Inhibition of Electrically-Induced Vocalization in Adjuvant Arthritic Rats as a Novel Method for Evaluating Analgesic Drugs

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Abstract—A new method for the evaluation of analgesic drugs in normal rats and in rats with hyperalgesia induced by adjuvant was developed using the vocalization response as an indicator of pain due to electrical shock. It was demonstrated that at ED50 doses, nonsteroidal anti-inflammatory drugs (NSAIDs), narcotic analgesic drugs and narcotic agonist/antagonist type analgesic drugs were effective, but pure narcotic antagonist drugs, CNS-acting drugs and anti-inflammatory steroid were ineffective. Acidic NSAIDs, except aspirin, were more effective in adjuvant arthritic rats than normals but other analgesic drugs had roughly the same effect in both. It was suggested that the acidic NSAIDs specifically inhibit inflammatory pain. Moreover, the vocalization response under adjuvant treatment is useful for the quantitative measurement of analgesics.

Recently, adjuvant arthritic rats have been used to evaluate the analgesic activity of narcotic and non-narcotic analgesics as an experimental model of chronic pain (1-8). Kuzuna and Kawai (2), Winter et al. (4) and Capetola et al. (7) reported that the vocalization response evoked by manipulation of the tarso-tibial joint or tail in adjuvant arthritic rats was inhibited by various analgesic drugs. Piccio et al. (3) reported that the vocalization of a group of adjuvant arthritic rats placed in close contact with each other was decreased by treatment with a wide variety of analgesic drugs. It appears, therefore, that the vocalization displayed by rats with adjuvant-induced polyarthritis may be regarded as an expression of pain.

Because the efficacies of weak analgesics such as NSAIDs and narcotic agonist/antagonist type analgesic drugs are not detectable in normal rats, the phenylquinone writhing test in mice (9), Randall-Selitto's test in rats (10) and the vocalization test in adjuvant arthritic rats should be superior to other methods. However, the vocalization response induced by manipulation of the tarso-tibial joint or tail and placing in close contact were observed only in adjuvant arthritic rats, but not observed in normal rats. Thus, none of the currently available techniques are sensitive enough to examine adjuvant arthritic rats and normals under the same experimental conditions.

Drugs which inhibit the pain response of adjuvant arthritic rats at the same dose level as in normals are not recognized as having alleviating effects on inflammatory pain. Thus it was decided to give electrical stimulation to adjuvant arthritic rats and/or normal rats and resulting precise and reproducible indications of algesia, the vocalization response, were quantitatively recorded.

Materials and Methods

Male Sprague-Dawley rats weighing 140-160 g at the time of Mycobacterium butyricum (adjuvant, Difco Laboratorones, Detroit, MI) injection were housed in an air-conditioned room at 22±1°C with a 12 hr light-dark schedule (light at 7:00). Food and water were given ad libitum during the experimental period.

Rats were injected intradermally at the base of the tail with 0.1 ml of a paraffin oil suspension of heat-killed adjuvant (0.5 mg) or the sterile vehicle (paraffin oil). The experimental equipment and its arrangement are schematically illustrated in
Fig. 1. The rat was placed in a test chamber made of acrylic resin (30 x 30 x 35 cm, inside dimensions). The floor grid was prepared for electrical stimulation and a nondirectional microphone with high impedance (10 KΩ) was attached to the ceiling. For electrical stimulation, the cathode was connected to the floor grid, and the anode was connected to the dorsal skin of the rat. Rectangular wave pulses, generated from a stimulator (SEN-7103, Nihon Kohden), were applied to the rat.

When the rat was stimulated, a pain response vocalization was detected with a microphone and relayed through a low cut filter (530 Hz), biophysical amplifier (AB-620G, Nihon Kohden), and an integrator (EI-600G, Nihon Kohden) for recording on an ink writing oscilloscope (WT-645G, Nihon Kohden).

