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Protective Effect of RC-3095, an Antagonist of the Gastrin-Releasing Peptide Receptor, in Experimental Arthritis

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Objective. **To evaluate the antiinflammatory effects of RC-3095 in 2 experimental models of arthritis, collagen-induced arthritis (CIA) and antigen-induced arthritis (AIA), and to determine the mechanisms of action involved.**

Methods. **RC-3095 was administered daily to mice with CIA and mice with AIA, after induction of disease with methylated bovine serum albumin. Disease incidence and severity were assessed using a clinical index and evaluation of histologic features, respectively. In mice with CIA, gastrin-releasing peptide receptor (GRPR) was detected by immunohistochemical analysis, while in mice with AIA, migration of neutrophils, presence of glycosaminoglycans, and lymphocyte proliferation, determined using the MTT assay, were assessed. Expression of cytokines interleukin-17 (IL-17), IL-1β, and tumor necrosis factor α (TNFα) was evaluated in all mouse knees using enzyme-linked immunosorbent assay. Treg cell production was assessed by flow cytometry in the joints of mice with AIA.**

Results. **In mice with AIA, administration of RC-3095 reduced neutrophil migration, mechanical hypernociception, and proteoglycan loss. These findings were associated with inhibition of the levels of all 3 proinflammatory cytokines, decreased lymphocyte proliferation, and increased Treg cell numbers. In the CIA model, treatment with RC-3095 led to a significant reduction in arthritis clinical scores and the severity of disease determined histologically. Synovial inflammation, synovial hyperplasia, pannus formation, and extensive erosive changes were all dramatically reduced in the arthritic mice treated with RC-3095. Furthermore, arthritic mice treated with RC-3095 showed a significant reduction in the concentrations of IL-17, IL-1β, and TNFα, and showed a diminished expression of GRPR.**

Conclusion. **These findings suggest that the GRP pathway has a significant role in chronic arthritis, and its inhibition can be explored as a possible therapeutic strategy in rheumatoid arthritis.**

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease characterized by inflammation of the synovium that leads to destruction of cartilage and bone (1). Its etiology is unknown but seems to involve both humoral (2,3) and cellular (4) immune mechanisms. There is now a growing awareness that RA is a heterogeneous disease composed of several distinct disease pathways (5). In spite of significant improvements in the treatment of RA, there is still a need for novel therapeutic strategies, and, to achieve this, identification of the many pathways involved in the modulation of inflammation is critical.

Although many different cell types, such as macrophages and synoviocytes, have long been known to be involved in RA, it has recently been realized that the peripheral nervous system has a key role in modulating the severity of RA (6). In addition, studies have shown that neuropeptides are secreted locally during immune responses, and that they are involved in vasodilation and

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Drs. Roesler and Schwartsmann are listed as inventors in a provisional patent application for the therapeutic use of gastrin-releasing peptide receptor antagonists in the treatment of inflammatory conditions.

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plasma extravasation, i.e., neurogenic inflammation. It is furthermore known that inflammatory cells can produce neuropeptides (7). Synovial tissue is richly innervated with neuropeptide-containing primary afferent and sympathetic neurons, and there is evidence that the release of these neuropeptides powerfully influences the severity of chronic inflammatory diseases, including RA (6).

The possible role of neuropeptides, including vasoactive intestinal peptide, substance P, and neuropeptide Y, in the mechanisms of joint inflammation has been investigated in RA (8,9). Another neuropeptide that has been studied is the gastrin-releasing peptide (GRP), which is the mammalian homolog of bombesin, a tetradecapeptide originally isolated from frog skin (10).

GRP affects several systems in mammals, including neuroendocrine regulation, gastrointestinal secretion, and cell proliferation. Furthermore, it is involved in the development and regulation of the immune response, acting directly on the immune cells that express the GRP receptor (GRPR) (11,12). Its presence in increased concentrations has been demonstrated in the joint fluid and chondrocytes of RA patients (13,14), in correlation with a raised erythrocyte sedimentation rate and increased levels of proinflammatory cytokines, such as tumor necrosis factor α (TNFα), interleukin-6 (IL-6), and monocyte chemoattractant protein (MCP), suggesting that this neuropeptide could be involved in modulation of the inflammatory process. The GRPR is a cell surface protein that binds, depending on the species, bombesin, GRP, or closely related peptides with high affinity, triggering intracellular changes that influence cell behavior (11). Moreover, recently the presence of GRPRs in articular chondrocytes and inflammatory infiltrates in mouse models of arthritis has been reported. Therefore, it has been suggested that this neuropeptide could be an interesting target for new therapeutic strategies in RA (15).

