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Spinal Antinociceptive Synergism between Morphine and Clonidine Persists in Mice Made Acutely or Chronically Tolerant to Morphine[1]

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ABSTRACT

Morphine (Mor) tolerance has been attributed to a reduction of opioid-adrenergic antinociceptive synergy at the spinal level. The present experiments tested the interaction of intrathecally (i.t.) administered Mor-clonidine (Clon) combinations in mice made *acutely* or *chronically* tolerant to Mor. ICR mice were pretreated with Mor either acutely (40 nmol i.t., 8 h; 100 mg/kg s.c., 4 h) or chronically (3 mg/kg s.c. every 6 h days 1 and 2; 5 mg/kg s.c. every 6 h days 3 and 4). Antinociception was detected via the hot water (52.5°C) tail-flick test. After the tail-flick latencies returned to baseline levels, dose-response curves were generated to Mor, Clon, and Mor-Clon combinations in tolerant and control mice. Development of tolerance was confirmed by significant rightward shifts of the Mor dose-response curves in tolerant mice compared with controls. Isobolographic analysis was conducted; the experimental combined ED_{50} values were compared statistically against their respective theoretical additive ED_{50} values. In all Mor-pretreated groups, the combination of Mor and Clon resulted in significant leftward shifts in the dose-response curves compared with those of each agonist administered separately. In all tolerant and control groups, the combination of Mor and Clon produced an ED_{50} value significantly less than the corresponding theoretical additive ED_{50} value. Mor and Clon synergized in Mor-tolerant as well as in control mice. Spinally administered adrenergic/opioid synergistic combinations may be effective therapeutic strategies to manage pain in patients apparently tolerant to the analgesic effects of Mor.

Coadministration of α_2 adrenergic and opioid receptor agonists results in a multiplicative or greater-than-additive effect, otherwise described as synergy (Ossipov et al., 1990a). That is, when delivered in combination, these drugs can be given in substantially lower doses than when they are administered separately to produce an equivalent antinociceptive effect. Intrathecal coadministration of α_2 adrenergic and opioid receptor agonists continues to be explored as a means to circumvent the disadvantages associated with opioid and adrenergic receptor agonists administered individually (Eisenach et al., 1994). Coactivation of α_2 adrenergic and opioid receptors on spinal neurons produces pronounced behavioral antinociceptive synergy in rat (Wilcox et al., 1987; Monasky et al., 1990; Ossipov et al., 1990a,b) and mouse (Roerig et al., 1992, Roerig, 1995). Morphine (Mor) concurrently applied supraspinally (intracerebroventricular, i.c.v.) and spinally (intrathecal, i.t.) results in antinociceptive synergy between the two sites of administration in rat (Yeung and Rudy, 1980) and mouse (Roerig et al., 1984; Wigdor and Wilcox, 1987; He and Lee, 1997). Systemically administered Mor will activate both supraspinal and spinal opioid receptors. Therefore, analgesia produced by systemic administration of Mor may involve a synergistic interaction between these two sites of opioid receptor activation. Mor applied supraspinally (i.c.v.) results in the release of noradrenaline at the level of the spinal cord (Kuraishi et al., 1978; Yaksh, 1979; Howe and Zieglgänsberger, 1984) presumably through activation of descending monoaminergic systems (Yaksh, 1979; Hammond and Yaksh, 1984; Yaksh, 1985; Wigdor and Wilcox, 1987). Noradrenaline, whether released from descending systems (Wigdor and Wilcox, 1987) or injected i.t. (Hylden and Wilcox, 1983), interacts synergistically with Mor in the spinal cord. Compatible with that proposal, intrathecal coadministration of the adrenergic blocker phentolamine with Mor prevented the synergistic interaction between Mor concurrently applied i.c.v. and i.t. (Wigdor and Wilcox, 1987).

Roerig and colleagues (1984) observed that the synergistic interaction between Mor concurrently administered spinally and supraspinally was reduced to additive in mice made tolerant to Mor by pellet implantation. These authors hy-

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ABBREVIATIONS: Clon, Clonidine; i.t., intrathecal; %MPE, percentage of maximum possible effect; Mor, morphine; i.c.v., intracerebroventricular.

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pothesized that the development of tolerance to systemically administered Mor might be due to a similar alteration in this spinal interaction. Consistent with that proposal, Roerig (1995) reported that the intrathecal antinociceptive synergy between Mor and clonidine (Clon), while present in placebo-pelleted subjects, was reduced to additivity in mice made chronically tolerant to Mor by pellet implantation. In light of the possibility that the outcome of these studies was affected by the presence of residual systemic, pellet-derived Mor at the time of testing, the present experiments sought to isolate this contribution using acute and chronic injection strategies both spinal and systemic. The present experiments tested the antinociceptive interaction between i.t. coadministered Mor and Clon in mice made *acutely* tolerant to both i.t. and systemically administered Mor and mice made *chronically* tolerant to Mor by repeated s.c. injection.

Materials and Methods

Animals. Experimental subjects were 20- to 25-g male ICR mice (Harlan, Madison, WI). These experiments were approved by the Institutional Animal Care and Use Committee. Subjects were housed in groups of 10 in a temperature- and humidity-controlled environment for at least 5 days before experimentation. Subjects were maintained on a 12-h light/dark cycle and had free access to food and water. Each animal was used only once.

Chemicals. Morphine sulfate was a gift from the National Institute on Drug Abuse, and Clon HCl was obtained from Boehringer-Ingelheim Ltd. (Ridgefield, CT). Both drugs were dissolved in 0.9% saline.

Antinociceptive Testing. Nociceptive responsiveness was determined using the warm water (52.5°C) immersion tail-flick test. The latency to the first rapid tail flick represented the behavioral endpoint (Janssen et al., 1963). Baseline measurements of tail-flick latencies were collected on all subjects (for a sample of $n = 1157$, mean = 3.5, S.D. = 1.0). Mice that failed to respond within 5 s to baseline tests were excluded from analysis (7%). To use each animal's baseline tail-flick latency as its own control, percentage of maximum possible effect (%MPE) was determined according to the following formula: %MPE = (postdrug latency − predrug latency)/(cutoff − predrug latency) × 100%. To avoid tissue injury, a maximum score of 100% was assigned to those animals not responding before the 12-s cutoff. Probe drugs were injected i.t. by direct lumbar puncture (Hylden and Wilcox, 1980). All behavioral testing was conducted by the same experimenter.

Induction of Acute Tolerance to i.t. Administered Mor. Mice were made acutely tolerant to Mor by a single i.t. injection of Mor (40 nmol) (Fairbanks and Wilcox, 1997). All acute toleragen (tolerance-inducing agent) injections were administered between 6:00 and 9:00 AM. Approximately 8 h after the injection, tail-flick latencies were collected on all subjects to determine that the tail-flick latencies had returned to baseline levels. Subjects were then tested with Mor (0.2, 0.6, 2, 8, 15, and 20 nmol, i.t.). The tail-flick test was performed 10 min after this probe Mor injection.

Induction of Acute Tolerance to Systemically Administered Mor. Mice were made acutely tolerant to Mor by a single s.c. injection of Mor (100 mg/kg s.c., 100 μl) according the method of Yano and Takemori (1977). Approximately 4 h after the injection, tail-flick latencies were collected on all subjects to confirm that they had returned to baseline levels. Subjects were then challenged with Mor (0.2, 0.6, 2, 8, 15, and 20 nmol, i.t.). The tail-flick test was performed 10 min after this probe Mor injection.