To evaluate the effect of drugs, 5 successive stimulations for 5 sec were applied at intervals of 15 sec every 30 min or 60 min. The average vocalization of each measurement was calculated by the mean values of 5 vocalization areas. The degree of vocalization was calculated by computing the area under the vocalization curve traced on an ink writing oscilloscope. The pre-drug vocalization magnitude was determined by means of the average of these 5 vocalization areas. The post-drug vocalization magnitude was calculated by the average vocalization of all measurements made on each of the animals over the whole post-drug period. The % inhibition was determined by comparing the pre- and post-drug vocalization magnitude. The dose which produced 50% reduction (ED50 values) was determined by probit analysis based on 3 logarithmically spaced doses per drug. Eighteen rats were used in each experimental group for determining the ED50.

The drugs used were morphine hydrochloride (Shionogi), codeine phosphate (Shionogi), pentazocine (Sankyo), levallorphan tartrate (Takeda), naloxone hydrochloride (Endo), indomethacin (Sigma), sodium diclofenac (Fujiwawa), ketoprofen (Hokuriku), naproxen (Tanabe), ibuprofen (Kaken), mfenamic acid (Sankyo), phenylbutazone (Sigma), aspirin (Sigma), aminopyrine (Sigma), acetaminophen (Sigma), mepirizole (Daichi), tiaramide hydrochloride (Fujiwawa), perisoxal citrate (Shionogi), benzodamine hydrochloride (Daichi), tinoridine hydrochloride (Yoshitomi), haloperidol (Taisho), diazepam (Hoffman-La Roche), atropine sulfate (Tokyo Kasei), imipramine hydrochloride (Kohei), diphenhydramine hydrochloride (Tokyo Kasei), cyproheptadine
hydrochloride (Merck), mephenesin (Sigma) and prednisolone (Shionogi). Water-insoluble drugs were suspended with 0.3% sodium carboxymethylcellulose in saline, and water-soluble drugs were dissolved in saline solution. Test drugs were administered in 0.2 ml/100 g body weight doses orally (p.o.) or subcutaneously (s.c.).

Statistical analysis was performed using Student's t-test for group comparison.

Results

Examination of electrical stimulation conditions: Adjuvant arthritic rats received electrical stimulation at a frequency of 0.5–500 Hz and pulse duration of 0.1–100 msec for 5 sec. The most intense vocalization response was obtained when 10 Hz and 5 msec pulses were applied (Fig. 2).

The intensity of electrical stimulation was gradually stepped up (0.1–0.25 mA/step). The vocalization response in adjuvant arthritic rats and normals increased in parallel with intensity of electrical stimulation and reached a plateau at 0.6–2.0 mA (m=1.8±0.1 mA) and 4.5–5.5 mA (m=5.0±0.2 mA), respectively. The animals were stimulated with submaximum intensity (about 80% of maximum intensity). About 20% of the animals showed running in the test chamber, biting of the floor grid or jumping during the electrical stimulation, which could interfere with measurement of the vocalization response. Such animals were avoided in our pharmacological investigation.

Adaptation of the vocalization response to successive stimulation was examined using 10 Hz, 5 msec at 0.5–1.5 mA (m=1.3±0.1 mA) pulse wave for 5 sec. Although no significant decrease in vocalization was observed when 10 successive stimulations were applied at 15 sec intervals, decreases were observed when stimulation was applied at 0 and 5–10 sec intervals.

Under these stimulation conditions, 5 successive stimulations for 5 sec were applied at intervals of 15 sec every 30 min–60 min and produced reproducible vocalization responses for up to 8 hr.

The same electrical stimulation conditions in adjuvant arthritic rats, normal rats and vehicle treated rats were also shown to give a stable vocalization response for 8 hr.

Development of hyperalgesia and arthritis after adjuvant injection: Thirty-one rats were used. Twenty-five randomly selected rats were injected with a suspension of adjuvant, and the remaining six rats were injected with only the sterile vehicle and used as controls. The pain threshold and the diameter of the tarso-tibial joint were measured once a day at the same hour (10:00 AM) throughout the experimental period.

The intensity of electrical stimulation which elicited a minimal vocalization response was defined as the pain threshold. The pain threshold of control rats was 3.8±0.2 mA which persisted evenly through the 33 days of the experimental period. In adjuvant
arthritic rats, the pain threshold was 3.9±0.1 mA before adjuvant injection, increased slightly from day 2 to day 4 after injection, and reached a peak of 4.9±0.2 mA on day 6. Thereafter, the threshold gradually dropped to 1.1±0.1 mA by day 15 and was maintained at this level until day 33. The diameter (mm) of the tarso-tibial joints was measured to determine the magnitude of arthritis. The swelling of paws began on day 10 and reached a peak on day 26 (Fig. 3).