The pseudononapeptide RC-3095 is an antagonist of the receptor of bombesin/GRP, originally synthesized by McKillop et al in 1990 (16), with the aim of inhibiting the action of bombesin/GRP. Using this specific antagonist of the GRPR, our group has demonstrated that inhibition of the GRPR could decrease the release of TNFα and IL-1β from activated macrophages in an experimental model of sepsis (17), elicit important action against oxidative damage in an experimental model of uveitis (18), and protect against gastric oxidative injury in an animal model of gastritis (19).

Recently, we demonstrated the effect of RC-3095 in a model of arthritis induced by Freund's complete adjuvant (CFA). This was the first study to demonstrate a protective effect of an antagonist of the receptor for bombesin /GRP, and to thus exhibit a possible role of GRP in the development of arthritis, but further studies are needed (20). However, this model has several limitations and is less representative of RA processes (21).

Antigen-induced arthritis (AIA) (22–24) and collagen-induced arthritis (CIA) (25–27) are 2 commonly used murine models of RA. AIA is induced by intradermal immunization and subsequent intraarticular (IA) injection of methylated bovine serum albumin (mBSA) (22–24). The disease is chronic and antigen-specific, and has been suggested to be T cell dependent (22). CIA is induced by intradermal immunizations with a major cartilage protein component, type II collagen (CII) (25–27). CIA is dependent on both B cells and T cells as well as the complement system (28,29).

Thus, in the present study, we studied the antiinflammatory and immunomodulatory effects of RC-3095 in 2 different animal models of arthritis, each of which better reflects the diverse pathophysiologic mechanisms involved in RA.

MATERIALS AND METHODS

Animals. AIA was induced in Balb/c wild-type mice, and CIA was induced in DBA/1J inbred mice. In both groups, only male mice, weighing 18–25 gm, were used. All experimental procedures involving animals were performed in accordance with the National Institutes of Health Guide for Care and Use of Animals and with the approval of our institutional ethics committee.

Drug. The GRPR antagonist RC-3095, or D-Tpi6-Leu13Ψ-(CH2NH)-Leu14 (30), was produced and donated by Æterna Zentaris.

Induction of AIA and CIA. For AIA studies, mice were sensitized by subcutaneous (SC) injection with 500 μg of mBSA (Sigma) in 0.2 ml of an emulsion containing 0.1 ml of 0.9% saline and 0.1 ml CFA (1 mg/ml of *Mycobacterium tuberculosis*; Sigma) on days 0 and 7. Twenty-one days after the initial injection, arthritis was induced in the immunized animals by IA injection of mBSA (30 μg/cavity), dissolved in 10 μl of saline, into the left tibiofemoral joint, and the contralateral joint was injected with 10 μl of saline alone as a control.

The Balb/c mice were injected with RC-3095 (1 mg/kg SC) or vehicle (0.9% saline) twice a day for a total of 2 or 10 days, starting on the second day before the induction of arthritis. Some mice were killed at 24 hours after IA injection, to study neutrophil migration, articular nociception, and lymphocyte proliferation. The remaining mice were reinduced with the same IA preparation after 4 days, and the mice were killed 4 days thereafter for studies on cytokines, glycosaminoglycans (GAGs), and the distribution of Treg cell populations.

For CIA studies, mice were randomly divided into 4 groups. Group 1 comprised mice that were not manipulated (nonimmunized), group 2 comprised immunized mice treated with vehicle (saline, administered SC at 5 ml/kg twice a day for

10 days after the onset of the disease), and groups 3 and 4 comprised immunized mice treated with either 0.3 mg/kg (group 3) or 1 mg/kg (group 4) RC-3095, administered SC twice a day for 10 days after the onset of the disease. The immunizations were administered by intradermal injection at the base of the tail with 100 μl of emulsion, consisting of equal parts of CFA containing 5 mg/ml heat-killed *M tuberculosis* antigen (strain H37Ra; Difco) and 2 mg/ml bovine CII (Sigma) in 10 m*M* acetic acid (day 0). Booster injections of 100 μl of emulsion consisting of equal parts of Freund's incomplete adjuvant and 2 mg/ml CII in 10 m*M* acetic acid were administered in the other site at the base of the tail 18 days later. Clinical evaluation was performed daily, through standardized scoring of arthritis, measurement of edema, and evaluation of articular nociception. The animals were killed on the eleventh day after treatment, by anesthesia overdose. The knees of the mice were collected to determine cytokine levels, and hind paws were collected for histology and immunohistochemistry analyses.