Induction of Chronic Tolerance to Systemically Administered Mor. Mice were made chronically tolerant to Mor by repeated s.c. injections of Mor (3 mg/kg every 6 h days 1 and 2; 5 mg/kg every 6 h days 3 and 4, s.c.). Injections were administered at 12:00 AM, 6:00 AM, 12:00 PM, and 6:00 PM for 4 consecutive days. Saline-pretreated controls (100 μl every 6 h, days 1–4) received equal numbers of injections as the Mor-treated subjects at the same times. Two (Fig. 4) or 6 h (Fig. 3) after the last injection, tail-flick latencies were collected on all subjects to confirm that the tail-flick latencies had returned to baseline levels. Subjects were then challenged with Mor (0.2, 0.6, 2, 8, 15, and 20 nmol, i.t.). The tail-flick test was performed 10 min after this probe Mor injection.

Statistical Analysis. Data describing antinociception are expressed as means of %MPE with S.E.M. Potency changes are presented as dose ratios between the ED_{50} values of different dose-response curves. Statistical comparisons of potencies are based on the confidence limits of the ED_{50} values. A dose-response shift is considered significant when the calculated ED_{50} value of one curve falls outside the confidence limits of the ED_{50} value of the curve to which it is being compared. The ED_{50} values and confidence limits were calculated according to the method of Tallarida and Murray (1987). Groups of 7 to 10 animals were used for each dose. For each experiment, six dose-response curves were generated. These included dose-response curves for Mor, Clon, and Mor-Clon coadministered in both Mor-tolerant and control groups. In the chronic studies, dose-response curves for the drugs administered separately were collected 1 week before the dose-response curves for drugs administered in combination. All Mor dose-response curves are displayed in Figs. 1-4A and Clon dose-response curves in Figs. 1–4B. The dose-response curves of the combination of Mor and Clon are represented in each figure twice: first in terms of the Mor dose in A, and second in terms of the Clon dose in B.

To test for synergistic interactions, the ED_{50} values and the 95% confidence intervals of all dose-response curves were arithmetically arranged around the ED_{50} value using the equation $(\ln(10) \times ED_{50}) \times (\text{S.E. of } \log ED_{50})$. Isobolographic analysis (the appropriate method for evaluating synergistic interactions) (Tallarida and Murray, 1987; Tallarida, 1992) necessitates this manipulation. An *additive* ED_{50} value would be derived from a dose-response curve where the interactions between Mor and Clon merely represent the sum of the effects of each drug when given alone. When testing an interaction between two drugs given in combination for synergy, additivity, or subadditivity, a *theoretical* additive ED_{50} value is calculated for the combination based on the dose-response curves of each drug administered separately. This theoretical value is then compared by a t test ($p < .05$) with the *observed* experimental ED_{50} value of the combination of Mor and Clon. These values are based on total dose of both drugs, in other words, the total dose of Clon plus the total dose of Mor. To compare the drug doses administered separately, we have separated the Clon and Mor components of the observed and theoretical ED_{50} values; these are presented in Tables 1 to 4. An interaction is considered *synergistic* if the observed ED_{50} value is significantly less ($p < .05$) than the calculated theoretical additive ED_{50} value (Tallarida and Murray, 1987; Tallarida, 1992). Additivity is indicated when the theoretical and experimental ED_{50} values do not differ. Drug interactions may also be illustrated through construction of isobolograms, such as are represented in Figs. 1 to 4, C and D. In these graphs the ED_{50} values of Clon and Mor are respectively plotted as the y- and x-axis intercepts. The thicker lines directed from each ED_{50} value toward zero represent the respective lower confidence limits of each ED_{50} value. The straight line connecting these two points is the theoretical additive line. The open circle that lies on or near the theoretical additive line represents the calculated theoretical ED_{50} value of the combination were the interaction merely additive. The closed circle represents the experimentally observed ED_{50} value of the combination of Clon-Mor. If the interaction is synergistic, the closed circle will be plotted significantly below the theoretical additive line and outside the lower confidence limits of ED_{50} values of Clon and Mor. In the present study, probe Mor in tolerant animals did not achieve full efficacy in experiments 2 and 4. Therefore, an ED_{50} value could not be calculated for Mor in those experiments. In those cases, to calculate a theoretical additive ED_{50}

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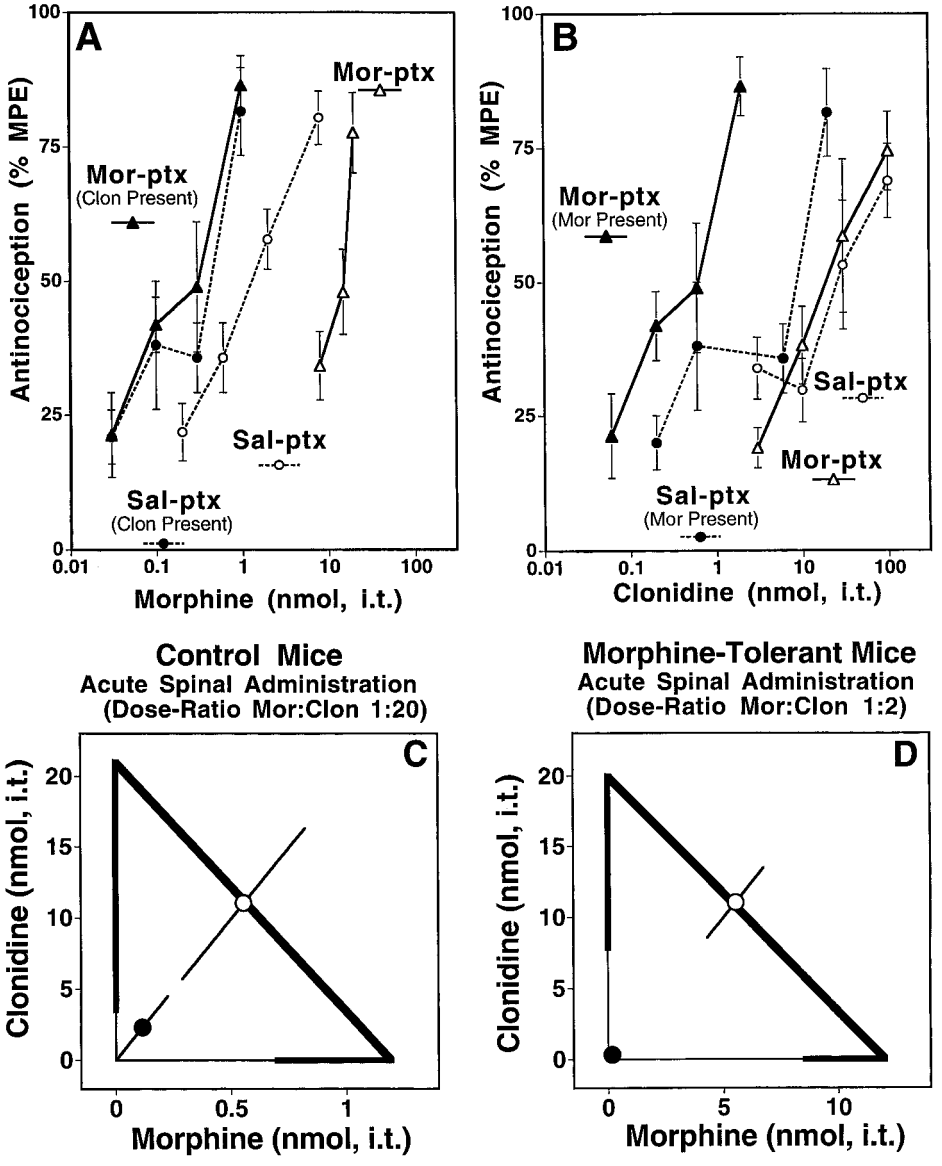

