A Monoclonal Antibody Specific for 6-Monoacetylmorphine Reduces Acute Heroin Effects in Mice

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ABSTRACT

Immunotherapy against drugs of abuse is being studied as an alternative treatment option in addiction medicine and is based on antibodies sequestering the drug in the bloodstream and blocking its entry into the brain. Producing an efficient vaccine against heroin has been considered particularly challenging because of the rapid metabolism of heroin to multiple psychoactive molecules. We have previously reported that heroin’s first metabolite, 6-monoacetylmorphine (6-MAM), is the predominant mediator for heroin’s acute behavioral effects and that heroin is metabolized to 6-MAM primarily prior to brain entry. On this basis, we hypothesized that antibody sequestration of 6-MAM is sufficient to impair heroin-induced effects and therefore examined the effects of a monoclonal antibody (mAb) specific for 6-MAM. In vitro experiments in human and rat blood revealed that the antibody was able to bind 6-MAM and block the metabolism to morphine almost completely, whereas the conversion of heroin to 6-MAM remained unaffected. Mice pretreated with the mAb toward 6-MAM displayed a reduction in heroin-induced locomotor activity that corresponded closely to the reduction in brain 6-MAM levels. Intraperitoneal and intravenous administration of the anti–6-MAM mAb gave equivalent protection against heroin effects, and the mAb was estimated to have a functional half-life of 8 to 9 days in mice. Our study implies that an antibody against 6-MAM is effective in counteracting heroin effects.

Introduction

The pharmacotherapies currently in use for the treatment of heroin abuse are agonists (methadone, buprenorphine) and antagonists (naloxone, naltrexone) to brain opioid receptors that either mimic or block the effects of heroin. The use of opioid maintenance therapy, including methadone and buprenorphine, has been successful in many aspects; however, these drugs are addictive and can contribute to overdose deaths (Simonsen et al., 2011; Hakkinen et al., 2012; Bernard et al., 2013). Concern may also be raised toward the reported negative effects of methadone on cognitive functions (Verdejo et al., 2013). Concern may also arise toward the reported negative effects of heroin’s first metabolite, 6-monoacetylmorphine (6-MAM), the predominant mediator for heroin’s acute behavioral effects and that heroin is metabolized to 6-MAM primarily prior to brain entry. On this basis, we hypothesized that antibody sequestration of 6-MAM is sufficient to impair heroin-induced effects and therefore examined the effects of a monoclonal antibody (mAb) specific for 6-MAM. In vitro experiments in human and rat blood revealed that the antibody was able to bind 6-MAM and block the metabolism to morphine almost completely, whereas the conversion of heroin to 6-MAM remained unaffected. Mice pretreated with the mAb toward 6-MAM displayed a reduction in heroin-induced locomotor activity that corresponded closely to the reduction in brain 6-MAM levels. Intraperitoneal and intravenous administration of the anti–6-MAM mAb gave equivalent protection against heroin effects, and the mAb was estimated to have a functional half-life of 8 to 9 days in mice. Our study implies that an antibody against 6-MAM is effective in counteracting heroin effects.

Upon administration, heroin displays low brain concentration and low affinity for μ-opioid receptors and is therefore presumed to be a prodrug mainly acting through its metabolites (Inturrisi et al., 1983; Selley et al., 2001; Andersen et al., 2009; Gottas et al., 2013). In rodents and humans, heroin is rapidly metabolized by sequential deacetylation to 6-monoacetylmorphine (6-MAM) and morphine (Rook et al., 2006a; Andersen et al., 2009; Gottas et al., 2013), mainly by esterase enzymes (Owen and Nakatsu, 1983; Salmon et al., 1999). In humans, morphine is further transformed by glucuronidation to pharmacologically active morphine-6-glucuronide (M6G) and inactive morphine-3-glucuronide (M3G) (Fig. 1A) (Glare and Walsh, 1991; Milne et al., 1996; Rook et al., 2006b), whereas M3G is normally the only morphine glucuronide found in rodents (Zuccaro et al., 1997; Handal et al., 2002). For decades, morphine was considered the metabolite responsible for heroin’s pharmacological effects (Way et al., 1965); however, in the 1980s, it was suggested that 6-MAM also could be of importance (Umans and Inturrisi, 1981; Inturrisi et al., 1983). Previous studies in our laboratory demonstrated that the immediate heroin response in mice is mediated by 6-MAM (Andersen et al., 2009) and that heroin is metabolized to 6-MAM mainly in the periphery before its transfer to the brain (Boix et al., 2013). These findings are in contrast to the traditional assumption that heroin is particularly addictive because of its high lipophilicity, which allows it to easily pass...
the blood-brain barrier and to be metabolized to active metabolites in the brain (Oldendorf et al., 1972). The predominant role of 6-MAM for the immediate heroin response appears to be valid regardless whether heroin is injected subcutaneously or intravenously (Andersen et al., 2009; Gottas et al., 2013; Raleigh et al., 2013).