As shown in Fig. 3, hypoalgesia was observed from day 2 to day 6 of the adjuvant injection. In 16 additional rats, three days after adjuvant (n=8) or vehicle (n=8) treatment, the pain threshold of the adjuvant and vehicle groups were 3.8±0.1 mA and 5.5±0.2 mA, respectively. Naloxone (2 mg/kg, s.c.) significantly lowered the threshold of the adjuvant treated group, but had no effect on the vehicle treated group (Fig. 4).

Analgesic effects of various drugs in adjuvant arthritic rats: The effects of drugs were determined on day 15-day 20 of adjuvant injection since the threshold for hyperalgesia was stable at this time. To evaluate the effect of NSAIDs in adjuvant arthritic rats, 5 successive stimulations of 15 sec intervals were applied hourly for 5 hr. Adjuvant arthritic rats in the narcotic and other drug groups received stimulation every 30 min for 3 hr. Stimulations were applied to

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Fig. 3. Development of hyperalgesia and arthritis after adjuvant injection. Twenty-five rats received intradermal injections of adjuvant (—○—). Six rats, used as controls, were injected with vehicle (—●—). Upper: Changes in the pain thresholds (mA) after adjuvant or vehicle injection. Lower: Development of arthritis in both hind paws after adjuvant or vehicle injection.

Fig. 4. Effect of naloxone on hypoalgesia observed on day 3 after adjuvant (—•—) or vehicle (—○—) injection. Statistically different from the vehicle treated group: **P<0.001. Statistically different from pre-naloxone values: *P<0.05, **P<0.01, ***P<0.001.
normal rats every 30 min for 2 hr for drugs. The intensities of electrical stimulation in adjuvant arthritic rats and normal rats were 0.5–1.5 mA (m=1.3±0.1 mA) and 3.5–4.5 mA (m=4.2±0.2 mA), respectively.

Morphine (0.25–1.0 mg/kg, s.c.) and pentazocine (1–4 mg/kg, s.c.) markedly and dose-dependently inhibited the vocalization response in adjuvant arthritic rats (Fig. 5). Codeine (1.25–5.0 mg/kg, s.c.) and levallophan (0.5–2.0 mg/kg, s.c.) also exhibited a dose-dependent analgesic action, but naloxone was inactive even at 10 mg/kg s.c.

The oral doses of indomethacin (0.5–2.0 mg/kg, p.o.), diclofenac (2.5–10.0 mg/kg, p.o.), aminopyrine (12.5–50.0 mg/kg, p.o.)

Fig. 5. Effects of morphine (2 mg/kg, s.c.) and pentazocine (4 mg/kg, s.c.) on the vocalization response in adjuvant arthritic rats.

Fig. 6. Effects of indomethacin (2 mg/kg, p.o.), diclofenac (10 mg/kg, p.o.), aminopyrine (50 mg/kg, p.o.) and acetaminophen (200 mg/kg, p.o.) on the vocalization response in adjuvant arthritic rats.
and acetaminophen (50–200 mg/kg, p.o.) showed dose-dependent inhibition of the vocalization response in adjuvant arthritic rats (Fig. 6). The analgesic action of these drugs reached a peak at 2 or 3 hr after administration and decreased slowly.

In contrast to the analgesic drugs, haloperidol (2–10 mg/kg, p.o.) slightly potentiated and diazepam (2–10 mg/kg, p.o.) failed to change the vocalization response in adjuvant arthritic rats. Atropine (2–10 mg/kg, s.c.), diphenhydramine (5–50 mg/kg, p.o.), imipramine (5–50 mg/kg, p.o.), mephenesin (300–600 mg/kg, p.o.) and cyproheptadine (2–20 mg/kg, p.o.) were all inactive at the doses tested. Prednisolone at a dose of 20 mg/kg p.o. exhibited a 13.14% anti-vocalization effect.