Fig. 1. Acute tolerance to i.t. administered Mor. Dose-response curves for i.t. administered Mor, Clon, and Mor-Clon combination. A, dose-response curves of the spinal antinociceptive effect of : 1) Mor on nontolerant (open circles, dashed line) and Mor-pretreated animals (open triangles, solid line) and 2) Mor in the presence of Clon on nontolerant (closed circles, dashed line) and Mor-pretreated animals (closed triangles, solid line). B, dose-response curves of the spinal antinociceptive effect of : 1) Clon on nontolerant (open circles, dashed line) and Mor-pretreated animals (open triangles, solid line) and 2) Clon in the presence of Mor on nontolerant (closed circles, dashed line) and Mor-pretreated animals (closed triangles, solid line). C, isobolographic representation of the antinociceptive (% inhibition) effect of the combination of Clon-Mor in control mice. The theoretical additive line connects the ED_{50} value of Clon (*y*-axis intercept) to the ED_{50} value of Mor (*x*-axis intercept). The white open circle represents the theoretical additive point where the ED_{50} value of the combination would fall were the interaction merely additive. The experimentally derived ED_{50} value of the combination of Clon-Mor is represented by the filled circle. The combination is considered synergistic when the experimental ED_{50} value differs significantly from that of the theoretical additive ED_{50} value (Student's *t* test). In this isobologram, the ED_{50} value of the combination of Clon-Mor falls below the theoretical additive line and differs significantly from that of the theoretical ED_{50} value; therefore, the combination is clearly synergistic in control mice. D, isobolographic analysis was applied to the data from Fig. 1, A and B, that represent responses to the combination of Clon-Mor in Mor-tolerant mice. In this isobologram, the ED_{50} value of the combination of Clon-Mor falls below the theoretical additive line and differs significantly from that of the theoretical ED_{50} value; therefore, the combination is clearly synergistic in Mor-tolerant mice.

value, we used the method described by Porreca and colleagues (1990) to evaluate synergistic interaction when one of the drugs in the combination is not fully efficacious.

Results

Confirmation of the Induction of Acute Tolerance to i.t. Administered Mor. We determined dose-response curves for the effects of Mor in the tail-flick test in naive, saline-pretreated, and Mor-pretreated (40 nmol, i.t.) mice. Morphine dose-response curves did not differ between naive or saline-pretreated mice (data not shown). Data from two naive and one saline-pretreated dose-response curves were pooled to generate a nontolerant dose-response curve (Fig. 1A, ED_{50}: 1.2 nmol, 0.7–1.7). Mor pretreatment increased the ED_{50} value in a dose-dependent manner (Fig. 1A). Data from three Mor-pretreated (40 nmol, i.t.) dose-response curves were pooled and are represented in Fig. 1A. Pretreatment with 40 nmol of Mor produced a 9.6-fold rightward shift in the Mor dose-response curve (ED_{50}: 12 nmol, 8.5–15). This dramatic rightward shift confirms the induction of Mor tolerance in this acute model. These data served to characterize our acute spinal tolerance model, were conducted concurrently with the present set of experiments, and have been presented previously (Fairbanks and Wilcox, 1997). They are plotted here for the purpose of comparison to the present results.

Synergy Detectable in Mice Made Acutely Tolerant by i.t. Administered Mor. Intrathecal administration of Clon produced an antinociceptive dose-response curve with an ED_{50} value of 21 nmol (11–30) in Mor-pretreated (40 nmol, i.t.) animals (Fig. 1B). This value is comparable to that of Clon administered to saline-pretreated mice (ED_{50}: 24 nmol, 6.2–42; Fig. 1B), indicating no apparent cross-tolerance. Based on these ED_{50} values, the Mor-Clon equieffective dose ratios were determined to be 1:20 in the nontolerant mice and 1:2 in the Mor-tolerant mice. Administration of Mor-Clon combinations in either Mor-tolerant or control mice produced leftward shifts in the dose-response curves for each drug administered in the presence of the other compared to each

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drug administered separately (Fig. 1, A and B). The shifts are significant; the ED_{50} values for each drug administered in combination is significantly lower than that of each drug administered separately (Table 1). This holds true for both pretreatment groups. In the isobolograms representing the drug interaction in controls (Fig. 1C) and Mor-tolerant animals (Fig. 1D), the experimental point (closed circle) falls significantly below the theoretical additive line and calculated theoretical additive ED_{50} value (open circle); these data illustrate synergism in both cases. Statistical analysis confirmed that the experimental ED_{50} values of the combinations in control and Mor-tolerant mice were significantly less than the respective calculated theoretical additive ED_{50} values (Table 1; $p < .05$). These results indicate a synergistic interaction between Mor and Clon in both controls and mice made acutely tolerant to spinally administered Mor.

Synergy Present in Mice Made Acutely Tolerant by Systemic Administration of Mor. Morphine pretreatment (100 mg/kg s.c.) reduced the efficacy of probe Mor to less than 50% MPE even at the highest doses tested (20 nmol, i.t.). To determine a combination equieffective dose ratio, we estimated the ED_{50} value to be 12 nmol (comparable to the previous acute tolerance experiments) and from that value we determined the combination equieffective dose ratio (1:2 Mor/Clon). Intrathecal administration of Clon revealed an antinociceptive dose-response curve with an ED_{50} value of 22 nmol (14–30) in Mor-tolerant mice (Fig. 2B). This value differs from that of Clon administered alone to saline-pretreated mice (ED_{50}: 7.5 nmol, 5.4–10; Fig. 2B). The observed shift indicates a 3-fold cross-tolerance to Clon (i.t.) in mice made acutely tolerant to systemically administered Mor. Based on these ED_{50} values, the Mor-Clon equieffective dose ratios were estimated to be 1:5 in the nontolerant mice and 1:2 in the Mor-tolerant mice. Administration of probe Mor-Clon combinations to Mor-tolerant (100 mg/kg s.c.) or control mice resulted in leftward shifts in the dose-response curves for each drug administered in the presence of the other compared with each drug administered separately (Fig. 2, A and B). The shifts are significant; the ED_{50} values for each drug administered in combination are significantly lower than those of each drug administered separately (Table 2). This is consistent for both pretreatment groups. In the isobolograms representing the drug interaction in controls (Fig. 2C) and Mor-tolerant animals (Fig. 2D), the experimental point (closed circle) falls significantly below the theoretical additive line and calculated theoretical additive ED_{50} value (open circle); these data depict synergism in both cases. Statistical analysis validated that the experimental ED_{50} values of the combinations in control and Mor-tolerant mice were significantly less than the respective calculated theoretical additive ED_{50} values (Table 2; $p < .05$). These results established a synergistic interaction between Mor and Clon in both controls and mice made acutely tolerant to systemically administered Mor.

