Rat Natural Killer Cell, T Cell and Macrophage Functions after Intracerebroventricular Injection of SNC 80

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ABSTRACT

We investigated the effects of (+)-4-[(α R)-α-(2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl]-3-methoxybenzyl]-N,N-diethylbenzamide (SNC 80), a nonpeptidic delta-opioid receptor-selective agonist, on rat leukocyte functions. Intracerebroventricular injection of SNC 80 (20 nmol) in Fischer 344N male rats did not affect splenic natural killer cell activity compared with intracebroventricular saline-injected controls. SNC 80 also had no effect on concanavalin A-, anti-T cell receptor-, interleukin-2- and anti-T cell receptor + interleukin-2-induced splenic and thymic lymphocyte proliferation in most experiments. In some experiments, however, SNC 80 significantly (P < .01) caused a 41 to 93% increase of concanavalin A-, anti-T cell receptor-, interleukin-2- and anti-T cell receptor + interleukin-2-induced splenic lymphocyte proliferation compared to controls. Additionally, SNC 80 did not significantly affect splenic T cell or natural killer cell populations as measured by the expression of T cell receptor<sub>α</sub> and T helper (CD4), T suppressor/cytotoxic (CD8) and natural killer cell surface markers. Finally, SNC 80 did not affect interferon-γ- or lipopolysaccharide (LPS)-induced splenic nitric oxide, and LPS-induced tumor necrosis factor-α production by splenic macrophages. These results suggest that SNC 80 could be useful in the treatment of pain without suppressing immune function. However, the potential immunoenhancing properties of SNC 80 may be also valuable in immunocompromised individuals.

Mu, delta and kappa opioid receptor classes have been identified in neural tissue (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973), and their stimulation has the potential to relieve pain. However, in addition to analgesic properties, mu opioid receptor agonists have been associated with the alteration of immune responses through central or peripheral pathways (Shavit et al., 1986; Weber and Pert, 1989; Bayer et al., 1992; Carr et al., 1993; Lysle et al., 1993; Flores et al., 1995). ICV injection of β-endorphin and DAMGO has been reported to enhance rat splenic NK cell activity (Jonsdottir et al., 1996) and nitric oxide production (Iuvone et al., 1995), respectively. In contrast, ICV injection of morphine and β-endorphin was shown to suppress Con A-, PHA- or LPS-induced rat splenic lymphocyte proliferative responses (Lysle et al., 1996; Panerai et al., 1994). In addition, morphine action in the periaqueductal gray matter of the mesencephalon has been linked to immunosuppression (Weber and Pert, 1989; Lysle et al., 1996), through central mu receptors (Band et al., 1992). Because of their effects on immune function, mu opioid agonists are not optimal for pain management in many clinical situations when suppression of immune function is undesired, such as AIDS patients, burn victims or cancer patients with intractable pain who opt for immunotherapy. We previously reported lack of immunosuppression following administration of buprenorphine, a partial agonist at mu opioid receptors (Williams et al., 1991).

Early studies with delta opioid agonists included naturally occurring peptidic ligands such as enkephalins and deltorphin, or exogenous analogues such as D-Pen<sub>2</sub>-D-Pen<sub>5</sub>-enkephalin (DPDPE). These peptidic compounds are rapidly degraded by the human body thus limiting their clinical application. In contrast, nonpeptide opioids are more stable, and their usefulness as analgesics without affecting immune function has been proven (Williams et al., 1991). Burroughs Wellcome synthesized the nonpeptide molecule BW373U86 which was shown to produce analgesia via the delta opioid receptor (Chang et al., 1993). This compound, however, had

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ABBREVIATIONS: SNC 80, (+)-4-[(α R)-α-(2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl]-3-methoxybenzyl]-N,N-diethylbenzamide; Con A, concanavalin A; FACS, fluorescent antibody cell sorter analysis; FITC, fluorescein isothiocyanate; HPLC, high-performance liquid chromatography; IL-2, interleukin-2; ICV, intracerebroventricular; NK, natural killer; antiTOR, IgG<sub>1</sub> monoclonal antibody to rat T cell receptor<sub>α</sub>; CD4, IgG<sub>1</sub> monoclonal antibody to rat T helper cells; CD8, IgG<sub>1</sub> monoclonal antibody to rat T suppressor/cytotoxic cells; NKCR, IgG<sub>1k</sub> monoclonal antibody to rat NK cells; LPS, lipopolysaccharide; DPDPE, D-Pen<sub>2</sub>-D-Pen<sub>5</sub>-enkephalin; DAMGO, H-Tyr-d-Ala-Gly-Phe(N-Me)-Gly-ol.

