Life Sciences, Vol. 41, pp. 1731-1738 Pergamon Journals
Printed in the U.S.A.

IMMUNOSUPPRESSIVE EFFECTS OF CHRONIC MORPHINE TREATMENT IN MICE

Henry U. Bryant, Edward W. Bernton and John W. Holaday

Neuropharmacology Branch, Department of Medical Neuroscience, Division of Neuropsychiatry
Walter Reed Army Institute of Research, Washington, D.C. 20307

(Received in final form August 3, 1987)

SUMMARY

In this report we describe the immunomodulatory effects of subcutaneous morphine pellets in mice, a model commonly used in the study of opiate tolerance and dependence. Mice given a single 75 mg morphine pellet displayed marked atrophy and reduced cellularity of the spleen and thymus, and an attenuated lymphocyte proliferative response to T- and B-cell mitogens (concanavalin A and bacterial lipopolysaccharide, respectively). These immunosuppressive effects were observed 72 hr following implantation of the pellet, a time point by which the mice also had developed tolerance to the antinociceptive effect of the pellet. Splenic and thymic atrophy with reduced mitogen-induced lymphocyte proliferative responses and opiate tolerance were also apparent in mice subjected to a multiple pellet implantation schedule. However, implantation of a pellet containing 37.5 mg morphine did not suppress mitogen-stimulated lymphocyte proliferation, which was slightly elevated in this group. These findings concur with other observations suggesting immunosuppression with morphine tolerance. Furthermore, we suggest that chronic morphine treatment acts as a pharmacologic stressor that mimics behavioral stress.

Opioids, such as morphine and their endogenous peptide counterparts produce multiple pharmacological effects and subserve many physiological functions. In addition to their well characterized effects on pain perception and gastrointestinal motility, opioid agonists and endogenous opioid peptides affect various cardiovascular (1), endocrine (2) and behavioral (3) parameters. Several lines of evidence suggest that opioids are also immunomodulatory agents. An increase in the incidence of infectious disease has been reported in opioid addicts (4), as well as a reduction of T-lymphocyte number (5) and function (6). In animal models, opioids can be shown to inhibit lymphocyte proliferative responses (7), natural killer cell activity (8), antibody production (9,10) and circulating levels of interferon (11). Specific binding sites for morphine and endogenous opioid peptides are also found on circulating lymphocytes (2).

Immunosuppressive effects of morphine have been observed following injection of a single dose (8) or after repeated daily injections of increasing doses (7). A complex interrelationship may exist between the immunosuppressive effects of morphine and the phenomenon of tolerance. For example, studies employing chronic morphine regimens show immunosuppression may be associated with tolerance. Additionally, suppression of the immune

0024-3205/87 $3.00 + .00

system inhibits the development of tolerance and physical dependence to chronic morphine administration (12).

One of the most common techniques for the induction of tolerance and physical dependence to morphine in laboratory animals is the subcutaneous implantation of morphine pellets. This method allows a relatively rapid induction of tolerance and dependence [12 to 24 hr (13)]. In spite of the reliable and rapid morphinization attainable with this technique, very little has been published regarding its effects on immune function. Therefore, in the studies reported here, we describe the effects of a 75 mg morphine pellet implant, a 37.5 mg morphine pellet implant and a multiple pellet implant schedule on immunocompetence in mice.

METHODS

Male C3H/HeN mice (Harlan S-D, Frederick, MD) were given subcutaneous implants of either a single 75 mg morphine pellet (NIDA), a halved morphine pellet (containing 37.5 mg morphine), a single microcrystalline cellulose placebo pellet (NIDA) or a halved placebo pellet. A separate group of mice received two additional morphine pellets 30 hr following implantation of the first morphine pellet, with controls receiving placebo pellets on the same schedule. Antinociceptive responses were measured at various intervals by determining the latency for the animal to lick its hind paws when placed on a hot plate (52°C, cut-off time of 45 sec). Daily body weights were also monitored.