TABLE 1
Acute tolerance to i.t. administered Mor (40 nmol, i.t.)

Probe Drug	Pretreatment	ED_{50} Clon (95% CL)	ED_{50} Mor (95% CL)
nmol, i.t.			
Mor	Saline		1.2 (0.7–1.7)
	Mor		12 (8.5–15)
Clon	Saline	21 (11–30)	
	Mor	24 (6.2–42)	
Mor + Clon (1:20 dose ratio)			
Observed (synergy)	Saline	3.6 (0.4–6.9)*	0.2 (0.02–0.4)*
Theoretical additive		12 (8.0–14)	0.6 (0.3–0.8)
Mor + Clon (1:2 dose ratio)			
Observed (synergy)	Mor	0.4 (0.2–0.5)*	0.2 (0.1–0.3)*
Theoretical additive		11 (6.9–17)	5.5 (4.0–7.1)

* Significant difference from theoretical additive by Student's t test, $p < .05$.

Synergy Detectable in Mice Made Chronically Tolerant by Systemic Mor (probe test at 6 h after the final injection). Morphine pretreatment (3 mg/kg every 6 h s.c. days 1 and 2; 5 mg/kg every 6 h s.c days 3 and 4) produced a 5-fold rightward shift (ED_{50}: 6 nmol, 2–10) in the probe Mor dose-response curve compared with that of saline-pretreated subjects (ED_{50}: 1.2 nmol, 0.7–1.9) (Fig. 3A). Intrathecal administration of Clon produced an antinociceptive dose-response curve with an ED_{50} value of 89 nmol (20–158) in animals pretreated with this Mor regimen (Fig. 3B). This value does not differ from that of Clon administered alone to saline-pretreated mice (ED_{50}: 64 nmol, 27–102; Fig. 3B). Based on these ED_{50} values, the Mor-Clon equieffective dose ratios were determined to be 1:50 in the nontolerant mice and 1:15 in the Mor-tolerant mice. Combination of Mor and Clon resulted in significant leftward shifts in the dose-response curves compared with those of each agonist administered separately (Fig. 3; Table 3). This observation indicates an increase in potency for each drug administered in the presence of the other compared with each drug administered alone. In the isobolograms representing the drug interaction in controls (Fig. 3C) and Mor-tolerant animals (Fig. 3D), the experimental point (closed circle) falls significantly below the theoretical additive line and calculated theoretical additive ED_{50} value (open circle); these data denote synergism in both cases. Statistical analysis verified that the experimental ED_{50} values of the combinations in control and Mor-tolerant mice were significantly less than the respective calculated theoretical additive ED_{50} values (Table 3; $p < .05$). These results signify a synergistic interaction between Mor and Clon in both controls and mice made chronically tolerant to systemically administered Mor.

Synergy Persists in Mice Made Chronically Tolerant by Systemic Mor (probe test at 2 h after the final injection). Mor pretreatment (3 mg/kg every 6 h s.c. days 1 and 2; 5 mg/kg every 6 h s.c days 3 and 4) prevented the ability of probe Mor to achieve full efficacy even at the highest doses tested (20 nmol, i.t.). An 8-nmol dose produced a 58% MPE. Based on that result and the data from the previous chronic tolerance experiment, we estimated that Mor pretreatment results in a 3-fold rightward shift (estimated ED_{50}: 6.5 nmol, i.t.) in the probe Mor dose-response curve when tested at 2 h after the final injection and compared with that of saline-pretreated subjects (ED_{50}: 2.1 nmol, 1.4–2.8; Fig. 4A). Intrathecal administration of Clon produced an antinociceptive dose-response curve with an ED_{50} value of 31 nmol (23–39; Fig. 4B) in animals pretreated with Mor (3 mg/kg every 6 h s.c. days 1 and 2; 5 mg/kg every 6 h s.c. days 3 and 4; Fig. 4B). This value differs from that of Clon administered alone to saline-pretreated mice (ED_{50} value of 62 nmol, 47–77; Fig. 4B), indicating some potentiation of Clon (i.t.), presumably from residual systemically administered Mor. Based on these ED_{50} values, the Mor-Clon equieffective

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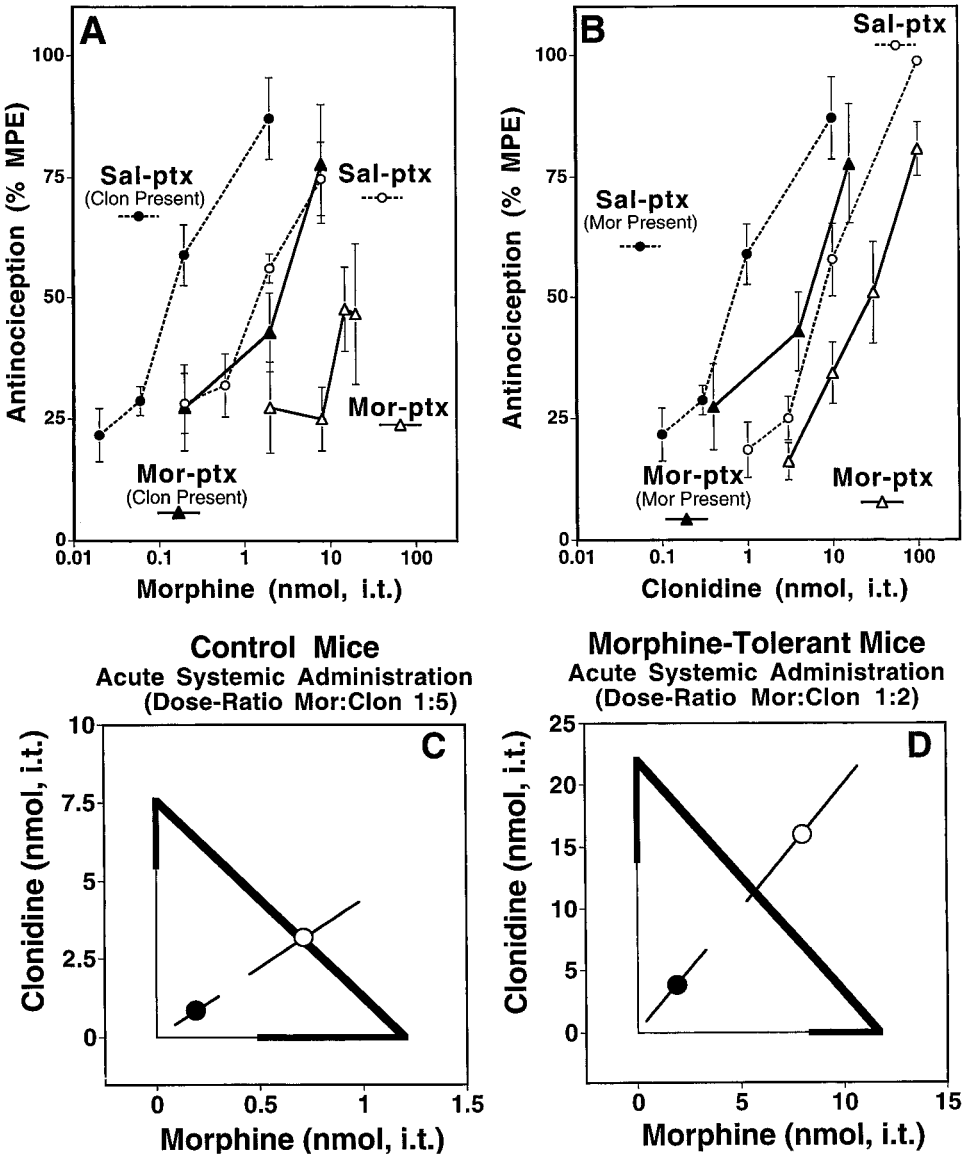