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several adverse effects, such as convulsions and barrel rolling (Comer et al., 1993). Calderon et al. (1994) were able to use BW373U56 to derive its optically pure methyl ether enantiomer SNC 80, a compound proven to be a potent δ-selective analgesic (Bilsky et al., 1995).

ICV therapy has been reported to be as effective as other neuraxial treatments to control pain (Ballantyne et al., 1996). ICV opioid treatment has been successfully used to control refractory pain due to cancer when systemic treatments have failed (Ballantyne et al., 1996). Our study was conducted to investigate the effects of acute ICV injection of SNC 80 on NK cell and T cell, and macrophage functions.

Materials and Methods

Reagents and culture media. Rat rIFN-γ (specific activity of 4 × 10^8 U/mg), penicillin-streptomycin solution, and DMEM/F12, RPMI 1640 and AIM-V media were obtained from Life Technologies (Grand Island, NY). SNC 80 was synthesized by Dr. Silvia N. Calderon at National Institutes of Health (NIH), and generously donated by Dr. Kenner Rice from the NIH. LPS from Escherichia coli serotype 0128:B12, methanol, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, sodium dodecyl sulfate, HCl, Con A, red blood cell lysing buffer and fetal bovine serum were purchased from Sigma Chemical Co. (St. Louis, MO). FITC-labeled monoclonal antibodies (IgG1) to αTCR, T helper cell (CD4) and T suppressor cell (CD8) surface markers were obtained from Harlan Bioproducts for Science (Indianapolis, IN). FITC-labeled monoclonal antibodies to NKR-P1 (anti NK cells) surface marker (Chambers et al., 1989) were kindly provided by Dr. William Chambers of the Pittsburgh Cancer Institute (Pittsburgh, PA). Cell sorter analysis was performed on a Becton Dickson FACScan (San Jose, CA). Actinomycin D was obtained from Sigma Chemical Co. (St. Louis, MO). FITC-labeled monoclonal antibodies to NKR-P1 (anti NK cells) surface marker (Chambers et al., 1989) were kindly provided by Dr. William Chambers of the Pittsburgh Cancer Institute (Pittsburgh, PA). Cell sorter analysis was performed on a Becton Dickson FACScan (San Jose, CA). Actinomycin D was obtained from Sigma Chemical Co. (St. Louis, MO).

Animals. Fischer 344N male rats (150–250 g), purchased from Harlan Sprague Dawley (Indianapolis, IN), were housed three to four per cage with water and rat food available ad libitum. Measures were taken to reduce micro-organism infestation of our colony by housing our animals in a room separate from the general University vivarium. Animals were anesthetized by i.m. injection of 100 mg/kg ketamine (Fort Dodge Laboratories, Inc., Fort Dodge, IA) and 20 mg/kg xylazine (Bayer Corporation, Shawnee Mission, KS) after which a double 23-gauge stainless steel guide cannula (outer diameter, 0.02 inches; inner diameter, 0.01 inches) was stereotaxically implanted 0.75 mm above the right lateral ventricle using the following coordinates: anterior-posterior, −1.0 mm; mediolateral, ±1.5 mm; dorsoventral, −3.5 mm in reference to the bregma. Rats were allowed 10 days for recovery from cannulation surgery before morphine injection and were adapted to handling daily by being picked up and held in the identical manner used during injections.

Drug preparation and administration. SNC 80 was dissolved in pyrogen-free saline to a concentration of 2 nmol/μl. Ten microliters of SNC 80 or vehicle (saline) was then administered ICV (total dose of SNC 80 was 20 nmol) at the speed of 10 μl/min with a microinjection pump (Harvard Apparatus, Southnatick, MA). Three hours after morphine injection, rats were killed by asphyxia with CO2.