Animals were sacrificed 72 hr following implantation of the first morphine or placebo pellet. Spleens and thymi were then aseptically removed and weighed. Adrenal glands were also removed and weighed in some experiments. The splenocytes were mechanically dissociated and suspended in RPMI 1640 culture medium (Gibco Laboratories, Grand Island NY). The spleen cells were then washed in an ammonium chloride buffer (155mM NH_4Cl; 7mM $KH(CO_3)$; 0.1 mM EDTA) for 90 sec to hemolyze red blood cells. The splenocytes were then resuspended to a concentration of 2.5 x 10^6 cells/ml in RPMI 1640 medium containing 5% fetal calf serum (HyClone Laboratories, Logan, Utah), 12 mM HEPES buffer (Gibco) and 0.05 mg/ml gentamycin (Gibco). Lymphocyte proliferative responses to various concentrations of concanavalin A (Con A; Sigma Chemical Co, St. Louis, MO) or bacterial lipopolysaccharide (LPS; from S. typhimurium, RIBI Immunochem Research Inc., Hamilton, MT) were assessed by aliquoting 100 µl of the cell suspension with 100 µl of mitogen (0.5 to 4.0 µg/ml Con A or 0.2 to 10 µg/ml LPS) to each well of a 96 well multi-titer plate. The stimulated cell suspensions were incubated at 37°C in humidified air containing 6% CO_2 for 24 hr. Each well was then pulsed with 0.5 µCi of methyl-^{3}H-thymidine (NEN Research, Boston, MA) and returned to the incubation environment. Twenty-four hr later, cells were harvested on glass fiber filters (Skatron Inc., Sterling, VA) and washed. The amount of radioactivity remaining on the filters was assessed by liquid scintillation spectrophotometry as a measure of the uptake of methyl-^{3}H-thymidine by the lymphocytes.

For any given replication of an experiment, spleen cells were pooled for each treatment group and triplicate wells were assayed at each concentration of mitogen. Each experiment (containing both a placebo and morphine pelletted group) was replicated two to five times (with the exception of the multiple pellet study). To normalize between replications, each point on the mitogen-induced dose response curve was multiplied by a factor such that the placebo proliferative response at 0.5 µg/ml Con A (final concentration in the well) yielded 23,500 cpm. Each corresponding morphine pellet curve was then adjusted by the same factor. Groups were evaluated for significant differences by Student's t-test; significance was ascribed if $p < 0.05$.

TABLE 1

	75 mg pellet		37.5 mg pellet		Multiple Pellet Regimen[a]	
	Placebo	Morphine	Placebo	Morphine	Placebo	Morphine
TIME TO RESPONSE (sec)[b]						
baseline	8.2 ± 0.5	9.6 ± 0.7	8.7 ± 0.5	10.2 ± 0.6		
2 hr	15.5 ± 4.3†	43.6 ± 0.9*	14.2 ± 1.4†	43.3 ± 1.1*	9.0 ± 1.0	41.0 ± 3.0*
24 hr	11.1 ± 1.0	35.5 ± 2.5*	11.8 ± 1.2	19.9 ± 3.3*	7.3 ± 1.0	22.4 ± 3.6*
48 hr	8.7 ± 0.9	18.3 ± 1.9*	9.5 ± 0.9	20.3 ± 3.9*	8.3 ± 0.8	15.9 ± 4.2*
72 hr	8.2 ± 0.9	12.3 ± 2.4	9.5 ± 1.4	11.4 ± 1.3	8.6 ± 2.0	10.8 ± 2.0
BODY WEIGHT (g)[c]	25.7 ± 0.4	20.7 ± 0.8*	23.9 ± 0.4	22.2 ± 0.5*	23.7 ± 0.5	17.2 ± 0.5*
LETHALITY[d]	0/27	4/30	0/19	1/11	0/8	5/8

All values represent mean ± S.E.M. * = $p < 0.05$ vs appropriate placebo group. † = $p < 0.05$ vs placebo baseline.

[a] single pellet implant at t=0, two additional pellets implanted at t=30 hr.

[b] latency to licking of hind paw following placement on a 52°C hot plate, 45 sec cut-off.

[c] pre-sacrifice body weight, animals were randomized at initiation of experiment such that there were no differences in body weight.

[d] total number dead prior to 72 hr termination point/total number pelleted.

RESULTS

As shown in table 1, implantation of a 75 mg morphine pellet in mice produced the expected analgesic response within 2 hr, which diminished in magnitude over the 72 hr implantation period as tolerance to the pellet developed. In accordance with the original report of Way et al. (13), these mice also displayed a temporary increase in locomotor activity and Straub tail shortly after implantation, and a reduced body weight following 72 hr. Interestingly, placebo mice also showed a small increase in the latency to respond on the hot plate 2 hr following implantation of the pellet (table 1) suggesting the liklihood of a slight stress-induced analgesia associated with the pelleting procedure.