**Analgesic potencies in adjuvant arthritic rats and normal rats:** As shown in Fig. 7, the typical analgesic drugs such as morphine, pentazocine, indomethacin, diclofenac, aminopyrine and acetaminophen exhibited dose-dependent inhibitory effects on the

![Fig. 7. Dose response curve of inhibitory effects of typical analgesic drugs in adjuvant arthritic rats (—○—) and normal rats (—●—).](image)
vocalization response in adjuvant arthritic rats and normals, but larger doses of these drugs were required in normals.

The ED50 values of drugs used in this experiment are shown in Table 1 which compares adjuvant arthritic rats with normal rats.

The ratio of each drug's ED50 values (ED50 values in normal rats/ED50 values in adjuvant arthritic rats) was calculated. The ratios of normals to adjuvant arthritic rats of narcotic analgesic drugs and narcotic agonist/antagonist type analgesic drugs were between 1.7 and 1.8. Those of nonacidic NSAIDs, on the other hand, were between 2 and 4.9, while those of acidic NSAIDs, except aspirin, were far beyond the limits of nonacidic NSAIDs at between 15.3 and 37.9 (Fig. 8).

| Test drugs | Route | N | ED50 (95% C.I.) mg/kg | N | ED50 (95% C.I.) mg/kg |
|------------|-------|---|----------------------|---|----------------------|
| Narcotic and narcotic antagonist analgesic drugs |       |   |                      |   |                      |
| Morphine s.c. | 18 | 0.52 (0.24− 1.32) | 18 | 0.88 (0.50− 1.56) |
| Codeine s.c. | 18 | 2.5 (1.6 − 4.0)   | 18 | 4.1 (2.2 − 7.6)   |
| Pentazocine s.c. | 18 | 1.8 (0.7 − 5.0)   | 18 | 3.4 (1.5 − 7.7) |
| Levalloprphan s.c. | 18 | 0.95 (0.52− 1.72) | 18 | 8.0 inactive |
| Naloxone s.c. | 18 | 10.0 inactive | 18 | 10.0 inactive |
| Acidic NSAIDs |       |   |                      |   |                      |
| Indomethacin p.o. | 18 | 0.91 (0.45− 1.91) | 18 | 23.4 (12.1 − 44.9) |
| Diclofenac p.o. | 18 | 4.1 (2.1 − 7.9)   | 18 | 64.8 (28.8 −146.0) |
| Ketoprofen p.o. | 18 | 0.89 (0.41− 1.54) | 18 | 21.6 (11.9 −39.3) |
| Naproxen p.o. | 18 | 4.6 (2.4 − 8.8)   | 18 | 172.1 (74.5 −397.5) |
| Ibuprofen p.o. | 18 | 11.3 (7.0 − 18.1) | 18 | 200.0 (95.2 −422.3) |
| Mefenamic acid p.o. | 18 | 15.2 (7.4 − 31.2) | 18 | 233.1 (99.1 −645.0) |
| Phenylbutazone p.o. | 18 | 10.6 (5.3 − 20.9) | 18 | 246.3 (156.2 −526.5) |
| Aspirin p.o. | 18 | 105.8 (50.7 −220.8) | 18 | 478.8 (247.6 −880.2) |
| Nonacidic NSAIDs |       |   |                      |   |                      |
| Aminopyrine p.o. | 18 | 21.9 (10.9 − 44.6) | 18 | 108.4 (61.3 −191.6) |
| Acetaminophen p.o. | 18 | 88.1 (46.0 −172.6) | 18 | 228.7 (122.5 −427.0) |
| Tiaramide p.o. | 18 | 46.9 (28.5 − 77.1) | 18 | 211.6 (117.4 −381.4) |
| Mepivacaine p.o. | 18 | 39.4 (22.2 − 70.0) | 18 | 78.8 (48.6 −127.8) |
| Perisoxal p.o. | 18 | 38.8 (18.0 − 61.3) | 18 | 138.6 (63.4 −303.8) |
| Benzoylamine p.o. | 18 | 30.8 (18.6 − 51.0) | 18 | 141.2 (65.9 −302.6) |
| Timoridine p.o. | 18 | 39.9 (22.7 − 70.2) | 18 | 126.9 (58.5 −275.2) |
| CNS acting drugs and steroids |       |   |                      |   |                      |
| Haloperidol p.o. | 12 | 10.0 inactive |     |                      |
| Diazepam p.o. | 12 | 10.0 inactive |     |                      |
| Atropine s.c. | 12 | 10.0 inactive |     |                      |
| Diphenhydramine p.o. | 12 | 50.0 inactive |     |                      |
| Imipramine p.o. | 12 | 50.0 inactive |     |                      |
| Mephenesin p.o. | 12 | 600.0 inactive |     |                      |
| Cypohypha tadin p.o. | 12 | 20.0 inactive |     |                      |
| Prednisolone p.o. | 12 | 20.0 inactive |     |                      |

