4) mediated reactions. A possible

explanation for the lack of demonstrable morphine-specific IgE is that the antibody (if present) may be cell-bound.

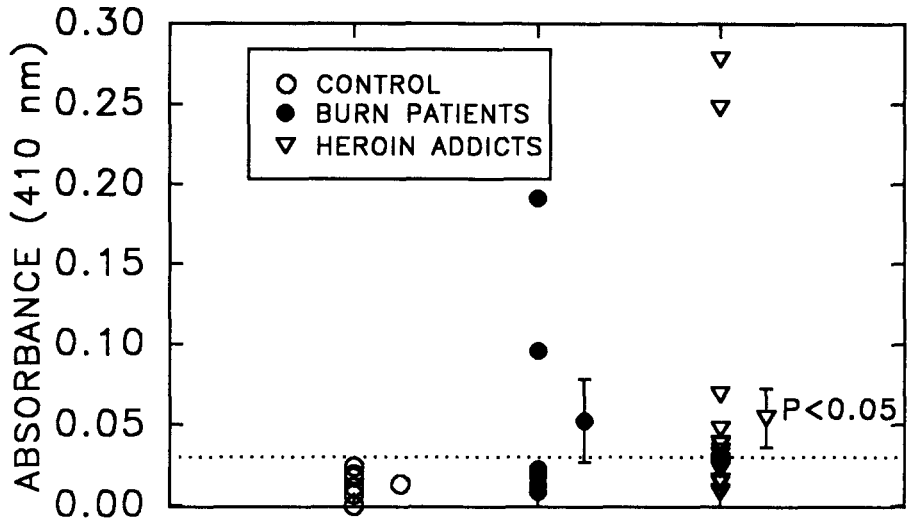

FIG 3.
NIDA ELISA Results. Individual absorbance values as well as group mean absorbances (± SE) are given on the Figure. The dotted line represents 2.5 times the control absorbance value.

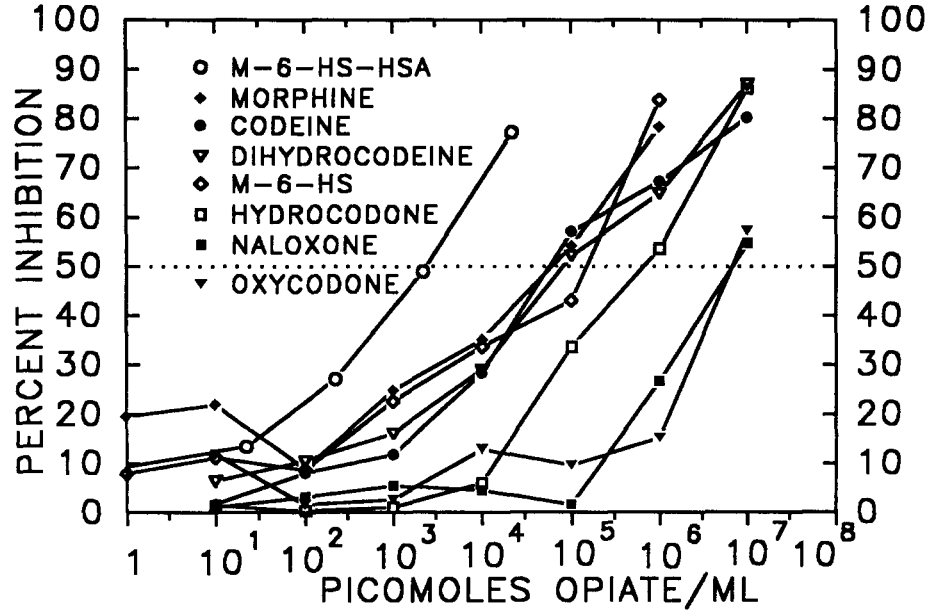

FIG. 4.
ELISA Inhibition by various opiates and structural analogues. IC_{50} values are given in the text. Standard error bars reside within the control mean point.

Failure to demonstrate IgE antibodies in subjects with asthmatic complaints and positive skin tests strongly suggesting an allergic etiology has been reported for other occupational allergens (17,40).

Inherent in the findings of the present report is putative evidence for the existence of macromolecularly bound opiates (or opiate metabolite[s]) in vivo, as the opiate nucleus itself is too small to be immunogenic in its own right (30). Antibody binding to M-6-HS-HSA could be inhibited by pretreatment with morphine sulfate, M-6-HS and numerous morphine analogues; however, analysis of the IC_{50}s suggest that the human anti-morphine antibody has an apparent low affinity for the native morphine nucleus, when compared to antibodies raised in immunized or hyperimmunized animals (7). Similar analysis, when applied to inhibition data using M-6-HS-HSA as inhibitor indicate that the conjugate is a better inhibitor (by 2-3 orders of magnitude) than the native morphine compounds. These findings are suggestivr of the formation of new antigenic determinants (NAD's) from morphine exposure.