The proof-of-concept for vaccines against drugs of abuse was first reported in the 1970s, demonstrating both active and passive immunization strategies (Berkowitz and Spector, 1972; Berkowitz et al., 1974; Bonese et al., 1974; Killian et al., 1978). Since then, several active vaccines (using a morphine conjugate) have been developed with effects toward heroin- or morphine-induced behaviors (Anton and Leff, 2006; Li et al., 2011; Stowe et al., 2011; Pravetoni et al., 2012; Raleigh et al., 2013; Schlosburg et al., 2013). Passive immunotherapy toward opioids has received minor attention. This immunization strategy has the advantages of being independent of the interindividual differences in immune response, the protection is immediate, the duration of action is predictable, and mAb or antibody fragments can be designed to fit the therapeutic application (Peterson et al., 2006; Peterson and Owens, 2009).

Based on the increasing amount of evidence that 6-MAM is the metabolite responsible for the immediate rewarding effects of heroin, we hypothesized that sequestration of this specific metabolite would be sufficient to markedly impair heroin-induced effects. Because most of the previously reported active vaccines toward heroin display a broad specificity toward heroin and its metabolites, further characterization of the effects of specific 6-MAM sequestration appeared warranted. The mAb examined in this study was generated using a 6-MAM derivatized hapten containing a linker at the 6-MAM N-bridge (Fig. 1B) (Moghaddam et al., 2003). We compared the effects of the anti–6-MAM mAb on brain levels of heroin and metabolites with locomotor activation, which share some of the brain structures implicated in drug reward (Wise and Bozarth, 1987) and can therefore be used as a measure of opioid’s psychostimulatory effects (Morland et al., 1994). We also studied the duration of the protective effect of the antibody and compared different routes of administration.

Materials and Methods

Animals. Male C57BL/6J-Bom mice (7–8 weeks old, 20–25 g; Taconic, Ejby, Denmark) were housed six to eight per cage in the animal facility at the Norwegian Institute of Public Health (22 ± 1°C, 50% ± 10% humidity, light period 7:00 AM–7:00 PM). The animals arrived at least 5 days before the experiments. Commercial mouse pellets and water were available ad libitum. The experimental protocol of the study was approved by the Norwegian Animal Research Authority.

Blood. Rat blood was collected from sacrificed male Sprague Dawley rats (200–250 g; Taconic, Ejby, Denmark) and human blood from healthy adult volunteers. To inhibit clotting, heparin (14–17 IU/ml) was used. The experiments were initiated within 30 minutes after blood collection.

Materials.

Antibody. The anti–6-MAM mAb was developed and synthesized by Affitech Research AS (Oslo, Norway) using a biotin-polyethylene glycol-6-MAM conjugate (Fig. 1B). The mAb was based on a previously reported single chain fragment variable (scFv) antibody fragment (6-MAM-214; Moghaddam et al., 2003). For the purpose of this study, an antibody format with a longer half-life (IgG) was customized by using the Fv regions of 6-MAM-214 (Moghaddam et al., 2003). For the purpose of this study, an antibody format with a longer half-life (IgG) was customized by using the Fv regions of 6-MAM-214. The anti–6-MAM mAb (human IgG1, l-light chain) was produced in stable transfected Chinese hamster ovary cells, purified via protein A (MabSelect) before anion-exchange chromatography. Thereafter, it was dialyzed against the formulation buffer (phosphate buffer). The antibody was checked for endotoxins (<0.5 EU/mg) and stored at 4°C.

Drugs. Heroin-HCl (mol. wt. 421.91), 6-MAM-HCl (mol. wt. 408.21), M6G-HCl (mol. wt. 498.61), and M3G-HCl (mol. wt. 488.53) were purchased from Lipomed AG (Arlesheim, Switzerland). Morphine-HCl (mol. wt. 377.32) was from Norsk Medisinaldepot AS (Oslo, Norway), and naltrexone (mol. wt. 377.86) was delivered by Sigma-Aldrich (Oslo, Norway). Heroin and its metabolites were dissolved in 0.9%
Immediately after the locomotor activity test, the injection analysis revealed that injection of saline does not induce changes in the total run distance during the session (Grung et al., 1998). The data were analyzed either as the distance traveled per 5-minute intervals or as the total distance traveled over the next 2.8 minutes and was maintained for 0.3 minute before returning to its initial conditions. The flow rate was 0.5 ml/min. Masses monitored were 270.10 and 242.10 for heroin and 282.10 for 6-MAM. The presence of the anti-6-MAM mAb d showed an interference in the results. In vitro, the gradient was increased to 100% over the next 2.8 minutes and was maintained for 0.3 minute before returning to its initial conditions. The flow rate was 0.5 ml/min. Masses monitored were 342.10 > 270.10 and 242.10 > 282.10 for naltrexone and 345.10 > 270.10 for naltrexone d3. Limit of detection was 0.001 μM. The interassay variability was lower than 15% for all compounds.