Arthritis scoring. We used a standardized method of arthritis scoring to evaluate the degree of swelling and erythema of the paws, in which 0 = normal, 1 = mild swelling and erythema, 2 = moderate swelling and erythema, 3 = severe swelling and erythema plus loss of function in 2 paws, and 4 = total loss of function in a minimum of 3 paws. Onset of disease, characterized by the development of erythema and/or paw swelling, was seen between day 25 and day 35.

Edema measurement. Paw thickness was measured with a plethysmometer (Ugo Basile) before the induction of CIA (V_o) and every day after the beginning of the disease (V_T), as previously described (31). The amount of paw swelling was determined for each mouse, and the difference between the V_T and the V_o was calculated to obtain the extent of edema (expressed in mm^3).

Evaluation of articular hypernociception. The articular hypernociception of the tibiofemoral joint was evaluated using a previously described method (32), with modification. In a quiet room, mice were placed in acrylic cages (12 × 10 × 17 cm high) with a wire-grid floor for 15–30 minutes before testing for environmental adaptation. In these experiments, an electronic pressure meter was used, consisting of a hand-held force transducer fitted with a polypropylene tip (IITC Inc.–Life Science Instruments). For this model, a large tip (4.15 mm^2) was adapted to the probe. An increasing perpendicular force was applied to the central area of the plantar surface of the hind paw to induce flexion of the tibiofemoral joint, followed by paw withdrawal. The electronic pressure-meter apparatus automatically recorded the intensity of the force applied when the paw was withdrawn. The test was repeated until 3 subsequent measurements that yielded consistent results (i.e., variation among these measurements was lower than 1 gm) were obtained. This represented the flexion-elicited mechanical threshold for evaluation of articular hypernociception, with results expressed in grams.

Evaluation of in vivo neutrophil migration in AIA. Neutrophil migration into the knee joints of mice in all groups was evaluated at the end of the experimental period. The mice were killed and the articular cavities were washed twice with 5 μl phosphate buffered saline (PBS) containing 1 m*M* EDTA, and then diluted to a final volume of 100 μl with PBS/EDTA to evaluate leukocyte migration at the indicated times. The total number of leukocytes was determined in a Neubauer chamber diluted in Turk's solution. Differential cell counts were determined in cytocentrifuge on Rosenfeld-stained slices (Cytospin 4; Shandon). Differential cell counts were performed with a light microscope, with results expressed as the mean ± SEM number of neutrophils per cavity.

Assessment of proteoglycan metabolism in patellar cartilage in AIA. Sulfated GAG content was evaluated in patellar cartilage with the 1,9-dimethylmethylene blue (DMMB) colorimetric assay (Sigma-Aldrich), as previously described (33). In brief, patellae were fixed overnight and decalcified in 5% (volume/volume) formic acid at room temperature for 4 hours before separation of the cartilage layer from underlying bone, and subsequently digested overnight at 60°C with 60 μl of 10 mg/ml of papain (type IV; Sigma) in 0.1*M* sodium acetate, pH 6.5, 10 m*M* L-cysteine, and 50 m*M* disodium EDTA per patella. The digested patellar tissue was then collected to measure GAGs using the DMMB assay. The assay was performed by monitoring the metachromatic reaction of sulfated GAGs with DMMB at 525 nm. The calibration curve ranged from 0 to 100 μg/ml chondroitin 6-sulfate, with results expressed as the μg of GAG per mg of cartilage.

Lymphocyte proliferation assay in AIA. In vitro proliferation of lymphocytes in the draining lymph nodes (DLNs) (popliteal and inguinal) of mice was examined using the MTT assay (34). Mice were killed, and their DLNs were removed aseptically. A single-cell suspension was prepared and cultured in triplicate wells (5×10^5 cells/well in a 96-well plate) and treated with RC-3095 (0.1 μ*M*) or GRP (100 n*M*) (35) for 72 hours at 37°C in 5% CO_2 in RPMI medium containing 100 μg/ml mBSA or only RPMI medium for control culture. On the day of the assay, the medium was removed and MTT (0.5 mg/ml) was added to the medium in each well. The plate was returned to the incubator for 4 hours, and the supernatants were then removed and 50 μl of DMSO (Sigma) was added. The plate was agitated in the dark for 10 minutes to dissolve the MTT formazan crystals. The optical density of each well was read at 480 nm.

