Liposome Encapsulation Prolongs Alfentanil Spinal Analgesia and Alters Systemic Redistribution in the Rat

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The effect of liposome encapsulation on the analgesia produced by intrathecally administered alfentanil was examined in the rat. In rats prepared with chronic intrathecal catheters, alfentanil in doses of 1–50 μg was administered intrathecally in either saline or in multimolecular liposomes (dipalmitoylphosphatidylcholine and cholesterol). Animals were then tested for analgesia by hot-plate and paw-pressure tests. A second group of animals received intrathecal injections of 30 μg alfentanil in saline or liposomes, and blood samples were obtained at 5, 15, 45, and 135 min thereafter for measurement of alfentanil plasma concentrations. The liposome preparation markedly prolonged spinal analgesia in the paw-pressure test and to a lesser extent in the hot-plate test. Neither the time to peak analgesia nor the intensity of analgesia differed between the saline and liposome groups. Liposome encapsulation significantly reduced the peak alfentanil plasma concentration at 5 min and prolonged the period in which low but measurable levels of alfentanil could be measured in plasma. These pharmacokinetic data demonstrate that liposome encapsulation resulted in a slow but prolonged appearance of free alfentanil into a diffusion pool available for uptake into the spinal cord. Consistent with the lower peak plasma concentration of alfentanil, the liposome group demonstrated a significantly lower incidence of catalepsy, indicating less systemic redistribution of alfentanil to supraspinal sites. Liposome encapsulation thus appears to produce a significant reduction in peak plasma concentration with a concomitant reduction in systemic side effects and an increase in the duration of action for a given intrathecal dose of the otherwise rapidly cleared alfentanil. (Key words: Analgesic techniques: intrathecal; spinal. Liposomes. Opioids: alfentanil.)

INTRATHecal ADMINISTRATION of opioids can provide selective spinal analgesia in a variety of clinical situations, including postoperative pain, cancer pain, and nonmalignant chronic pain.1,2 Morphine is commonly used for intrathecal administration because of its relatively long duration of action. However, a potential disadvantage is its tendency to spread rostrally in the cerebrospinal fluid (CSF) to reach brainstem opiate receptors, where it may cause significant respiratory depression and sedation. It has been argued that since the more lipid-soluble 4-ani-lopiperidines (e.g., fentanyl, alfentanil, sufentanil) are cleared more rapidly from the CSF, these drugs will be less likely to redistribute rostrally by bulk flow.3,4 The spinal use of these agents is, however, limited by their relatively short duration of action. While larger doses may be used to increase the duration of effect, there is a growing appreciation that the rapid movement of these agents into the vasculature results in significant plasma levels that also can evoke prominent supraspinal side effects.5-7

Two approaches may be used to overcome the pharmacokinetic limitations of these lipid-soluble agents. First, intrathecal catheterization techniques may be used to allow repeated or continuous dosing. However, this approach requires a continuous infusion pump or trained personnel to reinject the catheter at frequent intervals.

A second approach is to administer the spinal agent in a diffusion-modifier. For example, work with cycloextrin has resulted in some success in reducing the fraction of the injected dose available for diffusion.8,9 Recent studies have also reported success in prolonging the effect of rapidly cleared agents such as local anesthetics by incorporating them in a variety of encapsulation matrices, including liposomes,10,11 iophendylate,12 and polyanhydride polymers.13 These studies have been directed largely at peripheral sites of drug action. There are few studies aimed at developing a time-release vehicle for intrathecal opioid administration.

The goal of this investigation was to study the feasibility of using liposomes as a sustained-release vehicle for intrathecal administration of alfentanil, a highly diffusible 4-ani-lopiperidine opioid. This agent has been shown to produce a powerful dose-dependent analgesia that is extremely short-lasting and frequently accompanied by supraspinal effects, notably catalepsy, when administered intrathecally in an animal model.14

Materials and Methods

These experiments were approved by the University of California, San Diego Animal Care Committee, and American Association for Laboratory Animal Care guidelines were followed throughout.
Male Sprague-Dawley rats (250–300 g) were used for all experiments. The rats were housed in individual cages in a light-cycled room (12 h light, 12 h dark) and were provided free access to food and water.

