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Effect of Pain Management on Immunization Efficacy in Mice

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Immunization with complete Freund adjuvant (CFA) or incomplete Freund adjuvant (IFA) is commonly viewed as painful, yet rodents may not receive analgesics due to concerns that these drugs affect the desired immune responses. Here we tested the hypothesis that pain associated with immunization with CFA or IFA in mice can be relieved without compromising the effectiveness of the immune response. After subcutaneous immunization in the leg with antigen in CFA or IFA, mice were assessed for signs of pain by using behavioral tests, including unrestricted locomotion in an open field, forced running on an automated treadmill, and voluntary wheel running. Effects of the analgesics acetaminophen, meloxicam, and buprenorphine on behavioral and antibody responses were assessed after primary and secondary immunization with the model antigen ovalbumin and after repeated immunization with a limiting dose of recombinant protective antigen from *Bacillus anthracis*. Open field activity and the distance traveled during forced gait analysis and voluntary wheel running both decreased after immunization. Treatment with each of the analgesics normalized some but not all of these behaviors but did not decrease the mean or maximal antibody titer after primary or repeated immunization with a moderate dose of ovalbumin or after repeated immunization with a limiting dose of protective antigen. In summary, after immunization with CFA or IFA, mice showed behavioral responses suggestive of pain. Acetaminophen, meloxicam, and buprenorphine attenuated these effects without decreasing antibody responses. Therefore, the use of these analgesics for managing rodent pain associated with CFA- or IFA-containing vaccines can be encouraged.

Abbreviations: CFA, complete Freund adjuvant; IFA, incomplete Freund adjuvant; PA, protective antigen.

Over the past several years, increasing attention has been paid to recognizing and managing pain and distress in laboratory animals. The identification and effective treatment of pain are particularly important in rodents, because they are the most commonly used animals in biomedical research today. The recognition and management of pain are also important for animal welfare. Identifying pain in mice is particularly challenging because they frequently are group-housed, making observations of behavior and mobility difficult. In addition, mice use their ability to hide behavioral signs indicative of pain to maintain their social status[19] and to avoid attracting attention from predators.[18] No standard systems are currently in place to objectively assess pain and distress in laboratory rodents.[1] Assessment of pain and distress typically is based on observation of common clinical and behavioral signs[15,24] and can be quite subjective according to the observer's experience and interpretation.As defined by the US Department of Agriculture and the Animal Welfare Act,[3] a painful procedure is any procedure that would reasonably be expected to cause more than slight or momentary pain or distress in a human being to which that procedure is applied and is in excess of that caused by injections or other minor procedures. Immunization using complete Freund adjuvant (CFA) is given as an example of a painful procedure with the potential to cause a severe inflammatory reaction, depending on the species and route of administration.[3] Several agencies therefore have recommended that IACUCs should emphasize the use of adjuvants other than CFA due to its potential for inducing pain and distress.[3,8,11]

CFA has been used in a variety of animals for over 50 y and, despite many reports of the pathologic lesions resulting from the use of CFA,[7,32] the issues associated with pain or distress resulting from the lesions are not well described.[31] Some reports have concluded that the local inflammatory lesions likely resulted in pain and distress due to the use of excessively large innocula,[2] but others have only assumed the presence of pain or distress based entirely on the pathologic lesion without clinical or behavioral evaluation.[16,31] Several groups have documented clinical and behavioral signs indicative of pain and distress that lasted 2 to 3 d after intraperitoneal injection of mice with CFA.[20,21,34]

At our institution, securing IACUC approval to use CFA or IFA rather than other potentially less painful adjuvants requires strong scientific justification. Submitted justifications commonly claim that CFA is required due to its well-known effectiveness in enhancing immune responses to a wide variety of antigens and its position as the 'gold standard' for comparison to other immunization strategies.[31,33] In addition, the use of analgesics to relieve immunization-associated pain typically is refused due to a belief that this use would interfere with the efficacy of the immune response. Our review found a lack of specific published data that established the severity of pain associated with CFA or IFA immunization in mice, the ability of commonly used analgesics to relieve this pain, and the effects of analgesia on immune responses to CFA- or IFA-containing vaccines.

The current study was designed to address these issues. Wild type mice were subcutaneously immunized with antigen plus CFA followed by boosters of antigen plus IFA at 3-wk intervals. The mice were evaluated individually for 3 d after

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immunization for physical signs of inflammation and overall behavior. Mice immunized without analgesia participated in a battery of behavioral tests to identify responses suggestive of postimmunization pain. Three analgesics commonly used in laboratory rodents (meloxicam, buprenorphine, and acetaminophen) then were evaluated for their effects on normalization of vaccine-associated behavioral responses and immunization efficacy. These studies allowed us to develop an objective strategy for the assessment of immunization-associated pain and distress in mice and to refine the CFA model for immunization by identifying effective analgesics that do not negatively affect immunization efficacy.

Materials and Methods

Animals. Male C57BL/6J mice were purchased directly from The Jackson Laboratory (Bar Harbor, ME) or raised in house from breeders recently purchased from The Jackson Laboratory. Sentinel mice housed on soiled bedding from experimental mice were negative for endo- and ectoparasites and were negative for a comprehensive panel of murine viral and bacterial pathogens (including *Helicobacter* and *Pasteurella* spp.) by serology, PCR, and microbiologic assays (Research Animal Diagnostic Laboratory, Columbia, MO). All animal studies were approved by the Duke University IACUC. Mice were group-housed in solid-bottom cages on 1/8-in. corncob bedding (Bed-o'Cobs, The Andersons, Delphi, IN) and were provided cotton nest pads, except when participating in the voluntary running wheel studies. Mice in running wheel studies were singly housed in solid-bottom cages with a small amount of 1/8-in. corncob bedding and a stainless steel running wheel during data collection, after which they were returned to group housing. All mice were provided standard rodent chow (Purina Lab Diet 5001, PMI, St Louis, MO) and water ad libitum and were maintained on a 12:12-h light:dark cycle. All mice were housed, cared for, and used in compliance with the *Guide for the Care and Use of Laboratory Animals*[14] in an AAALAC-accredited program.

Immunizations. Mice received their first immunizations at 8 to 10 wk of age (n = 5 to 10 per experimental group). Vaccines were prepared by emulsifying antigen dissolved in saline with an equal volume of CFA or IFA (Sigma-Aldrich, St Louis, MO) via rapid transfer between 2 glass syringes connected by a stopcock for at least 10 min. Mice were immunized subcutaneously in 2 sites, by using 0.1 mL adjuvant in a total vaccine volume of 0.2 mL per mouse. Sites were shaved prior to immunization, but site preparation beyond alcohol swabbing was omitted in accordance with standard immunization practices in rodents. To facilitate behavioral testing by allowing lateralization, both sites were located on the left side of the mouse, over the upper leg and adjacent to tail. Immunization sites were separated sufficiently to prevent coalescence. For vaccines using ovalbumin (Sigma-Aldrich), 100 µg ovalbumin in CFA was administered on day 0, and 100 µg ovalbumin in IFA was administered 3 wk later (day 21). For vaccines using protective antigen (PA) from *Bacillus anthracis* (List Biologic Laboratories, Campbell, CA), 2 µg PA was given in CFA on day 0, and 2 subsequent doses each containing 2 µg PA in IFA were given at 3-wk intervals.