Fig. 2. Acute tolerance to systemically administered Mor. Dose-response curves for i.t. administered Mor and Clon and Mor-Clon combination. A, dose-response curves of the spinal antinociceptive effect of: 1) Mor on nontolerant (open circles, dashed line) and Mor-pretreated animals (open triangles, solid line) and 2) Mor in the presence of Clon on nontolerant (closed circles, dashed line) and Mor-pretreated animals (closed triangles, solid line). B, dose-response curves of the spinal antinociceptive effect of: 1) Clon on nontolerant (open circles, dashed line) and Mor-pretreated animals (open triangles, solid line) and 2) Clon in the presence of Mor on nontolerant (closed circles, dashed line) and Mor-pretreated animals (closed triangles, solid line). C, isobolographic analysis was applied to the data from Fig. 2, A and B, that represent responses to the combination of Clon-Mor in saline-treated mice. In this isobologram, the ED_{50} value of the combination of Clon-Mor (closed circle) falls below the theoretical additive line and differs significantly from that of the theoretical ED_{50} value (open circle) (Table 2); therefore, the combination is synergistic in control mice. D, isobolographic analysis was applied to the data from Fig. 2, A and B, that represent responses to the combination of Clon-Mor in Mor-tolerant mice. In this isobologram, the ED_{50} value of the combination of Clon-Mor (closed circle) falls below the theoretical additive line and differs significantly from that of the theoretical ED_{50} value (open circle) (Table 2); therefore, the combination is synergistic in Mor tolerant mice. It is noteworthy that in this instance the theoretical additive point does not line up precisely along the theoretical additive line. This is due to the fact that, in this case, the ED_{50} value of Mor in the tolerant state had to be estimated because full efficacy was not observed. The calculated theoretical additive value is based on the experimental data and therefore does not precisely match the theoretical additive line which connects the actual ED_{50} value from Clon to the *approximated* ED_{50} value from the Mor dose-response curve. Despite this imperfection, the observed difference between the observed ED_{50} value of the combination and the theoretical additive ED_{50} value is sufficiently great to strongly demonstrate a synergistic interaction.

dose ratios were determined to be 1:20 in the nontolerant mice and approximated at 1:5 in the Mor-tolerant mice. Administration of probe Mor-Clon combinations to Mor-tolerant (3 mg/kg every 6 h s.c. days 1 and 2; 5 mg/kg every 6 h s.c days 3 and 4) or control mice resulted in leftward shifts in the dose-response curves for each drug administered in the presence of the other compared to each drug administered separately (Fig. 4, A and B). These shifts are significant: the ED_{50} values for each drug administered in combination are significantly lower than that of each drug administered separately (Table 2). This observation indicates an increase in potency for each drug administered in the presence of the other compared with each drug administered alone. In the isobolograms representing the drug interaction in controls (Fig. 4C) and Mor-tolerant animals (Fig. 4D), the experimental point (closed circle) falls significantly below the theoretical addi-

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TABLE 2
Acute tolerance to systemically administered Mor (100 mg/kg)

Probe Drug	Pretreatment	ED_{50} Clon (95% CL)	ED_{50} Mor (95% CL)
nmol, i.t.			
Mor	Saline		1.2 (0.5–1.9)
	Mor		Not calculated
Clon	Saline	7.5 (5.4–10)	
	Mor	22.0 (14–30)	
Mor + Clon (1:5 dose ratio)			
Observed (synergy)	Saline	0.7 (0.4–1.1)*	0.2 (0.1–0.24)*
Theoretical additive		3.5 (2.6–4.5)	0.8 (0.6–1.0)
Mor + Clon (1:2 dose ratio)			
Observed (synergy)	Mor	3.0 (0.9–5.1)*	1.5 (0.4–2.5)*
Theoretical additive		9.2 (6.0–12)	4.6 (3.0–6.2)

* Significant difference from theoretical additive by Student's t test, $p < .05$.

tive line and calculated theoretical additive ED_{50} value (open circle); these data indicate synergism in both cases. Statistical analysis confirmed that the experimental ED_{50} values of the combinations in control and Mor-tolerant mice were significantly less than the respective calculated theoretical additive ED_{50} values (Table 4; $p < .05$). These results demonstrate a synergistic interaction between Mor and Clon in both controls and mice made chronically tolerant to systemically administered Mor.

Discussion

Conceptually, additivity refers to the interaction of two drugs such that when coadministered the resultant effect approaches the maximum effect or the sum of the effects of the two drugs administered individually (see Tallarida, 1992 for a more precise definition). Synergy describes the interaction of two drugs such that when coadministered the resultant efficacy or potency supports a greater-than-additive or multiplicative interaction compared to each drug administered alone (Gessner and Cabana, 1970; Tallarida and Murray, 1987). A rigorous mathematical distinction between these two phenomena has been published (Tallarida, 1992) and should be used as the ultimate scientific definition. Tolerance may be described as a decrease in agonist effect over time and/or a significant rightward shift in the agonist dose-response curve (Stevens and Yaksh, 1989), the opposite effect of synergy. This relationship formed the basis of the assertion that tolerance resulted from an absence of an ongoing synergistic interaction.

Concurrent administration of Mor i.c.v. and i.t. results in an antinociceptive synergistic interaction in mice (Roerig et al., 1984). This synergistic interaction may result in part from the interaction of the i.t. administered Mor with noradrenaline released from descending noradrenergic terminals in the spinal cord subsequent to i.c.v. administration of Mor. This synergistic interaction is reduced to additivity in mice made tolerant to Mor by pellet implantation (Roerig et al., 1984). That observation led to the proposal that the reduction in the synergistic interaction to additivity may be a mechanism by which apparent Mor tolerance develops (Roerig et al., 1984; Wigdor and Wilcox, 1987; Roerig, 1995). Roerig (1995) explored this proposal by testing the interactions of i.t. applied Mor-Clon combinations in Mor pellet- and placebo pellet-implanted ICR mice. Those experiments revealed that, although the interaction of Mor-Clon was synergistic in placebo-pelleted mice, it was merely additive in Mor-pelleted mice.

In our experimental model, Mor pretreatment produced significant and dose-related rightward shifts of the Mor dose-response curve (Figs. 1A, 2A, 3A, and 4A); these results confirm the induction of tolerance. The present study tested the interaction between i.t. coadministered Mor and Clon in both tolerant and control subjects. The results differ from those reported in the Mor pellet implantation model (Roerig, 1995). The present experiments do not support the proposal that reduction of the spinal adrenergic/opioid ligand synergistic interaction to additivity represents a mechanism underlying Mor analgesic tolerance.

