ANTAGONISM BYNALOXONE OF TOLERANCE AND DEPENDENCE IN MICE GIVEN A SINGLE DOSE OF MORPHINE

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Abstract—The effect of naloxone given at various times after morphine administration on the development of tolerance to and dependence on a single dose of morphine was studied. Naloxone antagonized the analgesic effect of morphine and the development of tolerance to and dependence on morphine, dose dependently. The time course of the development of tolerance to a single dose of morphine almost paralleled that of dependence on morphine but the time course of the disappearance of tolerance did not coincide with that of dependence. When start of the duration of action of morphine was blocked by naloxone for various time intervals, the degree of tolerance to and dependence on morphine was antagonized, time dependently. When the end of the duration of action of morphine was antagonized by naloxone for various time intervals, tolerance and dependence which developed up to that time was completely antagonized by naloxone.

Many investigators have reported that the narcotic antagonist, naloxone, antagonizes the analgesic effect of morphine and the development of tolerance to and dependence on morphine (1–9). But as the duration of action of naloxone is shorter than that of morphine, its utility as a narcotic antagonist is limited (10–13). Several investigators suggested that naloxone given before or simultaneous with morphine administration antagonized the development of tolerance to and dependence on morphine, dose dependently (2, 3, 4, 6). However, with respect to the antagonism of naloxone given after morphine, a definite conclusion has not been reached (7, 23).

We previously reported (9) that complete antagonism of tolerance and dependence in either acutely or chronically morphinized mice requires a continuous complete blockade of the narcotic receptors by naloxone, given before morphine, and even the slightest exposure of the narcotic to the receptor results in some degree of tolerance and dependence.

In the present work, we attempted to quantify the degree of tolerance to and dependence on a single dose of morphine when the analgesic effect of morphine is incompletely antagonized with varying doses of naloxone given simultaneously or after morphine administration.

MATERIALS AND METHODS

Male ddY mice weighing between 20 and 25 g used in all experiments were housed in
our laboratory for at least 3 days prior to experimentation and were used only once. Food and water were provided ad libitum. Haffner's tail-pinching method (14) which was modified by Takagi et al. (15) was used for determining the degree of analgesia. Mice were rendered tolerant to and dependent on morphine by a single s.c. dose of morphine hydrochloride (100 mg/kg) and analgesic assays using a test dose of morphine (s.c.) were performed 5 hr later. By this time the tail-pinching reaction time had returned to predrug control level. The control mean reaction time was 1.60±0.22 (mean±S.E.) sec. At least 30 animals were used to determine each dose response curve and ED50 value. The ED50 values, their 95% confidence limit and the significance of potency ratio between two ED50 values were determined by the method of Litchfield and Wilcoxon (16).

The degree of physical dependence on morphine was assessed by estimating the amount of naloxone required to induce withdrawal symptoms. Morphine-treated mice were placed singly into 30×30 cm clear plexiglass cylinders immediately after reinjection of various doses of naloxone (i.p.). The number of jumps and the degree of withdrawal symptoms during the succeeding 15 min period were recorded. Both values showed a similar pattern exclusive of the early stage after morphine administration. The following signs were mainly scored: jumping, wall climbing behavior in the plexiglass cylinder including rearing, restlessness, defecation including diarrhea and urination. These signs were given scores of 0 (absent), 1 (mild) or 2 (marked). Scores for all of the signs were summed to provide a total score representing withdrawal severity for each mouse. The ED50 of naloxone which induced withdrawal symptoms was estimated by the method of Dixon (17) using at least 4 groups with 4-5 animals to a group.

In order to quantify the antagonism of naloxone on the development of tolerance to and dependence on a single large dose of morphine, the following 4 experiments were carried out. In the first experiment, mice were treated with 100 mg/kg of morphine together with various doses of naloxone and the response times were measured every 30 min for 5 hr by the tail-pinching method. The experimental procedure of the 2nd experiment was the same as the above in which animals were given various doses of naloxone co-administered with 100 mg/kg of morphine. Five hours after injection of these drugs, the degree of tolerance and dependence was assessed as described above. In the next experiment, to estimate the degree of the development and the regression of tolerance and dependence after a single dose of morphine, the ED50 values of morphine and naloxone were measured at various times after morphine administration. To investigate the degree of development of tolerance to and dependence on a single dose of morphine, when the analgesic effect of morphine is antagonized with 2.5 mg/kg of naloxone given simultaneously or after morphine administration, we attempted to design a 4th experiment. As the time effect of 2.5 mg/kg of naloxone was about 1 hr, the duration of action of morphine was divided into 5 periods of 1 hr each and then it was blocked by naloxone for various intervals. ED50 values of morphine and naloxone were measured 5 hr after injection of morphine.