Naltrexone was analyzed by a UPLC-MS method. Internal standard (50 μl, 0.1 μM) and methanol (30 μl) were added to the diluate (70 μl). Separation was performed at 65°C on a HSS T3 column (Waters Corp.) using gradient elution with mobile phase consisting of methanol and ammonium formate buffer (10 mM, pH 3.1). The flow rate was 0.2 ml/min.
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Compared with the pronounced anti–6-MAM mAb effect seen when the mAb was given 1 hour before heroin injection, the reduction in heroin-induced run activity was no longer significant when the mAb had been in the circulation for 1 or 2 weeks before a single heroin injection (Fig. 5, A and B). One week after administration, the mAb was still able to reduce the brain concentration of 6-MAM upon heroin injection (35% reduction compared with saline-pretreated mice), but 2 weeks after mAb administration, this reduction was not significant (11% reduction, \( P > 0.05 \)) (Fig. 5C). The data from mice injected with heroin 1 hour after administration of mAb (Fig. 4) are included in Fig. 5 for comparison.

Both intraperitoneal and intravenous injections of the anti–6-MAM mAb significantly reduced the heroin-induced locomotor activity and brain concentrations of 6-MAM and morphine compared with their respective controls (Fig. 6). There were no differences in the mAb effect between the two ways of administration \( (P > 0.05) \).

The anti–6-MAM mAb displayed no cross-reactivity toward morphine. This was confirmed in vivo as brain morphine concentrations upon administration of 2.5 \( \mu \text{mol/kg} \) s.c. morphine were not different between antibody-treated and control mice \( (0.054 \pm 0.005 \ \text{nmol/g in control versus } 0.059 \pm 0.003 \ \text{nmol/g in antibody-treated mice}) \). This dose of morphine did not induce locomotor activity, as shown previously by Andersen et al. (2009).

Discussion

Producing an efficient vaccine against heroin has been considered particularly challenging because of the rapid metabolism of heroin to multiple psychoactive molecules. Based on the increasing amount of evidence that 6-MAM is the metabolite responsible for the immediate rewarding effects of heroin, we hypothesized that sequestration of this specific metabolite would be sufficient to markedly impair the acute behavioral effects of heroin. In this study, we therefore examined the effects of a mAb specific toward 6-MAM. We found a severe reduction in heroin-induced locomotor activity in mice pretreated with the anti–6-MAM mAb, which closely corresponded with the measured reductions in brain levels of active heroin metabolites.

The mAb used was based on a previously reported scFv antibody fragment recognizing 6-MAM with an affinity of 0.3 \( \mu \text{M} \) (6-MAM-214; Moghaddam et al., 2003). For the purpose of this study, an antibody format with a longer half-life (human IgG1) was customized. This mAb displayed no cross-reactivity to morphine, M3G, or M6G and only modest binding to heroin itself. The absence of cross-reactivity toward morphine \( (2.5 \ \mu \text{mol/kg s.c.}) \) was confirmed in vivo where no difference was found in morphine brain concentrations between control and mAb pretreated mice. In addition, a negligible cross-reactivity was found toward naltrexone, which is the drug in use for treatment of heroin addiction with the highest structure similarity to 6-MAM. The in vitro studies performed in blood confirmed the specificity of the mAb, showing an almost complete block of the metabolism of 6-MAM to morphine, whereas the conversion of heroin to 6-MAM remained unaffected.