Acute Induction of Tolerance by i.t. Administration of Mor. The pharmacology of spinal cord changes in acute and chronic opioid tolerance appears to be similar with respect to dependence on the *N*-methyl-D-aspartate/nitric oxide synthase cascade (Trujillo and Akil, 1991; Marek et al., 1991; Ben-Eliyahu et al., 1992; Tiseo and Inturissi, 1993; Elliott et al., 1994; Tiseo et al., 1994; Fairbanks and Wilcox, 1997). We initiated the present studies to ascertain whether the α_2-adrenergic receptor-mediated effects observed in a chronic Mor tolerance model (Roerig, 1995) would similarly be paralleled in an acute Mor tolerance paradigm. Morphine pretreatment by a single supramaximal i.t. injection (40 nmol) produced spinal antinociceptive Mor tolerance but no cross-tolerance to Clon (i.t.). In both the tolerant and nontolerant states, coadministration of Mor and Clon produced antinociceptive synergy (Fig. 1; Table 1).

Conceivably, the persistence of synergy observed in this first experiment could be specific to tolerance induction by *i.t.* Mor administration. *Systemic* administration of Mor activates supraspinal opioid receptors, an action which is correlated with a spinal release of noradrenaline (Wigdor and Wilcox, 1987). The prolonged presence of noradrenaline after repeated systemic Mor administration or Mor pellet implantation may lead to down-regulation or desensitization of spinal adrenergic receptors. Such an action could decrease the apparent potency of exogenously administered α_2 adrenergic receptor agonists (e.g., Clon cross-tolerance) and or the endogenous adrenergic contribution to the opioid-adrenergic synergy. To address this possibility, we tested the interaction of Mor-Clon (i.t.) coadministration in mice made acutely tolerant to systemically administered Mor (Fig. 2).

Acute Induction of Tolerance by Systemic Administration of Mor. Morphine pretreatment (100 mg/kg s.c.) produced acute tolerance to Mor (Fig. 2A) and acute cross-tolerance to Clon (Fig. 2B; Table 2). This cross-tolerance was consistent with the premise that activation of descending noradrenergic pathways by systemically administered Mor could result in an adrenergic receptor down-regulation or desensitization. However, in both the tolerant and nontolerant states, coadministration of Mor and Clon produced antinociceptive synergy (Fig. 2; Table 2). Therefore, the persistence of synergy in the tolerant state generalized to the presence of Mor tolerance after systemic administration. It is possible that the persistence of Mor-Clon antinociceptive synergy observed in Figs. 1 and 2 might be specific to *acute* induction of Mor. Therefore, we tested the interaction of i.t. coadministered Mor and Clon in mice made chronically tolerant to systemically administered Mor (Fig. 3).

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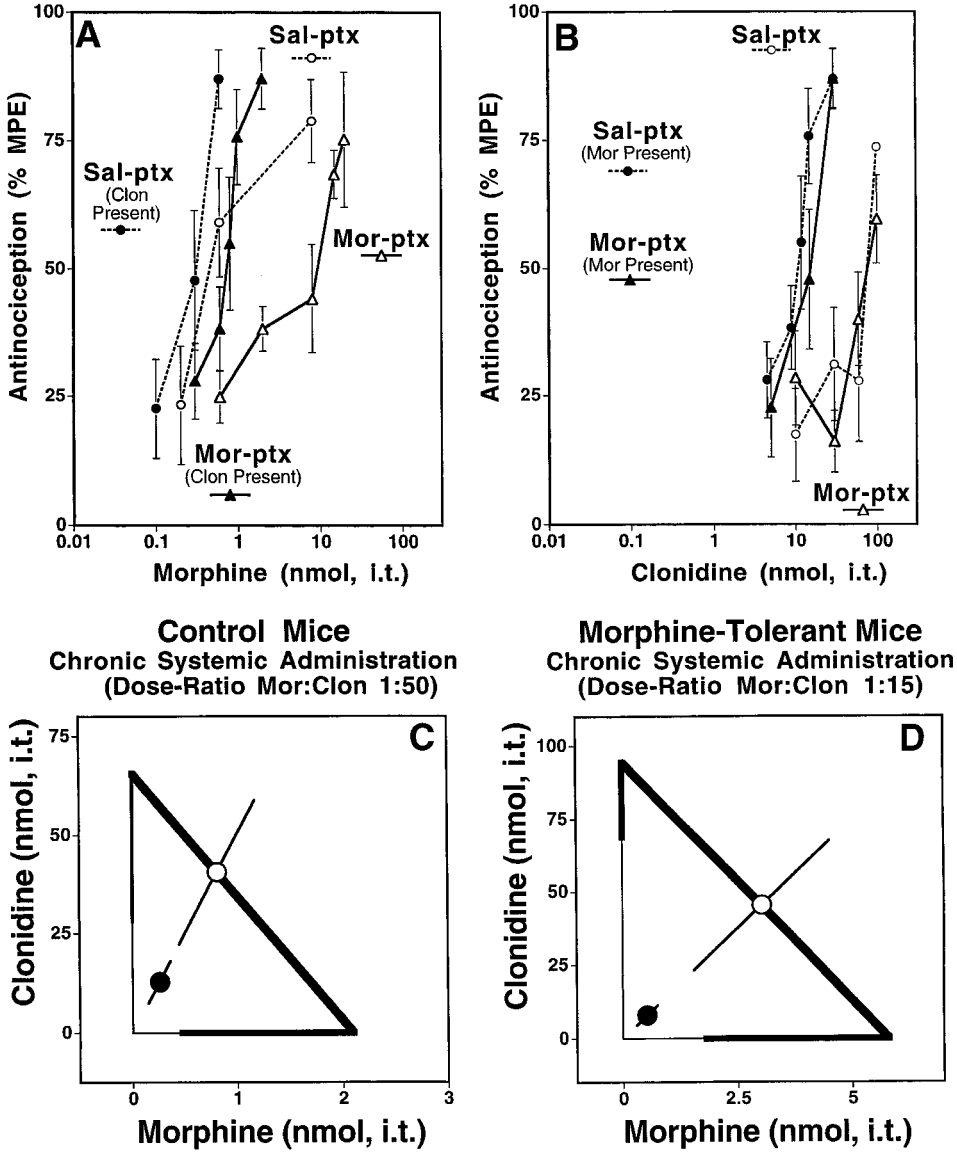

Fig. 3. Chronic tolerance to systemically administered Mor: probe test at 6 h after final injection. Dose-response curves for i.t. administered Mor and Clon and Mor-Clon combination. A, dose-response curves of the spinal antinociceptive effect of: 1) Mor on nontolerant (open circles, dashed line) and Mor-pretreated animals (open triangles, solid line) and 2) Mor in the presence of Clon on nontolerant (closed circles, dashed line) and Mor-pretreated animals (closed triangles, solid line). B, dose-response curves of the spinal antinociceptive effect of: 1) Clon on nontolerant (open circles, dashed line) and Mor-pretreated animals (open triangles, solid line) and 2) Clon in the presence of Mor on nontolerant (closed circles, dashed line) and Mor-pretreated animals (closed triangles, solid line). C, isobolographic analysis was applied to the data from Fig. 3, A and B, that represent responses to the combination of Clon-Mor in saline-treated mice. In this isobologram, the ED_{50} value of the combination of Clon-Mor (closed circle) falls below the theoretical additive line and differs significantly from that of the theoretical ED_{50} value (open circle) (Table 3); therefore, the combination is synergistic in control mice. D, isobolographic analysis was applied to the data from Fig. 3, A and B, that represent responses to the combination of Clon-Mor in Mor-tolerant mice. In this isobologram, the ED_{50} value of the combination of Clon-Mor (closed circle) falls below the theoretical additive line and differs significantly from that of the theoretical ED_{50} value (open circle) (Table 3); therefore, the combination is synergistic in Mor-tolerant mice.