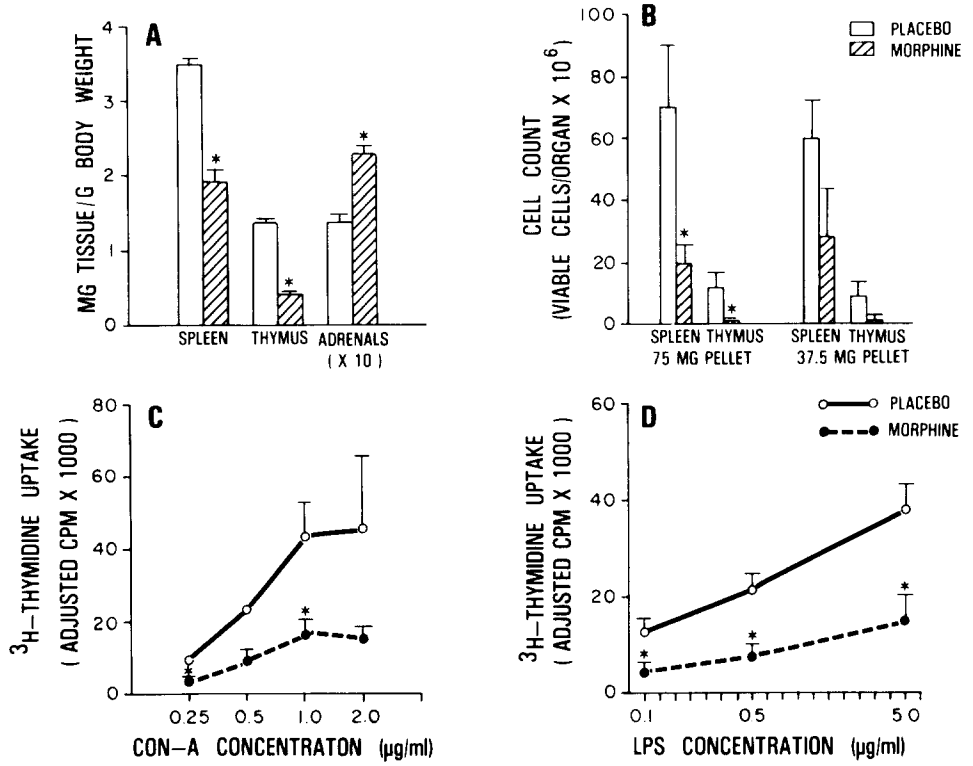

FIGURE 1

Effect of 75 mg morphine pellet implants on (A) spleen, thymus and adrenal size and (B) lymphocyte content and (C&D) lymphocyte proliferative responses to mitogenic stimuli. Each bar represents a mean value + S.E.M. for 26 or 27 mice. Each point represents a mean adjusted cpm + S.E.M. for five repetitions of the dose response curve to either Con A or LPS employing a total of 26 or 27 mice.
* = $p < 0.05$ vs the placebo group.

Implantation of a 75 mg morphine pellet for 72 hr was associated with a significant reduction of both spleen and thymus size (44 and 69% respectively, as compared to placebo pelletted controls) and a corresponding reduction in the number of immunocompetent cells in those organs (fig. 1A and 1B). There was also a marked hypertrophy of the adrenal glands obtained from mice that received the 75 mg morphine pellet. Lymphocyte proliferative responses to mitogenic stimulation were also significantly diminished in in the 75 mg morphine pelletted mice over the entire concentration range of Con A and LPS that was employed (fig. 1C and 1D).