Analgesics. Analgesics were administered according to a defined schedule to allow precise 12- and 24-h administration and to ensure adequate analgesic effectiveness during behavioral testing sessions. The initial dose of each analgesic was provided before immunization. Meloxicam was administered at a dose of 2 mg/kg subcutaneously once daily for 72 h (3 d) after immunization. Buprenorphine hydrochloride was administered at a dose of 0.1 mg/kg subcutaneously twice daily for 72 h after immunization. To prevent neophobic avoidance of acetaminophen, the analgesic was provided in drinking water to supply 300 mg/kg daily beginning 2 d prior to and continuing for 3 d after immunization. Logs of water consumption were maintained to ensure that acetaminophen consumption (on a per cage basis) remained within 10% of this targeted dose. Water containing drug was replaced daily. Control mice did not receive analgesic.

Postimmunization monitoring. Baseline core body temperatures were obtained rectally prior to immunization. After immunization with CFA or IFA, the mice were evaluated by a veterinarian daily for 3 d to assess core body temperature, body weight, local skin temperature, and erythema and swelling at the injection sites and overall movement and behavior in the home cages. Local skin temperatures were obtained by using an infrared thermometer, and the diameter of any nodules present was measured with a caliper. Erythema and swelling were scored as none, slight, moderate, or severe.

Behavioral assessments of pain. All behavioral tests were performed by the Duke University Mouse Behavioral and Neuroendocrine Analysis Core Facility. Baseline testing was performed 24 to 48 h before the primary immunization. The study was designed so that each mouse was tested both before and after immunization and thus served as its own control. Pre- and postimmunization measurements of a given type were performed by the same person at approximately the same time of day. The order of testing was similar for pre- and postimmunization time points to minimize any effects of testing order on test performance. Groups of nonanalgesic-treated control mice underwent the full test battery at either 48 or 72 h after primary and secondary immunizations. Based on results from these studies, a more limited test battery was applied to analgesic-treated mice at the time points 48 h after the primary immunization and 72 h after the secondary immunization.

For the open-field activity test,[27] mice were placed individually into an open-field arena (20 × 20 × 20 cm; AccuScan Instruments, Columbus, OH) and permitted free exploration for 30 min. Movement was monitored by 2 rows of infrared diodes that detected vertical and horizontal activity by the animal. Locomotor activity was defined as centimeters moved, upright rearing activity as the frequency of infrared beam breaks, and nonlocomotor activity as the frequency of repetitive beam breaks within a single location and less than 1 s apart.

An automated treadmill system (TreadScan, Cleversys, Reston, VA) was used for active gait testing, including forced treadmill running. The mice were placed into a clear acrylic glass runway with a flexible clear plastic floor that moved at a controlled speed (11 to 15 cm/s; walking area approximately 14 cm × 4 cm). A camera beneath the runway captured 20-s digital videos of the mouse's gait at 100 frames per second. Videos were analyzed by gait analysis software (Cleversys). An observer was present whenever a mouse was in the runway chamber. Gait measurements obtained from the data included running time and speed.

For voluntary wheel running, mice were housed in individual cages with running wheels (Coulbourn Instruments, Whitehall, PA). Cages were placed in 2-level circadian cabinets (Phenome Technologies, Lincolnshire, IL) that controlled the light cycle and air flow for the animals in a temperature- and humidity-controlled environment. Illumination during the light cycle was maintained at 526 nm with green-wavelength light-emitting diodes. Infrared light-emitting diodes with night vision cameras were used for monitoring animals during the dark cycle. Running wheel activity was monitored with Clock Lab data

collection and analysis software (Actimetrics, Wilmette, IL). Tau (τ), which is the average period of biologic rhythm or oscillation, and the average wheel running counts scored as revolutions per day were recorded for each animal as described.[17] Voluntary wheel running was assessed for 3 d before immunization to obtain baseline activity. Mice had access to the wheel for 3 d after both the primary and secondary immunizations.

For Von Frey testing, mice were acclimated to the Von Frey testing apparatus for 30 min. over 3 to 5 consecutive days prior to immunization. The apparatus was a 90 × 70 cm stainless steel platform painted black and elevated 40 cm above the table top, with 12 individual acrylic glass cubicles (12 cm^2), each of which held a single mouse. The floor of the platform on which the mice were standing was ventilated with a 2-mm black steel grid also painted black. This allowed for urine and feces to pass through and for stimulus filaments to be introduced. Von Frey filaments of ascending diameters (Bioseb InVivo Research Instruments, Vitrolles, France) were applied to the plantar surface of the hind paws of each animal through the steel grid. The filaments were touched at a right angle to the paw just until the fiber bent. Paw withdrawal was assessed, defined as flicking, raising, or licking of the paw. The fiber diameter threshold at which paw withdrawal occurred was recorded. The procedures were replicated 2 to 4 times for a given animal, with an intertrial interval of at least 10 s.

Grip strength was measured by allowing mice to grip a trapeze or small stainless steel grid that was fitted to a peak amplifier that recorded the pull force of the animal in grams (Grip Strength Meter for mice; SDI, San Diego, CA). The mice were gently pulled away from the trapeze or grid in a horizontal plane. The trapeze or grid was released when the pulling force overcame the grip strength of the mouse. Mice received 3 sequential trials, separated by approximately 15 s (± 5 s). A final score was calculated as the average of the 3 trials for each mouse.

All data from behavioral testing were analyzed by ANOVA with Bonferroni-corrected pairwise comparisons (version 19, SPSS Statistics, IBM, New York, NY). Comparisons were made between the same mouse, before and after immunization, and between groups of analgesic- compared with nonanalgesic-treated immunized mice to assess effects of immunization and the ability of analgesics to alleviate those effects, respectively.

Assessment of vaccine efficacy. The effect of analgesics on vaccine efficacy was determined by measuring serum IgG titers. For these assays, ovalbumin or PA in PBS was allowed to bind to 96-well microtiter plates by using 60 µL of 1 µg/mL antigen per well. Wells were blocked by incubation with 3% bovine serum albumin, and then antigen-specific antibodies were allowed to bind during incubation for 90 min with serial 2-fold dilutions of serum. Bound antibodies were detected by using goat antimouse horseradish peroxidase conjugates specific for murine IgG (Jackson ImmunoResearch Labs, West Grove, PA) followed by 3, 3′,5,5′-tetramethylbenzidine substrate (KPL, Gaithersburg, MD). The antibody titer was defined as the highest dilution at which color development in the immunoassay was 3 times the background signal obtained by using a similar dilution of pooled preimmunization serum. Reciprocal titers were log-transformed (base 2), and statistical evaluation was performed on the logarithmic data by using Student *t* test or ANOVA. A value of *P* value of 0.05 or less was defined as statistically significant. Upper and lower confidence intervals were generated by multiplying or dividing the geometric mean titer by 1 SD, respectively.