In the anti–6-MAM mAb pretreated mice (Figs. 4–6), the 60%–67% reduction in acute heroin-induced locomotor activity 20 minutes after heroin injection corresponded closely
with the 58%–60% reduction in brain 6-MAM levels. This strongly supports our previous finding that the immediate heroin response is mediated by heroin’s first metabolite, 6-MAM (Andersen et al., 2009). Reduced brain levels of morphine (29%−44%) were also found in the anti-6-MAM mAb-immunized mice (Figs. 4 and 6). This finding can be explained by the combined effect of reduced brain entrance of the morphine precursor 6-MAM and reduced metabolism of 6-MAM to morphine in the blood, both caused by 6-MAM binding to the antibody. We have previously shown that the brain morphine concentrations found after injection of 2.5 μmol/kg heroin are too low to induce locomotor activity in mice (Andersen et al., 2009), and therefore, the reduction in locomotor activity cannot be assigned to a reduction in morphine levels. Concerning heroin itself, which displays low affinity for μ-opioid receptors (Inturrisi et al., 1983; Selley et al., 2001) and is present in low concentrations in brain after administration (Andersen et al., 2009), it seems unlikely that the modest binding by the antibody can explain the observed ∼60% reduction in the behavioral response.

In two recently published studies using an active immunization strategy with a morphine conjugate producing antibodies targeting heroin, 6-MAM, and morphine, the reduction in brain 6-MAM levels was reported to be 44% and 69% after exposure to heroin or 6-MAM, respectively (Pravetoni et al., 2012; Raleigh et al., 2013). When comparing these results using a broad immune response with the effects of our specific anti-6-MAM mAb, the reductions in brain 6-MAM levels accomplished were of equivalent size. Our in vitro and in vivo experiments indicate that the 6-MAM sequestration could be even further increased by using higher doses of anti-6-MAM mAb.

In theory, the most efficient sequestration strategy for drug abuse vaccines should be to bind the first substance present in blood, in this case heroin, to avoid brain entrance and central effects. Raleigh et al. (2013) used an immunogen known to elicit antibodies with high affinity for both heroin and downstream metabolites and found a pronounced sequestration of heroin in the blood of immunized rats without affecting the brain levels of heroin. This result indicates that only a small fraction of the injected heroin enters the brain so rapidly that it escapes both antibody binding and metabolism by esterases in the blood. Raleigh et al. (2013) concluded that the most likely explanation for the reduced 6-MAM levels in brain of vaccinated animals was extensive binding and retention of 6-MAM in serum. This supports our previous study showing that the metabolism of heroin to 6-MAM is very fast and occurs primarily in the periphery prior to brain entry (Boix et al., 2013). Stowe et al. (2011) examined an antibody with high affinity for heroin and morphine, but no affinity for 6-MAM, and reported it to be inefficient in preventing acquisition of heroin self-administration, emphasizing the importance of 6-MAM sequestration. There are major differences in the presence of heroin-metabolizing enzymes in human and rodent blood (Berry et al., 2009; Bahar et al., 2012), which may complicate the extrapolation of opioid metabolism data across species. Despite these differences, the in vivo half-lives of heroin in rodents and humans have been shown to be remarkably similar (t1/2 ~2.5–4 minutes) (Way et al., 1960; Rook et al., 2006b; Boix et al., 2013; Gottas et al., 2013; Raleigh et al., 2013).

### Table 1

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Control</th>
<th>Anti-6-MAM mAb</th>
<th>mAb-Bound Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>μM</td>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heroin</td>
<td>0.088 ± 0.002</td>
<td>0.068 ± 0.001</td>
<td>23.4 ± 1.5</td>
</tr>
<tr>
<td>6-MAM</td>
<td>0.097 ± 0.002</td>
<td>0.008 ± 0.002</td>
<td>91.5 ± 2.3</td>
</tr>
<tr>
<td>Morphine</td>
<td>0.093 ± 0.004</td>
<td>0.091 ± 0.001</td>
<td>1.7 ± 1.6</td>
</tr>
<tr>
<td>Μ3G</td>
<td>0.106 ± 0.001</td>
<td>0.105 ± 0.001</td>
<td>0.3 ± 1.2</td>
</tr>
<tr>
<td>M6G</td>
<td>0.099 ± 0.004</td>
<td>0.098 ± 0.002</td>
<td>0.3 ± 2.2</td>
</tr>
<tr>
<td>Naltrexone</td>
<td>0.094 ± 0.002</td>
<td>0.088 ± 0.000</td>
<td>5.4 ± 0.3</td>
</tr>
</tbody>
</table>
Independent of an active or passive immunization strategy, it is of major importance that the antibodies have high specificity. Otherwise, the antibodies will rapidly be occupied with inactive or downstream metabolites and the binding capacity readily overcome by taking repeated doses of the abused drug over a short period of time (Kosten and Domingo, 2013). A high antibody specificity also has the advantage of not interfering with endogenous opioids, classic opioid analgesics, or conventional addiction treatment, which again opens for the possibility to combine current opioid substitution therapy with novel immunotherapeutics. A combined active and passive immunization against nicotine has been reported to enhance the nicotine vaccine efficacy (Roiko et al., 2008; Cornish et al., 2011), whereas combination of vaccines toward different drugs of abuse has been shown to be possible without compromising with the efficacy of the individual components (Pravetoni et al., 2012). The main advantages of passive immunization compared with active immunization are that pharmaceutical grade antibodies can be given to every patient in a precise dose, the protection is immediate, the duration of action is more predictable, and customized antibody forms can be selected relative to the therapeutic applications (e.g., long-acting for relapse prevention and short-acting for overdose) (Peterson et al., 2006; Peterson and Owens, 2009). Another advantage using therapies acting through a pharmacokinetic rather than pharmacodynamic blockade is that potential heroin effects mediated through other mechanisms than opioid receptor binding would also be blocked, such as opioid-induced activation of the central immune system through Toll-like receptor 4, suggested to be involved in drug reinforcement (Hutchinson et al., 2012; Wang et al., 2012; Theberge et al., 2013).