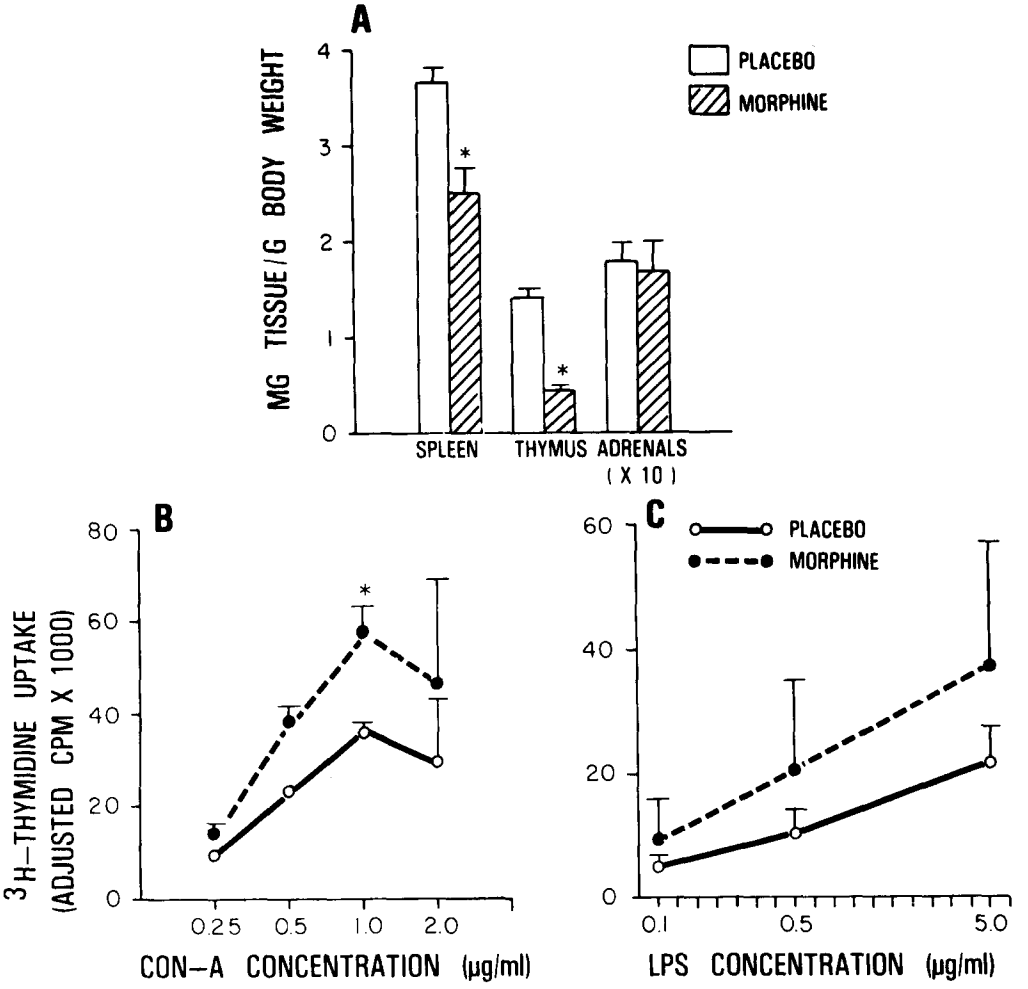

FIGURE 2

Effect of 37.5 mg morphine pellet implants on (A) spleen, thymus and adrenal size and (B&C) lymphocyte proliferative responses to mitogenic stimuli. Each bar represents mean organ size + S.E.M. for 10 mice. Each point represents a mean adjusted cpm + S.E.M. for two repetitions of the dose response curve to either Con A or LPS employing a total of 10 mice for each group. * = $p < 0.05$ vs the placebo group.

As with the 75 mg pellets, the 37.5 mg morphine implant resulted in an antinociceptive response (table 1), short term induction of increased locomotor activity and Straub tail, and decrease in body weight by the end of the 72 hr period. The 37.5 mg morphine pellet also led to a significant reduction of both spleen and thymus size (fig. 2A; 32 and 68% reduction respectively, relative to the placebo group), although the reduction in splenocyte and thymocyte cell counts was not significant (fig. 1B). In contrast to the 75 mg morphine implants, there was no discernable increase in the size of the adrenal glands in mice given a 37.5 mg morphine pellet. Also in contrast to the 75 mg implants, the 37.5 mg morphine pellet produced an increase in the proliferative responses of lymphocytes to Con A, although only significantly so at the lower concentrations (fig. 2B) There were no significant differences in LPS stimulated proliferation, although there was a trend for an increased response in the 37.5 mg morphine pelletted mice (fig. 2C).

Implantation of two additional 75 mg morphine pellets 30 hr following implantation of the first pellet led to an even greater reduction of spleen and thymus size when compared to the other pelletting regimens (61 and 72% reduction relative to a similarly treated placebo group; data not shown). Lymphocyte proliferative responses to Con A and LPS were markedly reduced (83 and 82% reduction of lymphocyte proliferation in response to 0.5 µg/ml Con A or LPS, respectively; data not shown) in mice subjected to the multiple pellet implantation regimen. However, the multiple pelletting regimen was also associated with a considerable increase in lethality (table 1). Implantation of the second and third pellets did not produce an increase in the antinociceptive effect of morphine (table 1) offering further evidence for the tolerant state of these animals.