Results

Physical responses to immunization. Mice were monitored daily for the first 3 d after immunization in an attempt to identify physical signs that might reflect immunization-related pain or intensity of immune responses. Core body temperature, skin temperature at the immunization site, and body weight did not vary significantly from baseline during the 72-h postimmunization period. Most mice did develop slight to moderate erythema, swelling, and nodules of various sizes at the immunization sites, especially by the 72-h time point. The erythema and swelling resolved by 1 wk after immunization, whereas the nodules of various sizes remained throughout the duration of the study in most of the mice. Mice appeared to maintain normal activity in their home cages after immunization.

Behavioral responses to immunization in absence of analgesic. A panel of behavioral tests was applied to nonanalgesic-treated control mice to identify behavioral changes that might potentially reflect immunization-related pain. All mice were studied at baseline (that is, prior to immunization). To minimize changes in test results due to habituation, mice were randomly selected for testing at either 48 or 72 h after primary or secondary immunization but not both points. Tests in which a significant difference emerged between pre- and postimmunization results in the absence of analgesia were considered to be potentially informative regarding immunization-related pain.

As previously reported for mice,[27] when tested prior to immunization, mice initially showed higher activity in the baseline open-field test, followed by reduced activity as they became acclimated to the test arena (Figure 1 A). The horizontal distance traveled in the open field was lower at both 48 and 72 h after primary immunization in the leg with antigen and CFA as compared with baseline ($P < 0.001$ for 48 and 72 h time points compared with preimmunization baseline; Figure 1 A). Distance traveled in the open field also was reduced after secondary immunization with antigen and IFA ($P < 0.001$ compared with baseline for both 48 and 72 h time points; Figure 1 A). Vertical activity (rearing on hindlegs) in the open field was reduced at 48 h after primary immunization with CFA ($P < 0.001$) but began to normalize by 72 h ($P = 0.03$; Figure 1 B). Vertical activity was below baseline at both 48 and 72 h after secondary immunization with IFA ($P = 0.004$ and 0.001, respectively, compared with baseline; Figure 1 B). Nonlocomotor behavior in the open field decreased after immunization, with reductions at both 48 and 72 h after primary immunization with CFA and at 48 and 72 h after secondary immunization with IFA ($P < 0.001$ compared with baseline at all 4 time points). Immunization-related decreases in nonlocomotor behavior were particularly apparent early in the open-field testing session (Figure 1 C).

Computerized analysis with tread-scan behavioral recognition systems was used to assess the effects of immunization on gait in nonanalgesic-treated control mice. These analyses examined the 4 phases of gait: stance, propel, swing, and brake. Stance is the phase at the beginning of a stride when the feet are in contact with the walking surface. No changes in stance time were observed for the front feet after immunization (data not shown). However, stance time for the rear feet was reduced at 48 h after primary immunization with CFA (baseline, 211 ± 7 ms; after immunization, 164 ± 17 ms; $P = 0.03$). Stance time for the rear feet normalized to 187 ± 18 ms by 72 h after primary immunization with CFA and was similar to baseline after secondary immunization with IFA. The propel phase of the stride when the front paws (nonimmunized legs) were leaving the walking surface was not affected by immunization (Figure 2 A). The propel time for rear paws showed a trend toward decreas-

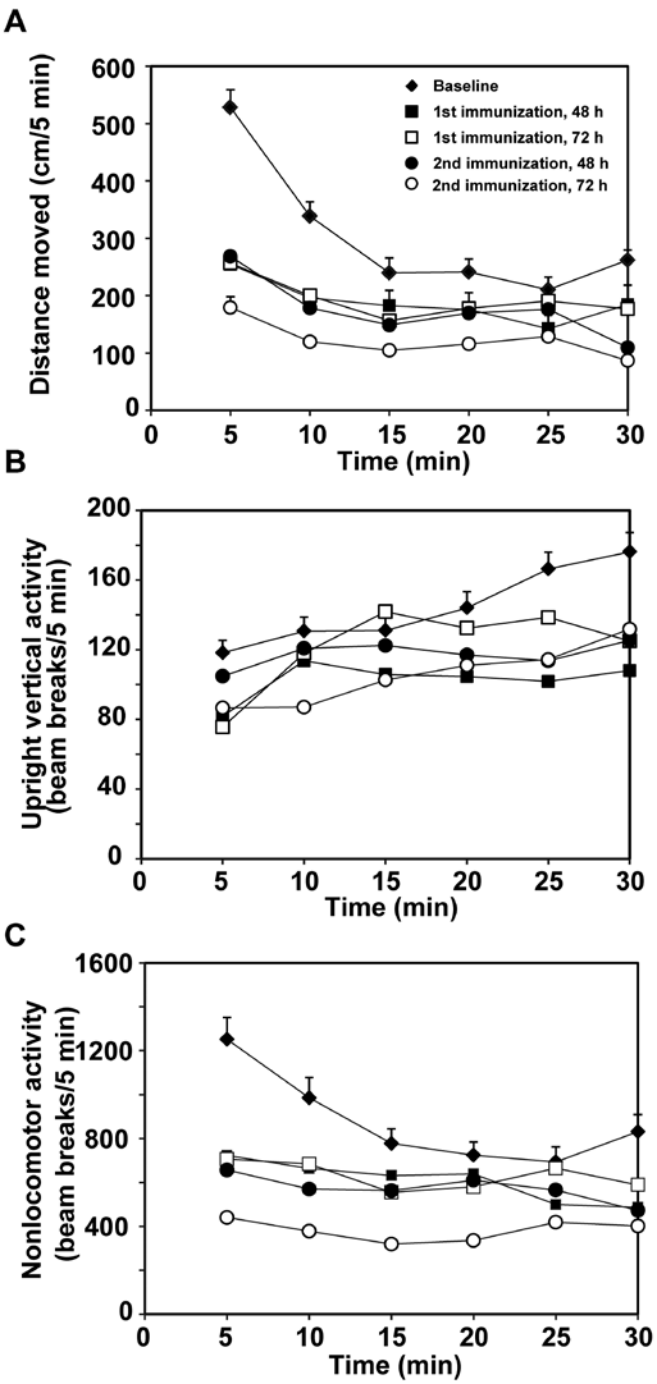

Figure 1. Effect of immunization on behavioral responses in the open field in nonanalgesic-treated control mice. (A) Horizontal locomotion, (B) vertical rearing, and (C) nonlocomotor activity were measured in an open field in nonanalgesic-treated control mice at 24 to 48 h before (baseline) and 48 and 72 h after primary (1st) immunization with antigen and CFA and secondary (2nd) immunization with antigen and IFA. Error bars that represent 1 SEM are shown for baseline measurements (n = 10 mice) but were similar or smaller for the postimmunization measurements (n = 5 mice each) and were omitted to simplify the figure. Each time point in each of the postimmunization studies was significantly different from baseline; P values ranged from 0.03 to less than 0.001.

ing at 48 h (P = 0.06) but had normalized by 72 h after primary immunization with CFA (Figure 2 A). However, propel time for the rear paws was significantly decreased at both the 48- and 72-h time points after secondary immunization with IFA (P = 0.02 and 0.05, respectively) compared with baseline (Figure 2 A). No immunization-related changes were noted in the swing phase, when the foot was free-floating during the active phase of the stride, or in the brake phase at the end of the stride (data not shown).