The presence of exogenous opiates or endogenous circulating opioids may explain why anti-opiate antibodies have not been readily observed previously, since the antigen binding sites could be occupied by these compounds, rendering the antibodies unmeasurable in indirect ELISA tests. The usually observed serum levels of morphine parent in patients treated for chronic pain is 2-200 ng/ml; for surgical anesthesia, morphine levels of 140-150 ng/ml are usually observed; and narcotic toxicity occurs with serum morphine levels of 200-500 ng/ml (31). Extrapolation of morphine serum levels to as high as 1,000 ng/ml have been reported in cases of "sudden death" in abusers (14). We have previously shown that free antigen competition could lead to lowered results for in vitro tests of antibody levels with other occupational allergens (18). Many of the methods used to screen for opiates (either for therapeutic, abuse or forensic uses), utilize radioimmunoassay techniques based on the reaction of opiates with anti-opiate antibodies produced in animals. Morphine clearance from M-6-HS-BSA immunized rabbits with circulating anti-morphine antibody has been shown to be significantly slower than in normal non-immunized animals (34). Human anti-morphine antibodies would be expected to interfere similarly, either extending the apparent half-life for excretion due to serum antibody binding of the parent compound or metabolite(s), or directly interfering with serum analyses by competitive antibody binding. Finally, autologous and homologous antiidiotypic anti-anti-β-endorphin IgG class immunoglobulins have been described (35) which compete with β-endorphin for opiate receptors, possibly regulating neuropeptide activity. Binding of opioid neuropeptides by anti-morphine antibodies or the production of anti-anti-morphine antibodies is also a consequence which could interfere with functions of endogenous neuropeptides.

The immunologic sequelae of occupational and abusive opiate exposure are not well documented in humans. Recently, asthma and dermatitis were reported in workers exposed to morphine dust at a Soviet pharmaceutical manufacturing facility (13). It is difficult to delineate between a pharmacologic, pseudoallergic or immunologic basis for morphine respiratory and dermal reactions, since opiates are known to directly degranulate mast cells (2). Complicating the description of opiate-mediated histamine release and bronchoconstriction is the finding of functional heterogeneity of mast cells (38,39), the presence of opiate receptors on smooth muscle (36,37) and the possibility of immunologically mediated release.

Asthmatic reactions have also been reported in heroin abusers (3,10,11,12).

Immunomodulating effects have also been described in these individuals that include lymphopenia with concurrent increased number of null cells and increased lymphocyte proliferation by phytohemagglutinin (PHA) (20). Results of functional immunologic studies in opiate abusers are seriously compromised due to the addition of adulterants (strychnine, arsenic, lactose and other substances of unknown origin) which may have immunotoxic and/or hypersensitizing properties themselves. Animal studies have shown that morphine exposure profoundly increases host susceptibility to bacterial and fungal infections (21,22) or tumor challenge (23), inhibits the primary antibody response to sheep red blood cells (24), and alters natural killer cell activity (25,26,27) and interferon production (28,29).

In Britain, the prevalence of asthmatic reactions in female heroin abusers is significantly greater than those reported for male heroin abusers (10). Female abusers in general have a propensity to initially smoke or "snort" heroin, in comparison to male users who appear to prefer intravenous administration. It has been shown that concomitant respiratory exposure to allergens and irritants can significantly increase sensitizing potential (32). It is also known that respiratory exposures are more likely to produce asthmatic symptoms when compared to parenteral exposures (33).

The workers in the present study generally had inhalation exposures to a variety of opiate compounds (some of which are known to have irritant and histamine releasing properties) in combination with exposure to irritant solvents. From this information, it is intriguing to speculate that respiratory exposure to irritating levels of opiates (as high as 10 mg/M^3; data not shown) or to opiates in combination with irritant solvents may enhance the immunogenic or asthmogenic properties of these compounds. In addition, the emerging street practice of smoking mixtures of heroin and cocaine (speedballs) may also increase the allergenicity or immunogenicity of opiates as cocaine (crack) smoking has been shown to be irritating to the lungs (43) and in some cases cause asthmatic responses (44). Also fascinating to speculate is the possibility that opiate abusers, in order to minimize their potential for AIDS infection by the use of shared needles and syringes, may change their preferred self-administation route to that of smoking opiates, with a possible increased potential for antibody production and allergic complications.

In conclusion, this present report gives statistically significant evidence for the existence of human anti-morphine antibodies in individuals who are occupationally exposed (mainly by inhalation) to opiate narcotic compounds (such as morphine and derivatives). Inhibition studies supported the ELISA findings that the IgG class antibodies were specifically directed to morphine with some cross-reactivity to morphine derivatives. Following the implementation of an effective workplace respiratory protection program, the levels of anti-morphine antibodies in these workers were reduced dramatically over a ten month period, indicating that antibody analyses could potentially be used as an immune biomarker of opiate narcotic exposure. In addition, evidence was also presented indicating that heroin abusers have significantly elevated levels of anti-morphine IgG in their sera when compared to normal controls. The existence of de facto synthesized anti-opiate antibodies in humans has far-reaching implications with regard to the potential control of narcotic abuse, further understanding of tolerance from chronic morphine exposure, and medicolegal implications regarding interpretations of the detection (or lack thereof) of opiates in forensic studies or in general screening for narcotic exposure.

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