**ANIMAL PREPARATION**

The details of implanting chronic intrathecal catheters in the rat have been described previously. Briefly, during halothane anesthesia a permanent lumbar intrathecal catheter (polyethylene, 0.8 mm OD, 9 cm length) was inserted into the spinal subarachnoid space through a slit made in the cisternal membrane. The tip of the catheter was positioned at the thoracolumbar level of the spinal cord. The external portion was tunneled subcutaneously and fixed on top of the skull. The catheter was plugged using a stainless steel wire. Only animals with normal behavior and motor function 4–7 days after catheter implantation were used for further study.

**LIPOSOME PREPARATION**

Liposomes were prepared by dissolving dipalmitoylphosphatidyl choline (190 mg, 0.26 mmol) and cholesterol (50 mg, 0.13 mmol) in 5 ml chloroform. The solution was then evaporated to dryness in a rotary evaporator at 60°C under low vacuum. Alfentanil hydrochloride (45 mg, 0.1 mmol) was dissolved in 1.5 ml normal saline and 3H-alfentanil (0.1 mCi, specific activity 12.5 mmol/Ci, radiochemical purity 98.6%) was added as a radiotracer. This solution was added to the dried lipid film, and the aqueous and lipid phases were rotated together at 60°C for 30 min and at room temperature for 1 h. The resultant liposome suspension was repeatedly frozen in an acetone dry-ice bath and thawed at room temperature six times. The liposomes were then diluted to 10 ml in saline and centrifuged at 22,000 × g and the supernatant decanted. This process was repeated until all the unencapsulated drug had been removed (three or four washings). Removal of the unencapsulated drug was verified by measuring the alfentanil concentration in the supernatant using radiotracer techniques. Removal was considered complete when the supernatant contained less than 0.5% as much alfentanil as was encapsulated. Encapsulation efficiency averaged 94%. These procedures resulted in the creation of a liposome preparation in which the typical particle profile was a series of multimamellar, fluid-filled vesicles. All procedures were carried out under sterile conditions. Alfentanil and 3H-alfentanil were gifts from Janssen Biochimica/Biotech. Dipalmitoylphosphatidyl choline and cholesterol were purchased from Sigma Chemical Company (St. Louis, MO).

After preparation, liposomes were spun into a pellet and stored under nitrogen at 25–30°C for 24–48 h until needed for the study. To assess the effect of storage on the liposomes, we studied a separate batch of liposomes that were not used for any of the animal tests in the study. We found that freezing and subsequent thawing resulted in leakage of approximately 30% of the encapsulated alfentanil and that this leakage was unaffected by duration of freezing. Therefore, the liposome preparations administered to the rats in this study contained approximately 70% of the dose encapsulated in the liposomes and 30% free in the saline-diluent.

**INTRATEHICAL DRUG ADMINISTRATION**

For the liposome group, liposomes containing alfentanil were resuspended in a suitable volume of 0.9% sodium chloride so that 10 μl contained the desired dose. Likewise, for the saline group, alfentanil hydrochloride was dissolved in 0.9% sodium chloride so that 10 μl contained the desired dose. The solutions were injected intrathecally over 20 s by hand. After each injection, the catheter was flushed by a subsequent injection of 10 μl 0.9% sodium chloride (catheter dead space less than 5 μl). The doses of alfentanil (plain and liposome-encapsulated) used in this study were 1, 3, 10, 30, and 50 μg. Each animal was tested with only one drug dose. The number of animals tested at each dose (distributed evenly between the two groups) was as follows: 1 μg, n = 12; 3 μg, n = 10; 10 μg, n = 10; 30 μg, n = 12; and 50 μg, n = 12.

**NOCICEPTIVE TESTING**

Nociceptive testing was performed in both groups using hot-plate and paw-pressure tests at time 0, before drug administration, and then at 2, 5, 15, 30, 60, and 120 min following intrathecal injection of each drug dose. The hot-plate test was performed by placing the animal on a metal plate heated to 52.5 ± 0.5°C. The test measured the latency between placement of the animal on the hot plate and the behavioral end-point. For this test, the behavioral end-point was hind paw licking or jumping. To prevent tissue damage, failure to respond by 60 s on the hot-plate test resulted in termination of the test, and the response latency was recorded as 60 s.