During forced running on an automated treadmill, mice showed no functionally significant differences in running speed (a value derived from average time required to complete a single stride) after immunization (data not shown). However, nonanalgesic-treated control mice showed a decrease in forced running time (the time a mouse ran before stopping) from 12.5 ± 0.9 s before immunization to 4.9 ± 0.6 s at 48 h (P < 0.001 compared with baseline) and 8.8 ± 1.1 s at 72 h (P = 0.018) after the primary immunization with CFA (Figure 2 B). Similar decreases in forced running time occurred at 48 and 72 h after the secondary immunization with IFA (P < 0.001 and P = 0.002, respectively; Figure 2 B). Because running speeds were the same, this result indicates that mice ran less distance after immunization than at baseline.

Mild shifts in posture associated with immunization were seen; these shifts were compensated for by the opposite or contralateral sides. Coupling ratios that reflect the coordination of front and rear paws or left and right paws were not affected by immunization. Stride width for front and rear paws also was unaffected by immunization. However, immunization resulted in a change in stride angle, the deflection angle of the foot relative to the central body axis and stride direction, such that feet were more inward facing. This change was most apparent at 48 h after primary immunization with CFA and at 72 h after secondary immunization with IFA (data not shown).

Prior to immunization, the rear paws and legs exerted more force on the grip meter than did the front paws and legs, indicating a stronger grip (Figure 2 C). The force generated by the rear paws and legs was lower after immunization at all 4 time points tested (Figure 2 C). The grip strength in the front (nonimmunized) limbs also was lower after primary immunization with CFA.

The Von Frey test measures paw flick response to probing with filaments of defined stiffness. In nonanalgesic-treated control mice at baseline, paw flick increased as the diameter of the filament increased, as has been reported previously.[9] Immunization reduced this paw flick response (Figure 2 D). Similar decreases in paw flick response were detected in both right paws (nonimmunized leg) and left paws (immunized leg).

Effects of analgesia on postimmunization behavior. Open-field testing, forced running time on an automated treadmill, and voluntary wheel running were used for evaluation of the effects of analgesia on behavior after immunization. Open-field measurements reflect responses to a novel environment and are more sensitive measures of wellbeing than are those obtained repeatedly or in the home cage.[27] Return of postimmunization results toward those measured before immunization in the same mice was considered to indicate effective analgesia.

Treatment with meloxicam minimized the pre- compared with postimmunization differences in distance traveled in the open field (Figure 3 B) and corrected post immunization decreases in open-field vertical rearing (Figure 3 F). Open-field distance traveled remained lowered after immunization in mice treated with either acetaminophen or buprenorphine (Figure 3 C and D) and neither of these analgesics affected the immunization-related decrease in open field vertical rearing (Figure 3 G and H). The decrease in nonlocomotor activity in the open field observed in nonanalgesic-treated control mice (Figure 3 I) was

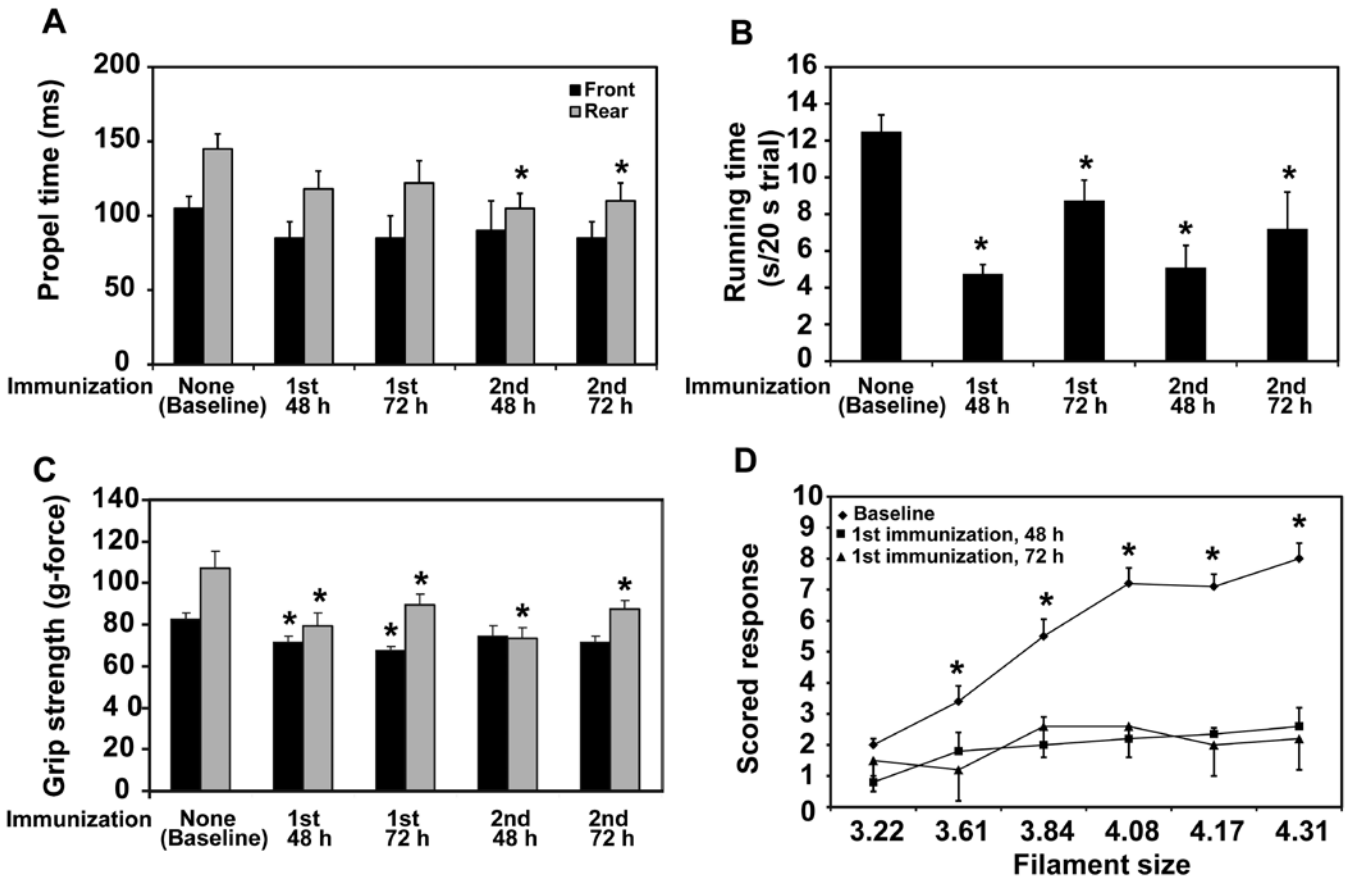

Figure 2. Effect of immunization on gait parameters, grip strength, and Von Frey responses in nonanalgesic-treated control mice. Computerized gait analysis revealed decreases in the propel phase of the gait for the rear (immunized) leg at both 48 and 72 h after secondary immunization with IFA. (B) Forced running time on an automated treadmill and (C) grip strength in rear legs also decreased after immunization in the leg. (D) Although mice had vigorous withdrawal responses of rear paws to probing with Von Frey filaments prior to immunization, their responses were decreased after primary immunization. No differences were observed in responses after probing feet on the immunized compared with nonimmunized side; the data shown therefore represent a composite of responses from both rear feet. Von Frey responses after secondary immunization did not differ from those seen after primary immunization and were omitted for clarity. *, Significant ($P < 0.05$) difference between baseline and values for experimental groups.

not affected by treatment with any of the 3 analgesics (Figure 3 J through L).