The chief obstacles in implementing passive immunotherapy for drug abuse are the need for large amounts of purified mAb and the cost and inconvenience of repeated injections (Brimijoin et al., 2013). The functional elimination half-life of the anti–6-MAM mAb used in this study was tested by injecting mice with mAb and expose to a single dose of heroin at different time points. When heroin was given 1 hour or 1 week after pretreatment, mice given mAb had brain concentrations of 6-MAM that were reduced by 60% and 35% compared with mice pretreated with saline. From these results (Fig. 5), a functional half-life of approximately 8 to 9 days may be estimated for the anti–6-MAM mAb in mice, which is in accordance with other studies reporting IgG half-lives of 5–12 days in rodents (Bazin-Redureau et al., 1997; Norman et al., 2009; Cornish et al., 2011; Treweek et al., 2011). In humans, the half-life of IgG has been reported to be as much as 3–4 weeks (Lobo et al., 2004; Peterson and Owens, 2009), which makes prophylactic prevention of drug abuse feasible, at least for limited time periods in subgroups of patients such as drug-abusing women during pregnancy or detoxified heroin addicts during periods with intense craving.

In the present study, the mice were given mAb in doses of 10–100 mg/kg, which corresponds to approximately 0.5–5 μM IgG, based on a distribution volume about twice the blood volume (Bazin-Redureau et al., 1997) and a molecular mass of 150 kDa. Because IgG has two binding sites per antibody molecule, 1–10 μM drug-binding sites were available. This is within a clinical relevant concentration range as Cmax of 6-MAM has been measured to be 5.0–17.5 μM after intravenous injection of heroin in heroin users (Rentsch et al., 2001; Girardin et al., 2003; Rook et al., 2006b). One of the advantages of passive immunization is the possibility to easily increase the antibody levels in the blood by giving higher doses of antidrug mAb.

Because mAbs are large molecules with relatively poor membrane permeability and stability in the conditions of the gastrointestinal tract, parenteral administration has been the preferred route of mAb administration (Dostalek et al., 2013).
Intravenous injections in mice are technically more challenging than intraperitoneal and subcutaneous injections, and therefore, the latter injection techniques are preferred in animal studies. We found that intraperitoneal administration...
of anti-6-MAM mAb in the mice gave equivalent protection against heroin effects compared with intravenous administration. To ensure that the mAb was absorbed into the blood before heroin exposure, a 4-hour delay was introduced between the intraperitoneal injection of mAb and heroin exposure.

In summary, we have tested a mAb specific toward 6-MAM with minor cross-reactivity toward heroin and no cross-reactivity toward heroin and metabolites. These findings strengthen the view that 6-MAM is the key mediator of acute heroin effects and imply that a vaccine against heroin, either active or passive, needs to sequester 6-MAM in the blood to be efficient.

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Authorship Contributions
Participated in research design: Bogen, Boix, Merrill, Andersen.
Conducted experiments: Bogen, Nerem, Andersen.
Performed data analysis: Bogen, Nerem, Boix.
Wrote or contributed to the writing of the manuscript: Bogen, Nerem, Boix, Merrill, Andersen.

References
Bazin-Redureau MI, Renard CB, and Scherrmann JM (1997) Pharmacokinetics of heroin, either active or passive, needs to sequester 6-MAM in a broad specificity toward heroin and metabolites. These levels. The efficacy of the specific passive anti-6-MAM mAb is equivalent with previously reported active vaccines with a broad specificity toward heroin and metabolites. These findings strengthen the view that 6-MAM is the key mediator of acute heroin effects and imply that a vaccine against heroin, either active or passive, needs to sequester 6-MAM in the blood to be efficient.

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