The paw-pressure test was performed using both right and left hind paws. The animal's paw was placed between two posts of the testing apparatus (Ugo Basale, Comerio, Italy), and the pressure on the paw was increased steadily from 0 to 400 g at the rate of 20 g/s. The test measured the amount of weight the animal would tolerate before withdrawing its paw. The results for the right and left hind paw then were averaged to yield the maximum tolerated weight. To prevent tissue damage, the maximum pressure applied was limited to 400 g. For animals that failed to respond at 400 g, a maximum tolerated weight of 400 g was recorded.
INTRATHecal ALFentanil IN LIPOSOMES

BEHAVIORAL OBSERVATIONS

In rats, systemic redistribution of opioids from spinal CSF to the brain results in a significant catalepsy. To assess this state we used the bar test. The forepaws of the rat were placed on a horizontal bar kept at a distance of 4 cm from the table surface. Failure to move from the bar within 20 s was considered a positive cataleptic response.

ALFENTANIL CONCENTRATIONS

Plasma Concentration

To determine the effect of liposome encapsulation on alfentanil plasma concentrations following intrathecal injection, a second group of animals was injected with 30 μg alfentanil in saline or in liposomes. At 5, 15, 45, and 135 min after intrathecal injection, four to six animals in each group were anesthetized with halothane; blood was collected by cardiac puncture for later measurement of alfentanil plasma concentrations; and the animals were sacrificed by decapitation. The blood samples were immediately frozen (−40°C) and stored at this temperature until analyzed.

Measurement of Alfentanil Concentration

Alfentanil concentration in liposomes and supernatant from liposome washings was determined by liquid scintillation counting. Ten-microliter samples of the liposome suspension or 10-ml samples of the washing supernatant were added to 10 ml Hydrofluor® scintillation cocktail and counted in a Packard scintillation counter (Tri Carb 2000) until the standard deviation of depurations per minute was ≤2% or until 50 min elapsed.

Alfentanil was analyzed by a modification of the technique of Woestenborghs et al. Briefly, this involves addition of an internal standard (R-38527), alkalinization of the sample with sodium hydroxide (1 ml, 0.1 M), and extraction into heptane:isoamyl alcohol (98.5:1.5). The organic layers were transferred to a clean tube and back-extracted with 2 ml 0.05 M sulfuric acid. The acid phase was alkalinized by addition of 200 μl concentrated ammonium hydroxide and reextracted with 4 ml heptane:isoamyl alcohol. The organic phase was then transferred to a clean tube and evaporated to dryness at 60°C under nitrogen, and the residue was dissolved in 50 μl methanol for gas chromatography—mass spectroscopy analysis.

Alfentanil concentrations were determined by injection (splitless) of 5–50 μl of the reconstituted extract onto the column of a Hewlett-Packard gas chromatograph with mass spectroscopy detector. Extraction recoveries averaged 94%. The limit of detection for the alfentanil assay was 0.1 ng/ml plasma, and assay variability was 6.5% at 5 ng/ml across days.

STATISTICAL ANALYSIS

Hot-plate resopne latencies (seconds) and paw-pressure tolerances (grams) are expressed as a percentage of the maximum possible effect (% MPE):

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\%MPE = \frac{(postdrug latency - predrug latency)}{(cutoff latency - predrug latency)} \times 100
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To calculate %MPE for the paw-pressure test, weight tolerance was substituted for latency in the above equation.

To compare the time course of analgesia produced by plain alfentanil and alfentanil in liposomes, the data were expressed as the area under the curve (AUC) of the time-effect (%MPE) plot. Area was calculated using the trapezoidal rule, in which height was the %MPE and the base was time.

Data are expressed as mean ± standard error of the mean. Intergroup comparisons for all data except alfentanil plasma concentration were performed using two-way analysis of variance. Student’s t test was used for post hoc testing. Differences between groups for plasma alfentanil concentration were assessed by unpaired Student’s t test. Differences between groups in the incidence of catalepsy (rigidity) were determined by Fisher’s exact test. Results of all tests were considered statistically significant at P < 0.05.

RESULTS

ANTINOCEPSION

The intrathecal injection of alfentanil resulted in rapid, short-lasting elevation in hot-plate latencies and paw-pressure tolerances with the duration of elevation being significantly prolonged when the agent was administered in liposome vehicle (fig. 1). The increase in hot-plate latencies and paw pressure tolerances (i.e., analgesia) was dose-dependent over the range of 1–50 μg (fig. 2). Comparison of the peak effect produced by a given dose of intrathecal alfentanil revealed no effect of vehicle.