Running time in the forced treadmill test was decreased in the control (no analgesia) group after both primary and secondary immunizations (Figure 4 A). Meloxicam did not prevent this decrease after primary immunization with CFA but did permit normalization of running time after secondary immunization with IFA. Both acetaminophen and buprenorphine prevented immunization-induced decreases in forced running time after both primary and secondary immunizations (Figure 4 A). Running speed did not decrease after immunization, with or without analgesic administration, and even increased slightly after immunization in mice treated with acetaminophen or buprenorphine (Figure 4 B).

Control and analgesic-treated mice showed a similar length of their circadian cycle while in the voluntary running wheel cages (data not shown), demonstrating that the analgesics used did not affect circadian rhythm. Voluntary running activity during the light cycle (normal resting period for the mouse) was similar for control and analgesic-treated mice and was not affected by immunization (data not shown). However, during the dark cycle (normal active period for the mouse), nonanalgesic-treated control mice as well as those treated with meloxicam showed significantly ($P < 0.05$) less voluntary wheel running as compared with baseline. Mice treated with acetaminophen or buprenorphine showed no decrease in voluntary running activity after either primary immunization (Figure 5) or secondary immunization (not shown).

Effects of analgesia on antibody production. Treatment with acetaminophen for 48 h before and 72 h after immunization or with meloxicam or buprenorphine for 72 h after immunization did not decrease primary antibody responses in response to moderate doses of ovalbumin in CFA (Figure 6 A) or secondary antibody responses to ovalbumin in IFA (Figure 6 B). As a more rigorous test of analgesic effects on antibody responses, additional groups of mice were immunized with a limiting amount of PA from *Bacillus anthracis* in CFA (first immunization) or IFA (second and third immunizations). Analgesic treatment did not affect the number of mice that mounted immune responses and did not alter the mean or maximum antibody titers that developed when mice were immunized under conditions where antigen was limited (Figure 6 C).

Effect of exercise on postimmunization behavior. Addition of voluntary homecage wheel running to our battery of postimmunization behavioral tests presented the opportunity to assess the combined effects of exercise before and after immunization on behavioral assessments in mice treated with acetaminophen or buprenorphine. Neither exercise nor analgesia caused significant differences in forced running speed after immunization (data not shown). Likewise, mice that could exercise in their

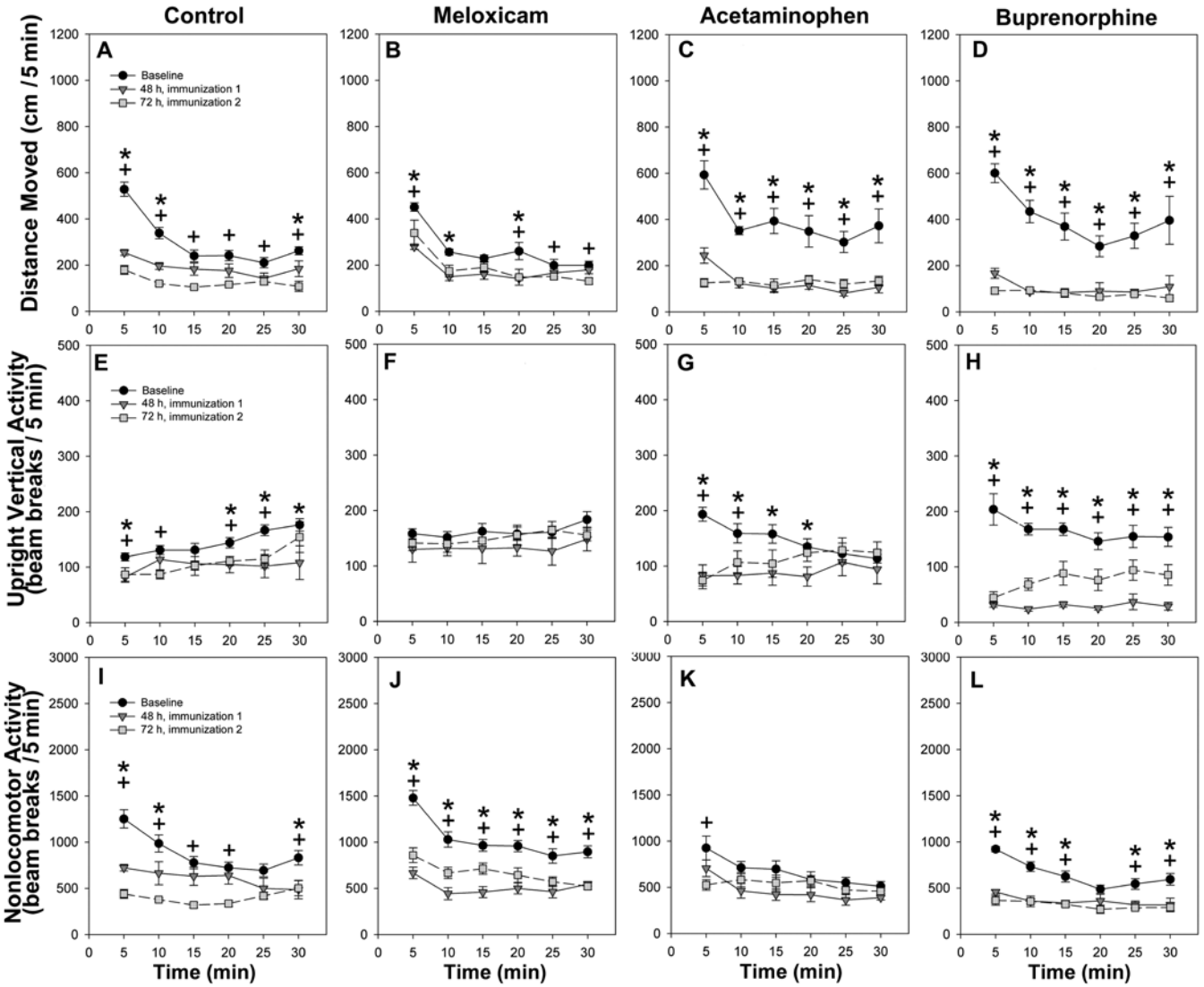

Figure 3. Effect of analgesia on open-field responses after immunization. For the open-field activity test, mice were placed individually into and permitted free exploration of the open-field arena. Locomotor activity was assessed by infrared sensors that recorded (A through D) horizontal locomotion, (E through H) vertical activity, and (I to L) nonlocomotor activity (repetitive but stationary movements) as a function of time in the arena. *, Significant ($P < 0.05$) difference compared with preimmunization baseline for primary immunization with CFA; +, significant ($P < 0.05$) difference compared with baseline for secondary immunization with IFA. In analgesic-treated mice, the lack of significant difference from baseline is an indicator that immunization-related changes had been alleviated by that drug.

homecage before immunization showed no changes in baseline open-field horizontal activity compared with that of nonexercised mice (compare solid black lines in Figure 7 with those in Figure 3). However, at 72 h after secondary immunization with IFA, exercised mice that also received acetaminophen or buprenorphine showed significantly ($P < 0.05$) more open-field activity than did nonexercised mice treated with the same analgesic (Figure 7 A and B).