Cell preparation and culture. Spleen and thymus were removed immediately after the rat was killed. Single-cell suspensions were prepared by disrupting the organs in RPMI 1640 medium supplemented with 0.5% penicillin-streptomycin solution. Lymphocyte suspensions were washed three times in this medium, and the final pellets were resuspended and adjusted at appropriate densities with AIM-V medium containing 0.5% penicillin-streptomycin solution. The culture medium was changed at this step to the serum-free medium AIM-V which has been observed to support cell culture (Kaldjian et al., 1992). For the macrophage assays, 2 ml spleen suspensions were centrifuged for 7 min at 1400 rpm and supernatants were discarded. Pellets were then resuspended in 2 ml of red blood cell lysing buffer; after homogenizing, 2 ml AIM-V medium was added, and suspensions centrifuged for 7 min at 1400 rpm. After this, supernatants were discarded, and pellets were resuspended in 3 ml AIM-V medium. Splenic cells were then counted visually, adjusted to a density of 4.5 × 10^6 cells/ml in this medium, and incubated for 2 hr in flat-bottomed 96-well plates (Becton Dickinson). Nonadherent cells were removed, and adherent cells were then incubated overnight in 100 μl AIM-V in the presence or absence of IFN-γ (50 U/ml) (higher doses of IFN-γ resulted in more than 30% reduction of macrophage viability, data not shown); the final monolayer consisted of >95% macrophages as judged by morphology and phagocytic activity.

NK cell assay. NK cell cytotoxic activity was assessed by the chromium release assay using 51Cr-labeled YAC-1 murine lymphoma cell line as reported previously (Weber and Pert, 1989). YAC-1 cells were labeled by incubating 10^7 cells with 200 μCi sodium 51chromate (NEN Research Products, Boston, MA) for 2 hr at 37°C, and then washed three times with RPMI 1640 medium and resuspended in this medium to a density of 5 × 10^4 cell/ml. YAC-1 cells were added to round-bottomed 96-well plates (Becton Dickinson, Lincoln Park, NJ) containing splenic cells at various concentrations to give effector/target ratios ranging from 25:1 to 400:1. Spontaneous and maximal 51chromium release were obtained by incubating 51Cr-labeled YAC-1 cells in AIM-V medium alone or medium containing 2% sodium dodecyl sulfate plus 0.1% HCl, respectively. After 4 hr of incubation, supernatants were harvested and 51Cr release was measured in a gamma counter (Packard, Downers Grove, IL). Four separate wells per animal per effector:target were analyzed; the mean of the four wells was used for final data analysis.

Cell proliferation assay. T cell proliferation was determined by [3H]-thymidine uptake as previously reported (Lysele et al., 1993). Thymic and splenic cells were adjusted to 5 × 10^6 cells/ml and cultured in round-bottomed 96-well plates (Becton Dickinson). Cell cultures were then incubated in the presence or absence of Con A, αTCR (5 μg/ml), IL-2 (5% of a 24-hr conditioned medium from Con A-stimulated splenic cells) and αTCR + IL-2 for 48 hr. [3H]-thymidine (1 μCi/well, ICN Pharmaceuticals Inc., Costa Mesa, CA) was added 4 hr before the end of the incubation period. Cell cultures were then harvested with a semi-automatic cell harvester (Tomtec, Orange, CT) and cell-incorporated radioactivity determined using a Microbeta Plus liquid scintillation counter (Wallac Oy, Turku, Finland). Three wells were analyzed for each animal with each stimulant studied; the mean of the three wells was used for final data analysis.

FACS. Spleen and thymus cell suspensions containing 1 × 10^7 cells/ml were incubated in ice for 20 min with 10 μl of FITC-labeled IgG1 monoclonal antibodies to TCR (rat T cell receptor-αβ), CD4 (rat T helper cells), CD8 (rat T suppressor cells) and NKR-P1 (anti NK cell) surface markers. Cells were washed two times with Hanks’ balanced salt solution containing 5% fetal bovine serum and 0.02% sodium azide, then washed one time with Hanks’ balanced salt solution alone. The cells were then fixed with 2% paraformaldehyde, and percent of cells with TCR, CD4, CD8 and NKR-P1 surface markers was determined by FACS analysis.