Histology and histologic scoring of arthritis in CIA. DBA/1J mice were killed at designated time points after the induction of arthritis, and the ankles were dissected and fixed in 10% buffered formalin for 7 days. Paws were then decalcified with 10% nitric acid for 27 hours. Tissues were sectioned and embedded in paraffin, and slides were prepared and stained with hematoxylin and eosin. We used a previously described comprehensive histologic scoring system. Briefly, the tibiotalar, talus-calcaneal, and midfoot joints were histologically scored for the following parameters. Synovial inflammation, determined in 5 high-power magnification fields (HMFs), was scored for the percentage of infiltrating mononuclear cells, as follows: 0 = absent, 1 = mild (1–10%), 2 = moderate (11–50%), and 3 = severe (51–100%). Synovial hyperplasia was scored for severity, as follows: 0 = absent, 1 = mild (5–10 layers), 2 = moderate (11–50 layers), and 3 = severe (>20 layers). Extension of pannus formation was determined on the basis of the reader's impression, as follows: 0 = absent, 1 = mild, 2 = moderate, and 3 = severe. Cartilage erosion was determined as the percentage of the cartilage surface that was eroded, as follows: 0 = absent, 1 = mild (1–10%), 2 = moderate (11–50%), and 3 = severe (51–100%). Finally, bone erosion was scored as follows: 0 = none, 1 = minor erosions observed only on HMFs, 2 = moderate erosions observed at low magnification, and 3 = severe transcortical erosions (36).

Staining of the tissue was interpreted by 2 blinded and independent investigators.

Immunohistochemical analysis for GRPR. Serial sections of the ankles of DBA/1J mice were subjected to immunohistochemistry for detection of GRPR, which consisted of deparaffinization and rehydration, inactivation of endogenous peroxidase activity, and blocking of nonspecific reactions (13). These sections were incubated for 12 hours at 4°C with a solution of the anti-GRPR primary antibody (Abcam) diluted 1:300. Identification of the primary antibody site was achieved by subsequent application of biotinylated antibody, streptavidin–horseradish peroxidase conjugate (LSAB; Dako), and exposure to diaminobenzidine tetrahydrochloride as chromogen (DAB Kit; Dako), and were counterstained with hematoxylin for visualization. Immunohistochemical staining was scored according to intensity, as follows: 1 = mild immunostaining, 2 = moderate immunostaining, and 3 = strong immunostaining. In addition, the area of distribution was expressed as the percentage of cells stained. We used a semiquantitative approach in which the percentage of stained cells was multiplied by the intensity of staining, and this number was used for data evaluation. A pathologist, who was blinded to the clinical and histopathologic information, independently analyzed the glass slides.

Measurement of cytokines. The knee joints of mice with AIA and mice with CIA were dissected, frozen with liquid nitrogen, crushed in a mortar and pestle, and then solubilized in PBS. The homogenates were centrifuged at 10,000*g* for 10 minutes, and the supernatants were used for detection of IL-1β, TNFα, and IL-17. The concentrations were assessed by enzyme-linked immunosorbent assay (ELISA; R&D Systems) using paired antibodies (detection limit 10 pg/ml).

Flow cytometry analysis. DLNs (popliteal and inguinal) from Balb/c mice were removed aseptically following immunization with mBSA and treatment with RC-3095. The cells were cultured in 12-well plates in RPMI medium (1×10^6/ml). Cells were stained with fluorescein isothiocyanate (FITC)–conjugated anti-mouse CD25 or phycoerythrin (PE)–conjugated anti-mouse CD4 and Cy5–conjugated anti-mouse CD3, FITC-conjugated anti-mouse or CD25 PE-conjugated anti-mouse FoxP3 and Cy5-conjugated anti-mouse CD4, and FITC-conjugated anti-mouse CD25 or PE-conjugated anti-mouse GITR and Cy5-conjugated anti-mouse CD4. After washing twice with buffer (1× PBS with 2% BSA), the cells were analyzed by flow cytometry. The acquisition and analysis of the samples were performed using FACSCalibur flow cytometry with CellQuest software (Becton Dickinson).

Statistical analysis. Values are the mean ± SEM and are representative of 2 or 3 separate experiments. The mean values in the different treatment groups were compared by analysis of variance with Tukey's adjustment for multiple comparisons or by Student's *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

Amelioration of the inflammatory reaction in AIA by treatment with RC-3095. IA injection of mBSA into the tibiofemoral joints of immunized mice induced a significant neutrophil migration to the articular cavity at 24 hours, as compared with that in the control contralateral joint in the same mice. Treatment with