As suggested by the time–effect curves in figure 1, there appeared to be a significant prolongation of analgesia in the liposome group, with no change in peak analgesia. This is confirmed by plotting the AUC versus dose. As indicated in figure 3, there was a statistically significant increase in the AUC as a function of dose when the agent was administered in the liposome vehicle. Thus, for the paw-pressure test, the dose-dependent increase in AUC was significantly greater for the liposome group as compared to the saline group (P < 0.0001), indicating marked prolongation of analgesia to paw pressure by encapsulating alfentanil in liposomes. For the hot-plate test, although the dose–response curve for alfentanil delivered in liposomes lay to the left of the curve obtained with saline vehicle, this difference did not achieve statistical signifi-
catalepsy in the liposome group, indicating less redistribution of alfentanil to brain.

**ALFENTANIL CONCENTRATIONS**

Figure 6 shows the plasma concentration of alfentanil in animals given 30 μg intrathecal alfentanil in saline or in liposomes. As anticipated, the peak plasma concentrations occurred during the first postinjection sample at 5 min for both the saline and liposome groups. However, after the same intrathecal dose, peak plasma concentration in the saline group was twice that observed in the liposome-treated animals. Consistent with the slower clearance from the spinal canal, plasma alfentanil concentrations in the liposome-treated group were significantly higher than those in the saline group at 45 and 135 min.

**Discussion**

In the present study, we observed that the intrathecal administration of alfentanil in a liposome-encapsulated...
preparation resulted in a prolongation in the duration of analgesic action, a reduction in peak plasma concentrations of the agent, and a concurrent reduction in the side effects that typically result when high doses of this diffusible agent are administered.

Drug release from multilamellar liposomes in a protected environment like the CSF may occur by 1) simple diffusion down a concentration gradient from the liposome into the CSF and/or 2) by the progressive dissolution of the liposome. In recent studies examining the resident time in rat lumbar CSF of multilamellar vesicles, it was shown that the half-life greatly exceeded that of inulin (approximately 100 h vs. 0.5 h), suggesting a prolonged residence time of the intact liposomes.** Given the time course of the alfentanil effects observed in the present experiments (i.e., approximately 2 h), we hypothesize that the appearance of free alfentanil reflects the movement of the drug through the vesicle walls.

Pharmacokinetic evidence that the liposomes in this study released alfentanil slowly over time is provided by the plasma concentration versus time data for the two groups (fig. 6). The peak plasma concentration of alfentanil in the saline group was more than twice that in the liposome group, indicating a rapid movement of alfentanil from the CSF to the plasma in the saline group. In the liposome group, alfentanil was presumably sequestered in the liposomes and was thus not available for free diffusion into spinal cord and then plasma. After the peak in plasma concentration, plasma alfentanil concentration decreased more slowly in the liposome group so that at the 45- and 135-min sample times the liposome group

** Yaksh TL, Jang JD, Kim S: Unpublished observations.

FIG. 3. Log dose versus area under the time–effect curve (AUC) curves for paw pressure (top) and hot plate (bottom) tests. *P < 0.05 compared to saline control.

FIG. 4. Plot of the area under the time–effect curves (AUC) versus the percent of the maximum possible effect (%MPE). *P < 0.05 compared to saline control.

FIG. 5. Log dose versus percent of animals developing rigidity following alfentanil in saline or in liposomes. *P < 0.05 compared to saline control.
had a significantly higher plasma alfentanil concentration compared to the saline group. This, too, suggests that alfentanil was being released slowly over time by the liposomes. We suspect that the plasma concentration differences between the two groups reflect concentration differences in the CSF and spinal cord, i.e., that the liposome group has a relatively lower peak but a more sustained alfentanil concentration in these two compartments as well. However, it would be overly simplified to assume that there was a linear relationship between plasma, spinal cord, and CSF concentrations.

In rats, rigidity following intrathecal injection of opioids results from systemic redistribution of opioid from the lumbar CSF to brain opiate receptors.19 The incidence of catalepsy in the saline group was greater than that in the liposome group after any given dose of alfentanil, although the difference reached statistical significance only at the 50-μg dose. This result is consistent with the higher peak plasma concentrations observed in the animals in the saline group. These observations provide functional data that emphasize the ability of liposomes to restrict the redistribution of the intrathecally injected agent.