Nitrite determination. Accumulation of nitrite in the supernatants of macrophage cultures was used as an indicator of nitric oxide production by resident or activated cells. Resident macrophages and macrophages activated with IFN-γ (50 U/ml) or LPS (25 ng/ml, higher doses do not significantly increase nitric oxide production, data not shown) were incubated at 37°C in an atmosphere of 5% CO2-95% air for 3 days in a total volume of 200 μl AIM-V medium per well. After incubation, supernatants were obtained and nitrite levels were determined with the Griess reagent as reported elsewhere (Gomez-Flores et al., 1997a), using NaNO2 as standard. Optical densities at 540 nm were then determined in a microplate reader (Molecular Devices Corporation, Palo Alto, CA).
TNF-α assay. TNF-α production by macrophages was determined by the L929 bioassay. In brief, macrophage monolayers were incubated in the presence or absence of 25 ng/ml LPS, in a total volume of 200 μl of AIM-V medium, for 4 hr after which supernatants were collected and kept at −80°C until use. TNF-α levels in the supernatants were then quantified by the L929 bioassay as described elsewhere (Gomez-Flores et al., 1997b). The bioassay was performed in D-MEM/F12 medium using ½ serial dilutions of the supernatants. Recombinant murine TNF-α (a gift from NCI Biological Resources Branch, Rockville, MD, lot 88/532) was used as standard. After 24 hr of incubation, cell viability of the L929 cells was determined by a colorimetric technique using methanol,3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide to a final concentration of 0.5 mg/ml, and incubating the cells for 1.5 hr at 37°C (Belkowski et al., 1995). Formazan crystals were dissolved in DMSO and optical densities at 540 nm were determined in a microplate reader (Molecular Devices Corporation). TNF-α levels represented the inverse of the dilution causing 50% cytotoxicity, and were expressed in U/ml (Kloステガール, 1985).

Histology. The brains were removed, fixed in isopentane on dry ice (−40°C) and kept at −80°C. Sequential 40-μm coronal sections through the injection site were obtained using a freezing-stage cryostat (−22°C) and the slides were Nissl-stained. The lateral-ventral placement of microinjection (Paxinos and Watson, 1986) was confirmed by light microscopy.

Statistical analysis. Each data point for animals within the same experimental group were pooled and expressed as the mean ± S.E.M. for each experiment. Four rats were used per experimental group in every experiment. Level of significance was assessed by Student’s t test, and by one-way analysis of variance, comparing the experimental group to the control group at each effector:target ratio for NK-cell analysis and each level of Con A, αTCR or IL-2 for T cell analysis.

Results

Effect of SNC 80 on NK-cell activity. As shown in figure 1 (experiments 1–3), ICV microinjection of SNC 80 did not affect splenic NK cell activity. However, in one experiment (experiment 3) SNC 80 caused a significant (P < .05) 12 ± 0.2% increase of NK cell activity at an effector:target ratio of 200:1 compared with cell response of ICV-injected saline control.

Effect of SNC 80 on splenic and thymic lymphocyte proliferation. ICV injection of SNC 80 was associated with either a significant increase or no effect on splenic and thymic lymphocyte proliferative response to Con A, antiTCR-, IL-2-, antiTCR + IL-2 as compared with ICV-injected saline control. As observed in figure 2 (experiment 1), SNC 80 caused significant (P < .01) 41 ± 1, 42 ± 1 and 55 ± 3% increase of splenic lymphocyte proliferation induced by Con A at doses of 1.25, 2.5 and 5 μg/ml, respectively, compared with cell response of ICV-injected saline control. Similarly, SNC 80 caused significant (P < .001) 93 ± 11, 106 ± 23 and 69 ± 16 percent increase of splenic lymphocyte proliferation in the presence of Con A at doses of 1.25, 2.5 and 5 μg/ml, respectively (figure 2, experiment 2), compared with cell response of ICV-injected saline control. In addition, significant (P < .001) 80 ± 10, 62 ± 7 and 75 ± 10% increase in splenic lymphocyte proliferation in the presence of antiTCR, IL-2 and antiTCR + IL-2 respectively, compared with cell response of ICV-injected saline control, were also observed. In some experiments, ICV injection of SNC 80 did not affect splenic lymphocyte proliferation induced by Con A (figure 2) or antiTCR, IL-2, and antiTCR + IL-2 in combination (experiments 1 and 3, figure 2). Similarly, SNC 80 did not affect thymic lymphocyte proliferation induced by Con A, antiTCR, IL-2 and antiTCR + IL-2 combination (experiments 1–3, figure 3).

Effect of SNC 80 on splenic T cell and NK cell populations. As observed in figure 4 (experiments 1–3), ICV injection of SNC 80 did not affect splenic T cell or NK cell populations as measured by the expression of TCR, CD4, CD8 and NKCR surface markers.
Effect of SNC 80 on nitric oxide and TNF-α production by splenic macrophages. As observed in figure 5, ICV injection of SNC 80 did not affect IFN-γ- or LPS-induced splenic nitrite and LPS-induced TNF-α production by splenic macrophages.