Chronic Induction of Tolerance by Systemic Administration of Mor. We used a schedule of repeated systemic Mor injections to induce tolerance. Morphine pretreatment produced tolerance to i.t. administered Mor (Fig. 3A) but no observable cross-tolerance to i.t. administered Clon (Fig. 3B). Coadministration of Mor and Clon produced antinociceptive synergy in both Mor-tolerant and control animals (Fig. 3; Table 3); this observation agrees with the observations made in acutely tolerant animals (Figs. 1 and 2). The presence of Mor-Clon antinociceptive synergy by the i.t. route remained in animals made chronically tolerant to Mor. Therefore, the observed persistence of tolerance was not attributable to a difference between acutely and chronically induced tolerance.

These data differ from the previous investigation of Mor-Clon antinociceptive interactions in mice made tolerant by Mor pellet implantation (Roerig, 1995). It is possible that as yet unidentified conditions associated with the development of subclinical systemic illness induced by the Mor pellet implantation (Sparber et al., 1979) may underlie the reduction of the synergy in Mor-pelleted mice. If so, the pellet model may better reflect the physiological barriers to effective pain management that exist in the clinical arena. Resolution of the specific conditions under which synergy is or is not

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TABLE 3
Chronic tolerance to systemically administered Mor (days 1 and 2: 3 mg/kg every 6 h; days 3 and 4: 5 mg/kg every 6 h) test at 6 h postfinal injection

Probe Drug	Pretreatment	ED_{50} Clon (95% CL)	ED_{50} Mor (95% CL)
nmol, i.t.			
Mor	Saline		1.2 (0.7–1.9)
	Mor		6.0 (2–10)
Clon	Saline	64 (27–102)	
	Mor	89 (20–158)	
Mor + Clon (1:50 dose ratio)			
Observed (synergy)	Saline	7 (2–13)*	0.15 (0.03–0.3)*
Theoretical additive		31 (15–47)	0.6 (0.3–0.9)
Mor + Clon (1:15 dose ratio)			
Observed (synergy)	Mor	9.5 (7.3–12)*	0.6 (0.5–0.8)*
Theoretical additive		46 (23–66)	3.0 (1.6–4.4)

* Significant difference froms theoretical additive by Student's t test, $p < .05$.

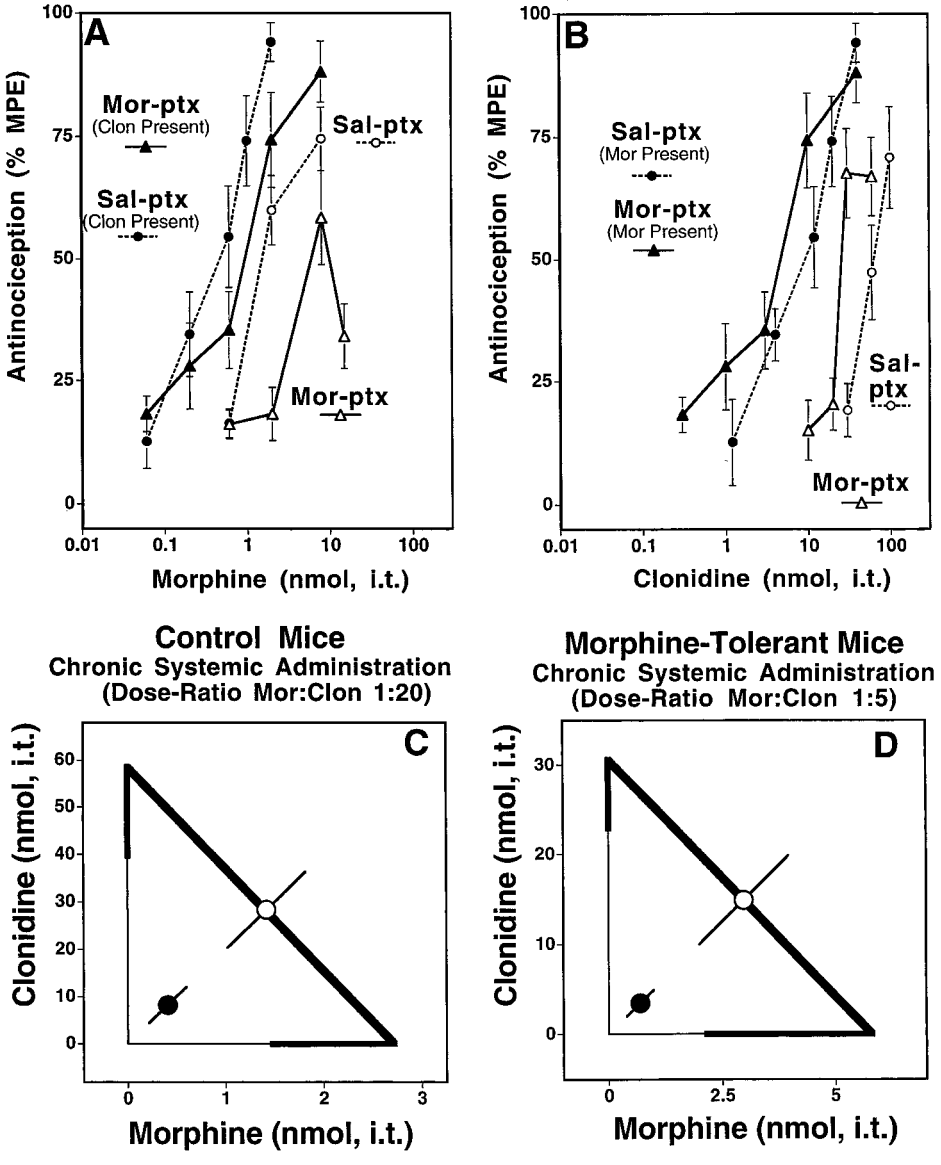

Fig. 4. Chronic tolerance to systemically administered Mor: probe test at 2 h after final injection. Dose-response curves for i.t. administered Mor and Clon and Mor-Clon combination. A, dose-response curves of the spinal antinociceptive effect of: 1) Mor on nontolerant (open circles, dashed line) and Mor-pretreated animals (open triangles, solid line) and 2) Mor in the presence of Clon on nontolerant (closed circles, dashed line) and Mor-pretreated animals (closed triangles, solid line). B, dose-response curves of the spinal antinociceptive effect of: 1) Clon on nontolerant (open circles, dashed line) and Mor-pretreated animals (open triangles, solid line) and 2) Clon in the presence of Mor on nontolerant (closed circles, dashed line) and Mor-pretreated animals (closed triangles, solid line). C, isobolographic analysis was applied to the data from Fig. 4, A and B, that represent responses to the combination of Clon-Mor in saline-treated mice. In this isobologram, the ED_{50} value of the combination of Clon-Mor (closed circle) falls below the theoretical additive line and differs significantly from that of the theoretical ED_{50} value (open circle) (Table 4); therefore, the combination is synergistic in control mice. D, isobolographic analysis was applied to the data from Fig. 4, A and B, that represent responses to the combination of Clon-Mor in Mor-tolerant mice. In this isobologram, the ED_{50} value of the combination of Clon-Mor (closed circle) falls below the theoretical additive line and differs significantly from that of the theoretical ED_{50} value (open circle) (Table 4); therefore, the combination is synergistic in Mor-tolerant mice.

present in tolerant states may facilitate improved and selective clinical pain management.