The antinociceptive data demonstrated that all doses of alfentanil resulted in similar peak analgesic effects in both groups. This similarity in peak effects was somewhat surprising, since it suggests that the local drug bioavailability producing the acute effect was comparable between the groups. The explanation for this equivalent initial analgesic effect lies, at least in part, in the fact that approximately 30% of the alfentanil in each administered dose was free in solution and not encapsulated in the liposome. Thus, some alfentanil was immediately available for uptake into the spinal cord. This unencapsulated fraction resulted from the release of some alfentanil from the liposomes as they were frozen for storage and thawed for use. The prolonged action and the lower incidence of catalepsy are, however, consistent with the plasma concentration data and suggest a continued release of alfentanil from the liposomes into a spinal biophase proximate to the spinal opioid receptor.

The magnitude of the prolongation of analgesia by liposomes was greater in the paw-pressure than the hot-plate test. This was not anticipated and is not readily explained. One explanation may lie in the fact that the dose-response curve for the hot-plate test lay to the right of the paw pressure dose–response curve. In other words, as the MPE has been defined for these two tests, a higher dose (i.e., spinal cord tissue concentration) of alfentanil was required to produce an equivalent %MPE in the hot-plate test as compared to the paw-pressure test. As a result, cord concentrations of alfentanil in the liposome group that were adequate to produce significant analgesia in the paw-pressure test were inadequate to produce significant analgesia in the hot-plate test.

In a study with similar aims, Langerman and co-workers reported that analgesia from intrathecal meperidine could be prolonged by administering the drug in a lipid solution (iophendylate, Pantopaque).20 In their study, iophendylate did produce a prolonged analgesic effect, but it also significantly reduced the peak analgesic effect and delayed the onset of analgesia. In contrast, the liposome vehicle used in this study has the advantage that the onset of analgesia was not delayed and the intensity of analgesia was not reduced in either analgesic test. This may be because the approximately 30% free fraction of alfentanil in the liposome solution acted essentially as a loading dose. In addition, the outer layers of the liposomes release drug relatively rapidly, and this contributes to early analgesia equivalent to that produced by alfentanil in the saline vehicle. Analgesia then persists in the liposome group because the inner layers of the liposomes continue to release drug more slowly.

The issue of potential neurotoxicity is important in evaluating any drug preparation for intrathecal use. The lipids chosen for this study occur naturally in mammalian cells, where, of course, they are not toxic in those concentrations. It is possible that the lipids might be toxic in the relatively large concentrations present in the liposomes we administered. However, histopathologic studies in animals have demonstrated no neurotoxicity following injections of dipalmitoylphosphatidylcholine/cholesterol liposomes into the brain.21 In addition, in a separate series of studies, we have administered five times the dose of liposomes used in this study with no change in the animal's response to hot-plate testing, paw-pressure testing, or motor function.†† We conclude from this that this lipo-

†† Yaksh TL, Jang JD: Unpublished observations.
some preparation is neither toxic nor itself analgesic. It is also possible that the liposomes will produce high local concentrations of drug that might be toxic at the site of drug release. However, that does not appear to be the case with alfentanil, in that all animals behaved normally neurologically after testing. Finally, it is likely that the liposomes will prolong both beneficial (e.g., analgesia) and detrimental (e.g., respiratory depression) effects of intrathecal opioids. Therefore, additional studies are necessary to define the therapeutic margin for liposomal opioids.

In summary, liposome encapsulation permits the delivery of relatively large concentrations of drug in a form that is sequestered and released slowly over time into the local biophase. This reversible sequestration allows the progressive exposure of the local tissue to relatively steady-state concentrations of agent over an extended period. By reducing the bioavailability of the encapsulated drug, the initial high-dose effects associated with redistribution (such as respiratory depression or allodynia), or direct high-dose tissue toxicity (e.g., neurotoxicity caused by high concentrations of local anesthetics or by chemotherapeutic agents) may be reduced. These possibilities are confirmed in the present studies with alfentanil and suggest that liposomes may be an effective method to prolong the otherwise short duration of analgesia produced by the 4-anilopiperidine opiates and other agents such as local anesthetics. Additional work will be necessary to define the optimal liposome composition, the relative toxicity, if any, of the liposome preparations, and the most appropriate opioid.

References

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