DISCUSSION

These studies demonstrate the immunomodulatory effect of a relatively short and simple chronic morphine regimen in mice. The immunosuppression induced by implantation of a single 75 mg morphine pellet was characterized by decreased lymphocyte content and marked atrophy of both the spleen and thymus, and attenuated lymphocyte proliferative responses to both T- and B-cell mitogens (Con A and LPS, respectively). These findings are in agreement with those of others (e.g. 7,11,14,15) that have employed different immunological markers in describing the immunosuppressive nature of relatively prolonged chronic morphine treatment regimens. However, this is the first indication that suppression of T- and B-lymphocyte proliferation occurs in animals morphinized by the commonly used morphine pellet implantation technique.

Altering the amount of morphine implanted produced effects somewhat different from those observed with the single 75 mg pellet. The lowest dose of morphine (37.5 mg in the pellet) produced a slight increase in the lymphocyte proliferative response to Con A (although, there was a significant degree of spleen and thymic atrophy in these mice). Halving the standard 75 mg morphine pellet (as was done in these studies) undoubtedly alters the release characteristics of the pellet in addition to decreasing the total amount of morphine available. This indicates the need for examination of the time course of morphine-induced immunosuppression. To this regard, we have found that mice given a 75 mg morphine pellet and sacrificed 120 hr after implantation (a time at which morphine levels in the blood are undoubtedly declining; B.C. Yoburn, personal communication) also show an increase in lymphocyte proliferative responses to Con A (16). This suggests that the increase in lymphocyte proliferation observed in the 37.5 mg pelletted mice may be related to declining morphine levels. Conversely, increasing the

quantity of morphine, as with the multiple pelletting regimen, yielded a greater degree of splenic and thymic atrophy and a more severe attenuation of mitogenic responsiveness of lymphocytes when compared to the single pellet regimen. However, the high rate of mortality with the multiple pellet regimen confounds interpretation of the results and was the primary reason for discontinuation of additional experimentation utilizing this dosing regimen.

Several reports have suggested that the phenomena of tolerance and dependence induced by repeated exposure to narcotic drugs may involve the immune system (17,18). Indeed, Meisheri and Isom have reported that regimens which suppress immune function effectively inhibit the development of tolerance and dependence to morphine (12). However, we observed the development of tolerance to the acute antinociceptive effects of the implanted pellet occurred concurrently with the reduction of spleen and thymus size and attenuation of mitogen-induced lymphocyte proliferation. Thus, in our model, this marked suppression of immune endpoints by morphine did not prevent the development of tolerance. These studies, therefore, suggest the possibility for a complex relationship between opiate induced immunological alterations and tolerance. For example, might regimens which stimulate the immune system and prevent the immunosuppressive effects of morphine, delay the development of tolerance to the analgesic effect? Further studies are necessary to delineate this possible link.

Precise mechanisms for the reduced immune organ weights and depressed mitogenic responsiveness of morphine pelletted mice are only speculative at this point. Since opiate receptors are found on the surface of lymphocytes (6), a direct action is possible. However, in vitro studies have generally found opioids to be stimulatory in nature (6,9,19,20). Although, in the T-cell E-rosetting assay, which is a measure of T-cell functionality, morphine has been shown inhibit rosetting by altering E-receptor kinetics and coupling within the cell membrane (21). Since implantation of either the single or halved morphine pellet was a lethal dose in some of the mice (approximately 10%), it is possible that the observed reduction of immune endpoints reflect a more generalized physiological disturbance. Morphine may also exert an indirect effect on the immune system secondary to its effects on neuro-endocrine function. For example, opiates activate the hypothalamo-pituitary-adrenal axis (2,22) and glucocorticoids are classic immunosuppressive agents (23). This mechanism seems likely in light of the enlarged adrenal glands we observed in the immunosuppressed, morphine-pelletted mice. The possibility that glucocorticoids mediate the immunosuppressive effect of morphine also merits further investigation.

Chronic behavioral stress is known to result in adrenal hypertrophy, thymic atrophy and increased levels of corticosterone and cholesterol in the circulation (24). In addition, stress results in decreased lymphocyte proliferative responses to mitogenic stimuli (25,26). Our observations reported here, and those of others (2,27) indicate that chronic morphine treatment produces the same pattern of responses. Taken collectively, these findings prompt us to suggest that chronic morphine treatment may be considered as a pharmacologic mimic of behavioral stress.

ACKNOWLEDGEMENTS

The authors would like to thank Tamara Shakarjian for her technical assistance and Jean M. Paige for her editoral assistance. This research was supported in part by a National Research Council Associateship to H. Bryant.

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