A notable difference between this set of experiments and that of the chronic Mor pellet implantation model (Roerig, 1995) is the relative dose ratios of Mor to Clon. This difference could be important because synergism is not solely a property of the drugs but is also dependent on the proportions of the drugs in the combination (Tallarida, 1992). In all three of our tolerance experiments discussed so far, the Mor dose-response curves were collected 4 to 8 h after the single

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TABLE 4
Chronic tolerance to systemically administered morphine (days 1 and 2: 3 mg/kg every 6 h; days 3 and 4: 5 mg/kg every 6 h) test at 2 h postfinal injection

Probe Drug	Pretreatment	ED_{50} Clon (95% CL)	ED_{50} Mor (95% CL)
nmol, i.t.			
Mor	Saline		2.1 (1.4–2.8)
	Mor		Not calculated
Clon	Saline	62 (47–77)	
	Mor	31 (23–39)	
Mor + Clon (1:20 dose ratio)			
Observed (synergy)	Saline	6.2 (1.7–11)*	0.3 (0.2–0.5)*
Theoretical additive		25 (20–31)	1.3 (1–1.5)
Mor + Clon (1:5 dose ratio)			
Observed (synergy)	Mor	0.7 (0.1–1.3)*	0.14 (0.02–0.3)*
Theoretical additive		7.0 (5.2–8.8)	1.4 (1.0–1.8)

* Significant difference from theoretical additive by Student's t test, $p < .05$.

supramaximal dose in the acute studies or after the final injection of tolerance-inducing Mor in the chronic model. These times represented the approximate times that the animals' tail-flick latencies had returned to baseline levels and presumably times at which residual Mor from the tolerance-inducing dose(s) had been cleared from the spinal cord. In the present experiments, in both Mor-tolerant and control mice, Mor was clearly more potent than Clon, a situation yielding Mor-Clon dose ratios that ranged from 1:2 to 1:50. The chronic pellet model consisted of a 3-day Mor pellet implant protocol (Roerig, 1995); the animals were tested 75 min after pellet removal, also a time at which the animals' tail-flick latencies had returned to baseline levels. In the placebo-pelleted animals, Mor was more potent than Clon resulting in dose ratios comparable to those observed in our experimental model. However, in the Mor-pelleted animals, the potency of Clon was higher than that of Mor, resulting in a reversal of the equieffective dose ratio (5:1, Mor/Clon); therefore, in the pellet model, Clon represented the smaller portion of the drug combination (Roerig, 1995). This increase in the potency of Clon in Mor-tolerant animals compared to placebo-pelleted controls suggests the presence of residual Mor in a concentration that is sufficient to potentiate the i.t. administered Clon but insufficient to prolong the tail-flick latency. This state of lingering synergy may artificially skew the observed ED_{50} values of drugs given alone and in combination, perhaps masking the statistical determination of synergy.

Accordingly, we conducted a second test of Mor-Clon interaction in animals made chronically tolerant to Mor by repeated systemic induction. In this experiment, all dose-response curves were collected 2 h after the final injection. The objective was to test for Mor-Clon synergy at a time when the Clon effect might be potentiated by presumably residual levels of Mor. Based on tail-flick latency tests conducted on several animals at various times after the final injection, this 2-h time appeared to be the earliest point at which the majority of the animals' tail-flick latencies had returned to baseline levels. This time was, therefore, the point at which we administered probe doses of Mor.

Chronic Induction of Tolerance by Systemic Administration of Mor (probe test at 2 h post final injection). Morphine pretreatment by a repeated bolus systemic injection produced tolerance to i.t. applied Mor (Fig. 4A). Testing 2 h after the final injection, there was a 2-fold increase in potency of Clon (i.t.) in Mor-tolerant mice compared to controls (Fig. 4B; Table 4). This observation would be consistent with the idea that the residual Mor potentiated the antinociceptive effect of Clon. However, this leftward shift was insufficient to reverse the relative dose ratios of Clon to Mor. Furthermore, in both the tolerant and control states, coadministration of Mor and Clon produced antinociceptive synergy (Fig. 4; Table 4).

Collectively, the present experiments demonstrate that the induction of Mor tolerance in mice, whether by acute i.t., by acute systemic, or by chronic repeated systemic injection of Mor, does not compromise the synergistic antinociceptive potential of Mor-Clon-induced antinociception. Sufficient receptor/effector interactions must remain functional to elicit the observed robust synergistic response. Interestingly, a collection of observations describing Mor tolerance appears to parallel findings related to the neuropathic pain state (Mao et al., 1995). Specifically, *N*-methyl-D-aspartate receptor antagonists appear to attenuate both states (Trujillo and Akil, 1991) as do nitric oxide synthase inhibitors (Kolesnikov et al., 1992; Kolesnikov et al., 1993; Babey et al., 1994; Elliott et al., 1994a,b; Meller et al., 1994; Bhargava, 1995; Mizoguchi et al., 1996); translocation of protein kinase C to the membrane also appears to be involved in both phenomena (Mao et al., 1994, 1995). It has been determined that Mor-Clon synergy is detectable in a state of neuropathic pain (Ossipov et al., 1997). This finding is of considerable interest given that it was previously thought that neuropathic pain was largely unresponsive to opioid therapy. The present experiments complement those observations and extend the parallel between neuropathic pain and Mor tolerance by demonstrating the effectiveness of Mor-Clon antinociceptive synergy in different states of Mor tolerance. These experiments support the potential utility of including Clon as a coadjuvant in spinal application of Mor for patients who appear to have become tolerant to Mor. It is interesting that a single, randomized, double-blind, clinical study (Eisenach et al., 1994) has isobolographically examined epidurally coadministered Clon and fentanyl; a synergistic interaction was not statistically detectable. These authors attributed this result to higher variability than expected; detection of a synergistic interaction would require a larger patient population than studied. However, in this study, patient needs for postoperative supplemental Mor were substantially reduced in those receiving the combination. Furthermore, in contrast to single drug administration, complete analgesia was obtainable in those patients receiving higher doses of the combination.

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Other clinical studies have shown that epidurally administered Clon decreases the required dose of Mor (Motsch et al., 1990) and prolongs the duration of action of (Rostaing et al., 1991). These studies and the recent Food and Drug Administration approval of Clon as an epidural analgesic and analgesic coadjuvant underscore the significance of our demonstration of Mor-Clon synergy in states of Mor tolerance.

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