Discussion

Opioid agonists selective for μ, δ, and κ opioid receptors have been shown to possess the ability to alleviate pain (Zaki et al., 1996). However, in vivo injection of μ opioid receptor selective agonists has been reported to suppress rat splenic B (ICV injection, Lysle et al., 1996) and T cell proliferation (s.c. injection, Lysle et al., 1993; Flores et al., 1996; ICV injection, Lysle et al., 1996). They have also been shown to suppress a variety of functions including murine T cell-mediated cytotoxicity (s.c. injection, Carr et al., 1995), production of rat (s.c. injection, Fecho et al., 1996) or murine (s.c. injection, Scott and Carr, 1996) interferon-γ, NK cell cytotoxic activity in rats (s.c. injection, Lysle et al., 1996; Fecho et al., 1996; PAG injection, Weber and Pert, 1989; Lysle et al., 1996; ICV injection, Lysle et al., 1996; Band et al., 1992), mice (s.c. injection, Scott and Carr, 1996; Carr et al., 1994), Rhesus monkeys (s.c.
injection, Carr et al., 1993) and humans (Provinciali et al., 1996), and phagocytosis of Candida albicans by murine (s.c. injection, Rojavin et al., 1993) or human (s.c. injection, Tubaro et al., 1987) macrophages.

Delta selective opioid compounds are devoid of many of the adverse effects seen with mu selective opioid agonists, including, in most cases, immunosuppression. They also have been shown to have diminished abuse potential. For these reasons, it is essential to develop analgesics which are selective for delta rather than mu opioid receptors (Rapaka and Porreca, 1991). Early studies were performed with derivatives of the naturally synthesized delta opioid receptor-selective enkephalin peptides, such as DPDPE, in search of new analgesics. It was found that certain delta selective agonists enhance lymphocyte proliferation even in the absence of mitogen (Hucklebridge et al., 1990). Band et al. (1992) reported that ICV injection of DPDPE did not significantly alter NK cell function. However, DPDPE and other peptidic opioids were shown to be very unstable in animal models and had a low potential to cross the blood brain barrier, thus limiting its use (Hambrook et al., 1976).

SNC 80 has been tested and proven to be a potent analge-
sic, acting at the delta opioid receptor, in both rats and Rhesus monkeys (Calderon et al., 1994; Bilsky et al., 1995). In the results reported in this study, we generally observed that ICV injection of SNC 80 did not affect NK cell, T cell and macrophage functions (figures 1–5). In some experiments we showed that this opioid increased T cell proliferative responses to various stimulus (figures 1 and 2). Therefore, the delta opioid receptor selective agonist SNC 80 could potentially be used in many different clinical situations where immunosuppression is undesirable as shown for mu selective ligands such as morphine (Weber and Pert, 1989; Bayer et al., 1992; Carr et al., 1993; Lysle et al., 1993; Flores et al., 1995). Furthermore, SNC 80 was observed to enhance T cell proliferative responses in some instances thus making this compound potentially suitable in treating not only pain, but also ameliorating the immune status of immunocompromised individuals. SNC 80 could be used as a reference to further develop analgesics with minimal or no impact, even with enhancing properties, on immune functions.

Acknowledgments

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References


Band LC, Pert A, Williams W, de Costa BR, Rice KC and Weber RJ (1992) Central Fig. 4. Effects of SNC 80 on splenic cell populations. Expression of splenic lymphocyte and NK-cell surface markers was determined 3 hr after ICV acute microinjection of SNC 80 or saline, by using FITC-labeled IgG1 monoclonal antibodies to TCR (rat T cell receptor), CD4 (rat T helper cells), CD8 (rat T suppressor cells) and NKCR (rat NK cell receptor) surface markers, as explained in the text. Data represent mean ± S.E.M. of three replicate determinations per treatment (four rats per treatment).

Fig. 5. Nitrite release and TNF-α production by splenic macrophages after ICV injection of SNC 80. Rat splenic macrophages were obtained 3 hr after ICV acute microinjection of SNC 80 as explained in the text. Macrophages were then cultured overnight with or without IFN-γ (50 U/ml). Resident macrophages, IFN-γ-(50 U/ml) primed macrophages, and macrophages activated with 25 ng/ml LPS were incubated for 3 days. After incubation, accumulation of nitrite (a) in the culture medium was determined by using the Griess reagent. Resident macrophages were also incubated in the presence or absence of 25 ng/ml of LPS for 4 hr after which levels of TNF-α (b) in culture supernatants were determined by the L929 bioassay, as explained in the text. Data represent mean ± S.E.M. of triplicates from one experiment. TNF-α production by untreated macrophages from SNC 80-treated or control animals was negligible.