Discussion

Assessment of pain experienced by animals is challenging, particularly for prey species such as mice that may attempt to hide pain or discomfort. The goal of the current study was to identify and validate objective methods to assess and relieve pain related to immunization in mice. Our results show that responses suggestive of pain after immunization with CFA or IFA can be identified and objectively assessed in mice by using commonly available behavioral tests and can be alleviated by administration of analgesics.

These conclusions rely on 2 critical assumptions. First, changes in behavior before and after immunization for a given mouse were assumed to reflect pain or discomfort associated with the immunization. Second, interventions that restored behavior toward what occurred before immunization were assumed to relieve such pain or discomfort. Although these assumptions may not be universally valid, they nonetheless provide a starting point that allowed us to begin to address this important but otherwise intractable animal welfare question. Although we did not evaluate a group of mice subjected to 'sham immunization,' our study design allowed within-animal comparisons that control for interindividual variation. Our study was designed specifically to evaluate the effects of immunization with vaccines containing CFA or IFA and therefore cannot be used to differentiate effects of the adjuvants used or effects of the immunization itself.

Robust and highly significant immunization-associated changes were detected in horizontal locomotion, vertical rearing, and nonlocomotor activity in the open field for

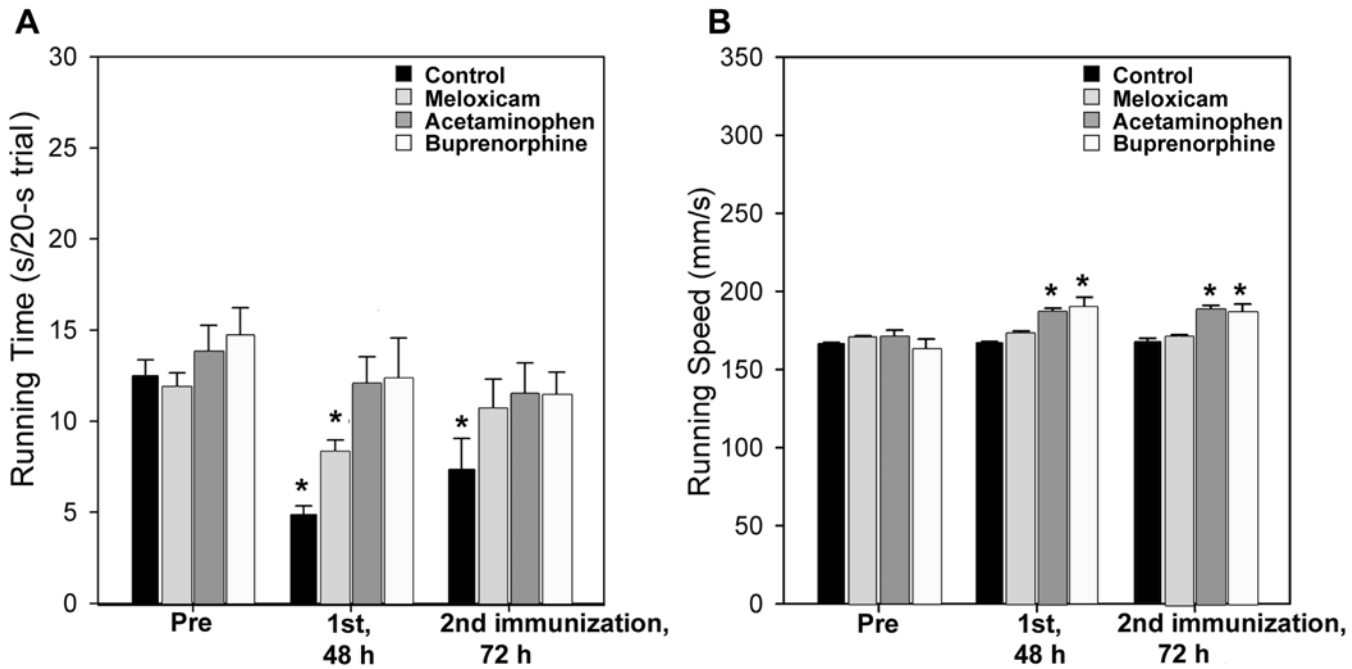

Figure 4. Effect of analgesia on forced treadmill running after immunization. Active gait scans were performed by using an automated treadmill. Videos of gait were digitized and analyzed to obtain (A) running time and (B) speed in control and analgesic-treated mice. *, Significant ($P < 0.05$) difference compared with preimmunization baseline (Pre) value. In analgesic-treated mice, the lack of a significant difference from baseline is an indicator that immunization-related changes had been alleviated by that drug.

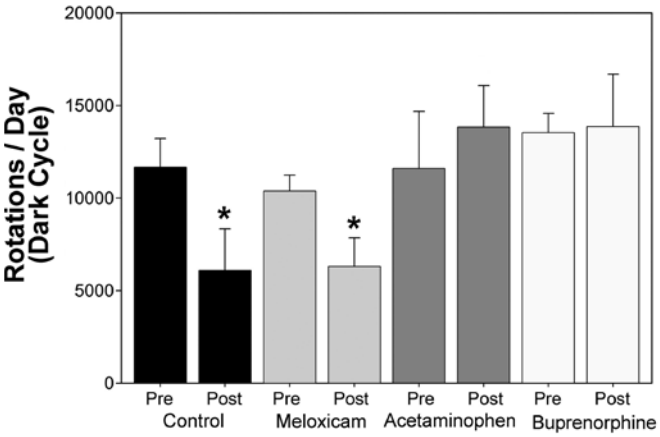

Figure 5. Effect of analgesia on voluntary running after immunization. Mice were placed individually in housing cages with a computerized running wheel for 3 d before (Pre) and 3 d after (Post) primary immunization with CFA to assess the effect of immunization and analgesia on voluntary running behavior. C, control (no analgesia); M, meloxicam; A, acetaminophen; B, buprenorphine; *, significant ($P < 0.05$) difference compared with preimmunization baseline value. In analgesic-treated mice, the lack of a significant difference from baseline is an indicator that immunization-related changes had been alleviated by that drug.

nonanalgesic-treated control mice. Gait changes (reduction in stance and propel times for the rear feet and changes in stride angle), increased thresholds for moving lower limbs, decreased running time, and decreased grip strength also were observed after immunization. A relation of these changes to pain is plausible, because mice experiencing pain might be less likely to explore a novel area or would decrease weight-bearing (for example, vertical rearing) on a painful limb. The observed gait changes are consistent with a mild limp that would be expected to minimize local discomfort by repositioning or minimizing weight bearing on the immunized limb. Responses in the Von Frey paw flick tests are a composite of sensitivity of the probed foot (pain) and motivation and ability to move that foot. The ability of the mice to rapidly move all limbs after immunization was confirmed based on other tests. Direct changes in pain experienced in the foot itself are not expected, because immunization was performed much higher in the leg. Although our data cannot rule out the possibility that immunization globally reduces pain sensation, the interpretation that the mice have less motivation to move their paws after immunization seems more likely. The concept that overall motivation for limb movement is decreased due to immunization-associated pain or malaise is plausible based on anecdotal human experience. Because immunization-related changes in grip strength and paw-flick responses were similar in immunized and nonimmunized limbs, we speculate that those changes may be due to systemic effects of immunization rather than simply due to local discomfort. Taken together, the changes in these behavioral measures after immunization consistently indicated a reluctance to move or to bear weight on the immunized compared with nonimmunized limbs. These measures may therefore accurately reflect pain after immunization.