Morphine hydrochloride was obtained from Takeda Pharmaceutical Co. Ltd. (Osaka). Naloxone hydrochloride was a gift from Sankyo Pharmaceutical Co. (Tokyo), Dr. E.L.
Way's Laboratory and Endo Laboratories (Garden City, N.Y.). The drugs were dissolved in saline solution and the volume of 10 ml/kg was injected into each animal.

RESULTS

1. Antagonism by naloxone of morphine analgesia

When various doses of naloxone were co-administered with 100 mg/kg of morphine (s.c.), the analgesic response time of morphine was inhibited according to the doses of administered naloxone as shown in Fig. 1.

Since the duration of action of naloxone lasted for only 1.5 hr when 20 mg/kg of naloxone was administered, the longer lasting analgesic effect of morphine became manifest after this time period. In order to suppress the analgesic effect of morphine for over 5 hr periods, therefore, continuous complete blockade of the narcotic receptor was required, i.e. naloxone had to be given more than 3 times.

2. Effect of naloxone on the development of single dose tolerance and dependence

A single large dose of morphine could produce a considerable tolerance and dependence in mice as is shown in Table 1. About 3.5 fold increase in the ED50 of morphine and a considerable decrease in ED50 of naloxone were seen. Less than 1.25 mg/kg of naloxone

![Fig. 1. Antagonism by various doses of naloxone on a single dose of morphine analgesia. Mice were given a single dose of morphine 100 mg/kg, s.c, together with various doses of naloxone and the analgesic response times were measured every 30 min for 5 hr. The figures under the curves refer to the doses of naloxone. The group of 0 (saline) were given saline instead of naloxone together with morphine. Each group included 10-12 animals.](image-url)
### Table 1. Antagonism by various doses of naloxone in development of tolerance to and dependence on a single dose of morphine

<table>
<thead>
<tr>
<th>Morphine (mg/kg)</th>
<th>Naloxone (mg/kg)</th>
<th>ED50 of morphine: mg/kg (95% confidence limits)</th>
<th>ED50 of naloxone mg/kg (mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
<td>a13.1 (9.35–18.34)</td>
<td>2.08 ± 0.41</td>
</tr>
<tr>
<td>100</td>
<td>0.6</td>
<td>a12.0 (8.51–16.92)</td>
<td>3.43 ± 0.92</td>
</tr>
<tr>
<td>100</td>
<td>1.25</td>
<td>a10.2 (8.29–12.54)</td>
<td>4.94 ± 1.21</td>
</tr>
<tr>
<td>100</td>
<td>2.5</td>
<td>a9.4 (7.46–11.84)</td>
<td>9.68 ± 2.61c</td>
</tr>
<tr>
<td>100</td>
<td>5</td>
<td>a7.5 (5.18–11.05)c</td>
<td>12.06 ± 2.06c</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>6.6 (4.83–9.06)c</td>
<td>38.59 ± 5.73c</td>
</tr>
<tr>
<td>100</td>
<td>20</td>
<td>6.5 (4.74–8.90)c</td>
<td>59.96 ± 7.39c</td>
</tr>
<tr>
<td>100</td>
<td>25</td>
<td>6.1 (4.50–8.32)c</td>
<td>73.29 ± 8.35c</td>
</tr>
<tr>
<td>100</td>
<td>30</td>
<td>5.4 (4.24–6.95)c</td>
<td>&gt;80</td>
</tr>
<tr>
<td>100</td>
<td>40</td>
<td>5.1 (4.16–6.41)c</td>
<td>&gt;80</td>
</tr>
<tr>
<td>0</td>
<td>0, 40</td>
<td>4.1 (2.57–6.38)c</td>
<td>&gt;80</td>
</tr>
</tbody>
</table>

- a: Injections were given 5 hr before testing the animals.
- b: Various doses of naloxone were concomitantly administered with morphine.
- c: Values are significantly different from those of animals given morphine alone.
- d: Values are significantly different from that of control.