N: Number of animals
A method utilizing the inflammatory condition of adjuvant arthritic rats for evaluating analgesic drugs has been described. These rats resemble the human condition with respect to both inflammation and hyperalgesia (11). Thus, adjuvant arthritic rats represent a unique model for pathologically induced pain.

Kuzuna and Kawai (2), Winter et al. (4), and Capetola et al. (7) demonstrated that the vocalization response in adjuvant arthritic rats induced by manipulation of the tarsotibial joints or tail can be used as an indicator of nociception. However, the quantitative measurement of the vocalization response has not been established.

In the present study, the vocalization response was detected with a microphone, passed through an integrator connected to a biophysical amplifier, low cut filter and recorded on an ink writing oscilloscope. A low cut filter was used to exclude frequencies below 530 Hz since the frequency of vocalization from rats was about 2000 Hz, as shown in Fig. 1. The most intensive vocalization response was obtained, and adaptation was not observed when 10 Hz, 5 msec for 5 sec stimulation was given at 15 sec intervals. The same electrical stimulation conditions in adjuvant arthritic rats, normals and vehicle treated rats were shown to give a stable vocalization response. Thus we have established the electrical circuit and experimental conditions for quantitative measurement.

Guzman et al. (12), Lim et al. (13), Taira et al. (14, 15) and Adachi and Ishii (16) have reported that the vocalization response for screening analgesic drugs is more advantageous as an indicator of pain than conventional screening methods which use the escape response to thermal, mechanical or electrical stimulation. The major advantage of the vocalization response is that it yields quantitative and reproducible results.

In adult dogs, the vocalization response have been reported to show tachyphylaxis (17) and spontaneous diminution (18). Our results with rats, however, show that neither of these phenomena occurs.

Pircio et al. (3), using a group of adjuvant arthritic rats placed in close contact, reported that haloperidol and chlorpromazine produced anti-vocalization. These results, however, may be explained by the inhibitory action of antipsychotic drugs on the emotional stress of close contact. In our tests, despite the high sensitivity of adjuvant arthritic rats to pain, electrical stimulation was specific for analgesic drugs, whereas antipsychotic, anti-anxiety, antidepressant, anticholinergic, antihistaminic, antiserotonergic and centrally acting muscle relaxants were all inactive.

The development of hyperalgesia and arthritis after adjuvant injection was examined from day 0 to day 33. The pain threshold was increased from day 2 to day 6 after adjuvant injection, thereafter gradually decreased from day 6 to day 15, and maintained at a low level until day 33. The initial increased threshold was markedly lowered, but the later decreased threshold was not affected by

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**Fig. 8.** The difference between adjuvant arthritic rats and normal rats in the pattern of analgesic potency. The ratio of ED50 values: ED50 values of normal rats/ED50 values of adjuvant arthritic rats.

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**Table:**

| Drug            | Ratio ED50 (Normal/Adjuvant) |
|-----------------|-----------------------------|
| Morphine        | 0                            |
| Codeine         | 10                           |
| Pentazocine     | 20                           |
| Indomethacin    | 30                           |
| Diclofenac      | 40                           |
| Ketoprofen      |                              |
| Naproxen        |                              |
| Ibufrofen       |                              |
| Mefenamic acid  |                              |
| Phenylbutazone  |                              |
| Aspirin         |                              |
| Aminopyrine     |                              |
| Acetaminophen   |                              |
| Tiaramide       |                              |
| Mepirizole      |                              |
| Perlasoxal      |                              |
| Benzydamine     |                              |
| Tinoridine      |                              |

- Narcotic and narcotic antagonist drugs
- Acidic NSAIDs
- Nonacidic NSAIDs
naloxone. Recently, Yonehara et al. (19) found that the pain threshold and the turnover ratio of endogenous opiate peptide were increased in the brain and spinal cord at the initial stage in adjuvant arthritic rats. This finding may be related to our results.