Our studies showed that readily observable physical signs such as body temperature, erythema or swelling at the immunization site, and general physical activity were not sensitive indicators of pain or distress after immunization in the mice. Immunization-associated changes in the open-field tests of locomotor activity, vertical rearing, and stereotypic behavior and forced treadmill running in nonanalgesic-treated mice seemed to be consistently highest at the time points of 48 h after primary immunization with CFA and 72 h after secondary immunization with IFA; we therefore used these time points for the analgesia studies. The 72-h time point correlates with the maximum expected time of peak T cell infiltrates in a recall response.

Treatment with the common analgesics meloxicam, acetaminophen, and buprenorphine normalized many—but not all—of

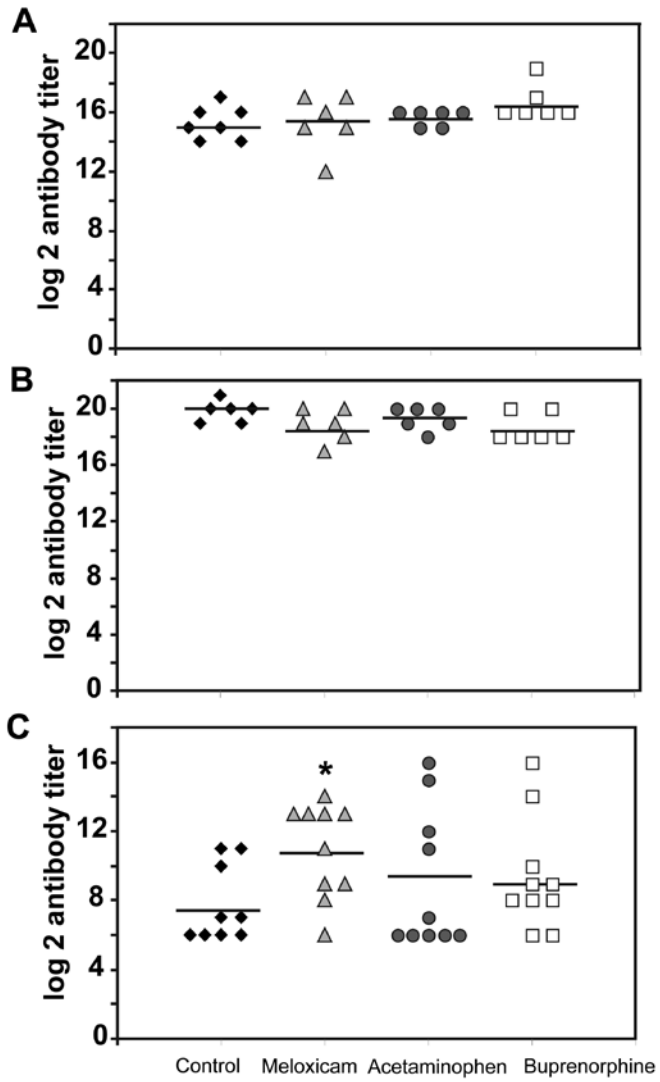

Figure 6. Effect of analgesia on antibody responses after primary immunization with CFA or repeated immunization with CFA and IFA. Each point represents the titer from a single mouse; the line indicates the geometric mean. (A) Ovalbumin-specific IgG responses on day 21 after primary immunization on day 0 with 100 mg ovalbumin and CFA. (B) Ovalbumin-specific IgG responses on day 42 after primary immunization on day 0 with 100 mg ovalbumin and CFA and secondary immunization on day 21 with 100 mg ovalbumin and IFA. (C) PA-specific antibody responses are shown on day 56 following immunization with 2 mg PA and CFA on day 0 and with 2 mg PA and IFA on days 21 and 42. The line indicates the geometric mean titer for each group. No significant differences in antibody titer were observed unless noted. *, Significant ($P < 0.01$) difference from value for control (no analgesia) group.

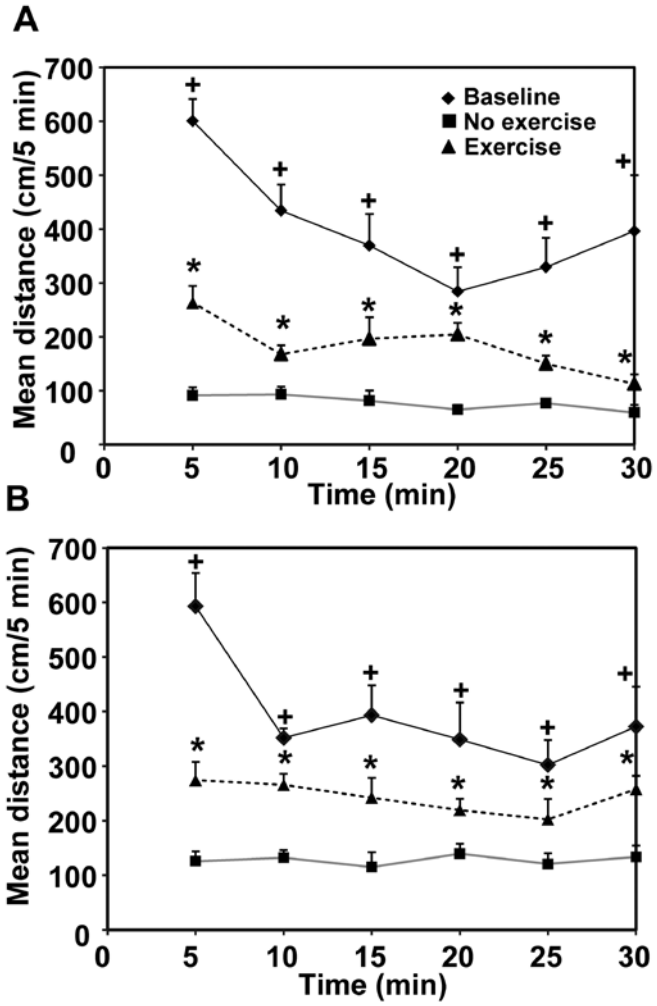

Figure 7. Effect of voluntary exercise on locomotion in an open field. Baseline horizontal locomotion in the open field test for mice allowed to exercise voluntarily on running wheels for 2 d prior to testing (solid black lines in panels A [acetaminophen treatment] and B [buprenorphine treatment]) did not differ from that observed for nonexercised mice (Figure 3 C and D). Mice allowed to exercise after immunization (dotted black lines in panels A and B) showed significantly (*, $P < 0.001$) increased locomotion at 48 h after primary immunization compared with immunized mice that remained sedentary (solid gray lines). Despite this beneficial effect of exercise and administration of analgesic, locomotion remained significantly ($P < 0.001$) decreased compared with baseline for both exercised and nonexercised mice after immunization.

the immunization-related behavioral changes but did not inhibit antibody responses to vaccines containing CFA or IFA, even when the dose of antigen was limiting. Allowing voluntary exercise before and after immunization further decreased immunization-associated behavioral changes in analgesic-treated mice. Exercise of the immunized limb therefore may represent an additional method to minimize pain associated with CFA- or IFA-containing immunizations. Because studies requiring immunization are common, these results provide methods for enhancing humane animal use through appropriate assessment and management of immunization-associated pain.