**Fig. 2.** Time course of development and regression of tolerance to and dependence on a single dose of morphine given s.c. Ordinate: ED50 of morphine (right) and ED50 of naloxone (left) (mg/kg). As the ED50 of morphine from 20 min to 5 hr was not measured because of the significant analgesic effect of morphine, broken line is an estimated value.
had no effect on the development of tolerance to and dependence on 100 mg/kg of morphine. When more than 5 mg/kg of naloxone was co-administered with morphine, ED50 values of morphine and naloxone were significantly different from those of animals given morphine alone. With increasing doses of naloxone, ED50 values of morphine and naloxone gradually approached those of normal levels.

3. *Time course of the appearance and disappearance of tolerance and dependence*

ED50 value of naloxone (degree of dependence) was not detectable within 20 min, but some abstinence symptoms (restlessness, climbing behavior) were observed 5–10 min after morphine injection. After that time period, a considerable increase in development of dependence was seen, and a maximum state was reached 4 hr after injection of morphine, thereafter decreasing gradually and disappearing after 36 hr (Fig. 2).

ED50 value of morphine (degree of tolerance), however, could not be detected from 20 min to 5 hr because of the significant analgesic effect. Estimated values of the development of tolerance are indicated by the broken line in Fig. 2, peaking at 4 hr after morphine injection. ED50 of morphine decreased gradually thereafter but was still observed 72 hr later. Thus, the tolerance to and dependence on morphine may rapidly develop in a short period in acutely morphinized mice, but the time course of the regression of tolerance does not approach normal levels.

![Graph](image)

**Fig. 3.** Antagonism of naloxone given at various times, on the analgesic effect of a single dose of morphine given s.c. Morphine was administered at time 0. 2.5 mg/kg of naloxone was given at time 0, 1 hr, 2 hr and 3 hr after injection of morphine (arrow parts). Morphine analgesia was suppressed for about 1 hr by 2.5 mg/kg of naloxone.
not coincide with that of dependence.

4. Development of tolerance to and dependence on a single dose of morphine which was incompletely antagonized by 2.5 mg/kg of naloxone at various periods of the duration of action of morphine

When mice were challenged with 2.5 mg/kg of naloxone at the same time, 1 hr, 2 hr or 3 hr after 100 mg/kg administration of morphine, the analgesic effect of morphine was antagonized for about 1 hr before returning to the normal level (Fig. 3).

Using this method, we measured the degree of development of tolerance and dependence in mice in which the analgesic effect of morphine was antagonized by 2.5 mg/kg of naloxone at various intervals following a single large dose of morphine. Results are summarized in Fig. 4. ED50 values of morphine and naloxone in mice treated with morphine alone

**Fig. 4. Development of tolerance to and dependence on a single dose of morphine given s.c. and which was incompletely antagonized by 2.5 mg/kg of naloxone for various time intervals.**

As the duration of action of 2.5 mg/kg of naloxone was about 1 hr as is shown in Fig. 3, the duration of action of morphine was divided into 5 periods of 1 hr each, and then it was blocked by naloxone for various intervals. ED50 values of morphine and naloxone in mice treated with morphine alone 100 mg/kg of morphine was injected at time 0.

**A:** The beginning period of the duration of action of morphine was blocked by naloxone at various intervals. 2.5 mg/kg of naloxone was given s.c. and hourly at these points (*), B: One hr inhibition by naloxone was carried out at various times. C: The latter period of the duration of action of morphine was antagonized by naloxone at various time intervals.

**a:** Values are significantly different from those of animals given morphine alone.

**b:** Values are significantly different from that of control.
were 13.1 (9.35-18.34), 2.08±0.41 mg/kg, respectively. When the duration of action of morphine was blocked by naloxone for initial periods of 1 hr, 2 hr, 3 hr and 4 hr, the ED50 values of morphine and naloxone were affected, time dependently in proportion to the period of morphine exposure after naloxone blockade (Fig. 4A). A considerable degree of tolerance and dependence was seen when naloxone blockade was performed for the initial 1 or 2 hr periods of the duration of action of morphine and the greater part of development of tolerance and dependence was inhibited when naloxone blockade was carried out for the initial 3 hr period.

Next, one hr inhibition using the same dose of naloxone was performed at 1 hr, 2 hr, 3 hr, 4 hr and 5 hr after injection of morphine (Fig. 4B). When mice sustained 1 hr inhibition at 2 hr after morphine, the ED50 values of morphine and naloxone were similar to those seen in animals antagonized by naloxone for the initial 2 hr period of morphine effect. In mice sustaining a 1 hr inhibition at 3 hr after morphine, these values resembled those seen in mice in the 3 hr period inhibition, etc. These results indicate that 1 hr inhibition with naloxone at various periods can antagonize the tolerance and dependence developed in the animals up to that time.