As an indicator of the severity of arthritis, the diameter of the tarso-tibial joints with both hind paws was measured. Swelling of both hind paws was observed from day 10 and reached the maximum on day 26. This swelling was negatively correlated with changes in the pain threshold.

Our method was also able to distinguish between narcotic agonist/antagonist type analgesic drugs which are clinically effective analgesic drugs such as levallorphan (20) and pentazocine (21) and the pure narcotic antagonist drug naloxone which does not produce analgesia clinically (22).

Prednisolone showed mild anti-vocalization activity. Pircio et al. (3) found dexamethasone to be inactive, and Kuzuna and Kawai (2) reported that prednisolone at a dose of 5 to 20 mg/kg caused mild analgesia, but no dose response relationship was found. At present, the mechanism of the analgesic action of steroids remains unclear.

NSAIDs produced effective analgesia at relatively low doses in adjuvant arthritic rats. The analgesic activity exhibited by NSAIDs in adjuvant arthritic rats does not appear to be due to its anti-inflammatory action because the doses of NSAIDs found effective in the present method are well below those required for effective anti-inflammatory and anti-arthritis activity in rodents. Moreover, NSAIDs generally do not have any effect on the edema produced by various agents after it has been allowed to develop (23–25).

In Fig. 9, the known, clinically effective doses of various analgesic drugs are compared to the doses estimated in the present study. The ED50 values of all analgesic drugs in adjuvant arthritic rats were highly correlated with their clinical doses ($r=0.7389$, $P<0.001$). There was no correlation between the clinically effective doses and the ED50 values in normal rats ($r=0.5852$, N.S.).

Comparisons of the ED50 value in adjuvant arthritic rats with the ED50 value in normals were elucidated characteristically in the experimental model of chronic pain. The ED50 ratios for narcotic analgesic drugs, narcotic agonist/antagonist type analgesic drugs and nonacidic NSAIDs were not over 5, but acidic NSAIDs, except aspirin, were 15–38. Compared with normal rats, adjuvant arthritic rats were specifically affected by acidic NSAIDs. From these observations, it would appear that acidic NSAIDs are stronger than some narcotic analgesic drugs used clinically (26–28).

Acidic NSAIDs inhibit the biosynthesis of prostaglandins, suggesting that prostaglandins are involved as a mediator of inflammatory pain (29), while narcotic analgesic drugs, narcotic agonist/antagonist type analgesic drugs and nonacidic NSAIDs have little or no effect on prostaglandin biosynthesis. The results of the present study suggest that the analgesic action of acidic NSAIDs in adjuvant arthritic rats is associated with their inhibitory effect on prostaglandin biosynthesis.

Aspirin is a potent inhibitor of prostaglandin biosynthesis (29). Nevertheless, the ratio of the ED50 values of aspirin was much lower than those of the other acidic NSAIDs. In our experimental model, the reason for the exceptional action of aspirin was unknown.

![Fig. 9. Correlation between the clinical doses and the ED50 values for analgesic drugs in adjuvant arthritic rats. The dose was the acute clinically effective dose. Each point represents individual values. The correlation coefficient ($r$) was 0.7389 ($P<0.001$). Key: (0), acidic NSAIDs; (40), non-acidic NSAIDs; (\), narcotic analgesic drugs and narcotic agonist/antagonist type analgesic drugs.](image-url)
The phenylquinone writhing test in mice (19) was nonspecific for analgesic evaluation, and the Randall-Selitto’s test in rats (10) relatively gave a variance in the results. Our results have shown that this new method was specific for analgesic drugs and was able to detect the analgesic action of both narcotic agonist/antagonist type analgesic drugs and NSAIDs as well as narcotic analgesic drugs. Moreover, the quantitative measurement of the vocalization response has been established. This method should be useful for the evaluation of analgesic drugs.

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