The behavioral differences that we observed before and after immunization were not likely simply due to repeated testing. Mice have been shown to modify their behavior when repeatedly exposed to the open field.[6] In particular, novelty-related anxiety during the initial exposure may result in a small to moderate reduction in activity between first and second exposures. The interval between exposures appears to have less consequence than the number of exposures, particularly for relatively short periods of time between exposures used in this study. However, the significant differences that were observed based on presence or absence of analgesic treatment or different times after immunization in mice exposed to the open field for the second time clearly indicate that those differences cannot simply be due to reexposure. Novelty has not been reported as a factor in determining gait, grip strength, or response to von Frey testing. Running wheel activity in mice is known to remain stable and synchronized with light cycle when the environment remains stable over test days,[5] and the multiple

days studied prevented any novelty-related effects on voluntary running behavior.

The finding that some measures of open-field activity remained lower after immunization despite administration of analgesics indicates that the analgesics tested cannot restore the novelty-seeking and exploratory behavior characteristic of nonimmunized mice. Alternatively, these results may reflect lassitude associated with psychologic or sedative effects of some analgesics rather than continued pain. The increased voluntary home cage wheel running observed in analgesic-treated mice suggests that analgesic treatment, particularly with acetaminophen or buprenorphine, does effectively relieve local discomfort after immunization. Opioids in general have the potential to cause some CNS depression,[19] and buprenorphine in particular has been shown to cause sedation as a side effect of administration in some animals.[26] Administration of buprenorphine has also been shown to produce a progressive increase in locomotor activity.[30] These seemingly conflicting drug effects may account for both the failure to normalize in the open-field tests (Figure 3 D, H, and L) and the increased running speed observed in buprenorphine-treated mice after immunization (Figure 4 B). Meloxicam was the only analgesic tested that normalized vertical activity in the open field. Given that vertical activity in particular requires both muscle strength of the lower body and motivation to put weight on the immunized limb, this finding likely reflects improved control of immunization-associated pain in the immunized leg in meloxicam-treated compared with control mice. The generally similar activity curves for mice that received acetaminophen during baseline testing to prevent neophobic avoidance after immunization and for animals that were drug-free during baseline testing indicates that acetaminophen administration alone does not significantly affect most behavioral measures tested.

Nonlocomotor activity during open-field testing was defined by multiple beam breaks in a single location, less than 1 s apart. This definition likely limits the movements measured to rearing and grooming.[27] In performing these behaviors, the mouse bears weight on its hindlimbs and does repetitive movements with its forelimbs or tries to stand upright repeatedly in a short period of time. Nonanalgesic-treated control mice showed a decrease in nonlocomotor movements after immunization, consistent with discomfort generated if these activities focus weight on the immunized limb. However, the nonlocomotor activity observed in the acetaminophen group after immunization did not significantly differ from baseline at either of the time points measured (Figure 3 K). Because acetaminophen was provided 2 d prior to immunization to prevent neophobic avoidance, some nonlocomotor movements may have been suppressed during baseline testing as compared with those of the control group. Nonlocomotor activity in the buprenorphine and meloxicam groups appeared to be similar to that seen in control groups. Immunization may have different effects on movements that require bearing pressure through the hips and pelvis as compared with movements that require all 4 limbs.[23] Therefore, tests such as the forced treadmill, running wheel, and open field may have different thresholds for sensitivity to pain or inflammation and thus different responses to the different analgesics.

The doses, route, and administration schedules that were used for meloxicam and buprenorphine in the current study were within the ranges recommended for analgesia by veterinary reference texts.[10,26] The buprenorphine normalized results in forced and voluntary running assays, although not in the open field. The sedative or motivational effects of these analgesics clearly may affect results of behavioral tests but were not assessed in the current study. In this study, meloxicam and buprenorphine were administered by subcutaneous injection to ensure rapid action. Buprenorphine can also be provided orally in flavored gelatin cubes, although the oral route is associated with a questionable level of analgesia[10,19] and difficulty in ensuring uniform intake in group-housed animals. Meloxicam is available as an oral liquid form and can also be administered as a topical gel that has been shown to produce therapeutic plasma levels.[12] Providing either meloxicam or buprenorphine in food or water could reduce both the need for handling and potential pain associated with injections but may not produce efficacious levels of analgesia. The strict criteria for storage and record keeping of buprenorphine as a controlled substance may produce additional obstacles to administration of this drug.

Our results may appear to conflict with some studies in humans that have shown a decreased immune response when analgesics such as nonsteroidal antiinflammatory drugs and opioids are administered.[4,13,25,28] However, the human studies may not be comparable to our study, because they included subjects such as young children, elderly adults with a history of chronic use of antiinflammatory drugs, and immune-compromised patients, who may be less likely to mount effective immune responses as compared with those of the general population. Furthermore, in these cited studies, the types and dosing parameters of analgesics varied, a variety of vaccine compositions were used, and CFA and IFA were not used. Our data clearly show that providing analgesics for 3 d after immunization as described here does not decrease antibody responses in mice that are immunized by using CFA or IFA.

The current study was designed to mimic the conditions typically used when mice in research studies are immunized with vaccines that contain CFA and IFA. The antibody response was not negatively affected in our studies when analgesics were provided over a period of 3 to 5 d relative to immunization. Buprenorphine, in particular, has been shown to produce little to no negative immune alteration, and it may actually enhance immune function in some settings.[22,25,29] Our study found that mice treated with meloxicam had significantly ($P = 0.01$) higher antibody titers when antigen was limiting, whereas antibody titers from mice treated with acetaminophen or buprenorphine did not significantly differ from those of nonanalgesic control mice (Figure 6 C). The reason for this outcome remains unclear. Nevertheless, the data strongly refute the hypothesis that providing analgesia after immunization of mice with CFA- or IFA-containing vaccines will prevent or negatively affect antibody responses.

In summary, we identified objective behavioral measures that appear to sensitively detect immunization-related pain in mice and refined the CFA–IFA model for immunization by identifying effective analgesics that do not negatively affect immunization efficacy. Therefore, use of any of these analgesics can be encouraged for the management of rodent pain associated with vaccines that use CFA or IFA as adjuvants. These results likely will enhance animal welfare in immunization studies and facilitate IACUC decision-making when evaluating animal use protocols and monitoring compliance in protocols that use CFA.

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