To investigate the above results in detail, mice treated with a single dose of morphine were given naloxone for 1 hr, 2 hr, 3 hr and 4 hr of the latter period of the duration of action of morphine, respectively. As indicated in Fig. 4C, ED50 values of morphine and naloxone were much the same and were not significantly different from those seen in non-treated mice, despite previous exposure of morphine to the receptor.

DISCUSSION

Since Way et al. (18) reported an inverse relationship between the degree of dependence and the amount of naloxone required to induce jumping responses, the naloxone induced jumping method for assessment of the degree of dependence has been widely used in many laboratories. It has been previously reported that naloxone induces jumping in mice given only a single injection of morphine (3, 4, 6, 9, 19, 20). These results indicate that a degree of physical dependence on narcotic drugs can develop within hours after an acute dose of the narcotic.

With respect to the onset of the development of tolerance to and dependence on a single dose of morphine, Kaneto et al. (3), Kosersky et al. (4), Smits (6), Huang et al. (21) and Tremblay et al. (22) reported that the time course for the development of the naloxone induced jumping behavior, after an acute dose of morphine, was relatively short (20-30 min). Our results in this experiment are in good agreement with these data.

Barthelemy and Jacob (23) and Kosersky et al. (4) suggested that the extremely rapid onset of jumping activity following morphine might involve factors other than physical dependence. On the other hand, Smits (6) declared that naloxone induced jumping is not due to an agonist effect of morphine but is actually a sensitive index of withdrawal in a morphine dependent mouse. In our experiment herein, restlessness and climbing behavior observed after naloxone challenge were manifest even 5-10 min after morphine injection.
while defecation, urination and jumping behavior were not observed at such an early stage. All of these withdrawal symptoms were clearly observed 30 min after morphine administration.

The rapid onset of tolerance to narcotics in these mice was not specifically determined owing to the significant analgesic effect of morphine, but it does appear that the development of tolerance to morphine may occur together with that of dependence. Yamamoto et al. (24) gave a continuous infusion of morphine to rats and found that the regressive time course of tolerance did not always coincide with that of dependence. In the present work, the regressive time course of tolerance and dependence showed no parallelism. The ED50 of morphine was undiscernible 36 hr after morphine, while the ED50 of naloxone was observed even 72 hr later.

Several investigators have reported the effects of naloxone given before or simultaneous with morphine administration, on the development of tolerance to and dependence on morphine (2, 5, 6, 9). However, there are few reports related to the effect of naloxone given at various times after morphine administration, on the degree of the development of tolerance and dependence. Mushlin and Cochin (7) suggested that naloxone blocks the development of tolerance to morphine even if given after the morphine-receptor interaction responsible for analgesia has been initiated. Based on the finding that injected naloxone was effective when the proper antinociceptive effect of morphine had regressed almost completely, Barthelemy and Jacob (23) reported that the antagonism of tolerance by naloxone is not only due to the reduction in the analgesic effect of morphine but also to other mechanisms.

Although the duration and the magnitude of the action of naloxone may not be fixed according to the elapsed time after morphine administration, in the present experiment, we designed the experimental procedure described above in such a way so as to estimate the effect of naloxone on the development of tolerance to and dependence on a single dose of morphine.

Naloxone given at the same time or after morphine administration antagonized not only the analgesic effect of morphine and the development of tolerance to and dependence on morphine but also the tolerance and dependence developed in animals up to that time. Our results suggest that the development of tolerance to and dependence on morphine was antagonized time dependently by naloxone when the analgesic effect of morphine was antagonized at earlier periods of the duration of action of morphine. In other words, when the analgesic effect of morphine was blocked by naloxone for 1 hr, 2 hr, 3 hr and 4 hr periods in an initial time, the ED50 values of morphine and naloxone were affected time dependently, in proportion to the period of morphine exposure, after naloxone blockade. When the latter duration of action of morphine was antagonized by naloxone for various time intervals, the development of tolerance to and dependence on morphine was completely antagonized. In addition, our experiments suggest the possibility that the tolerance and dependence developed in mice up to that time were antagonized by naloxone injected at the time of latter duration of the action of morphine. Related experiments are ongoing in our laboratory.

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