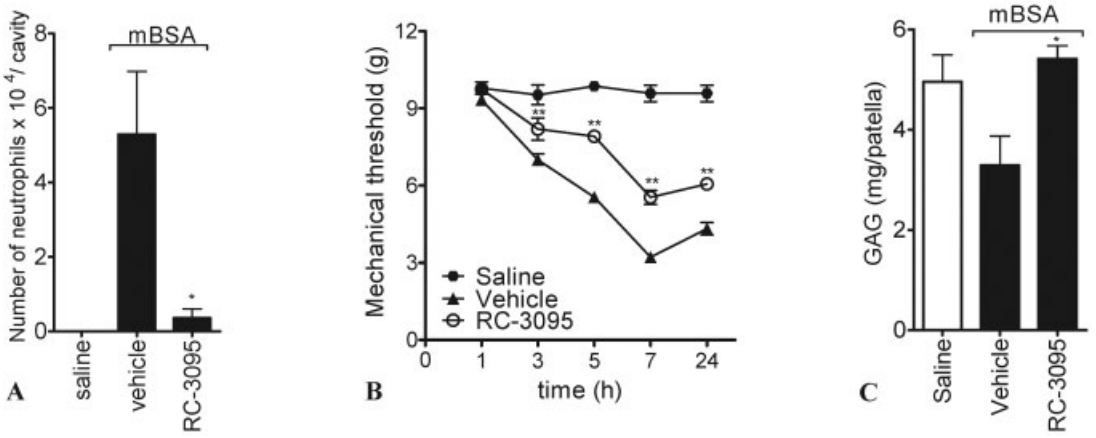

Figure 1. Inhibitory effect of RC-3095 on disease progression in mice with antigen-induced arthritis (AIA). **A,** Neutrophil migration into the articular cavity of the knee joints of mice immunized with methylated bovine serum albumin (mBSA) was assessed 24 hours after treatment with vehicle or RC-3095 (1 mg/kg). Injection of saline alone was used as a control. $* = P < 0.045$ versus vehicle treatment, by Student's unpaired *t*-test. **B,** Hypernociceptive responses were evaluated 1, 3, 5, 7, and 24 hours after intraarticular injection of mBSA. Treatment with RC-3095 inhibited mechanical hypernociception at 3, 5, 7, and 24 hours, as assessed by the paw pressure test. $** = P < 0.01$ versus vehicle treatment, by two-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test. **C,** Cartilage damage was assessed by measurement of the glycosaminoglycan (GAG) content using a colorimetric assay. Arthritis-induced loss of proteoglycans was significantly reduced in mice treated with RC-3095 (1 mg/kg twice per day), preventing cartilage erosion. $* = P < 0.05$ versus vehicle treatment, by one-way ANOVA followed by Tukey's post hoc test. Bars show the mean ± SEM results in 6 mice per group.

Table 1. Effects of RC-3095 on the tissue concentration of interleukin-17 (IL-17), IL-1β, and tumor necrosis factor α (TNFα) in mice with antigen-induced arthritis and mice with collagen-induced arthritis*

	Saline-injected or nonimmunized controls	RC-3095–treated immunized mice	Vehicle-treated immunized mice
Antigen-induced arthritis			
IL-17	41.72 ± 4.38	81.67 ± 11.15†	175.3 ± 23.15
IL-1β	30.63 ± 0.85	37.24 ± 0.64†	53.24 ± 5.54
TNFα	7.45 ± 1.82	27.21 ± 7.16†	128.3 ± 48.78
Collagen-induced arthritis			
IL-17	0.0 ± 0.0	0.88 ± 0.88†	25.11 ± 6.31
IL-1β	23.2 ± 0.57	40.37 ± 3.09†	67.8 ± 3.82
TNFα	0.0 ± 0.0	3.34 ± 1.58†	16.58 ± 2.73

* Values are the mean ± SEM pg/joint in 10 mice per group. Comparisons between treatment groups were made using one-way analysis of variance followed by Tukey's post hoc test. RC-3095 (1 mg/kg) or vehicle (5 mg/kg of 0.9% saline) was administered subcutaneously after arthritis induction in antigen-induced arthritis and after the onset of arthritis in collagen-induced arthritis. The cytokine concentrations in the joint tissue were assessed by enzyme-linked immunosorbent assay at 24 hours after arthritis induction.
† $P < 0.05$ versus vehicle-treated immunized mice.

RC-3095 greatly reduced antigen-induced recruitment of neutrophils into the synovial cavity during joint inflammation (Figure 1A).

Inflammation-related hypernociception was first noticeable at 3 hours after antigen challenge, and then became more intense by 5 hours, peaked at 7 hours, and was persisting at 24 hours. Treatment with RC-3095 (1 mg/kg, twice per day) diminished the extent of inflammation-related hypernociception observed at 5, 7, and 24 hours after antigen challenge in immunized mice compared to that in vehicle-treated immunized mice (Figure 1B). The GAG content, an indicator of turnover of proteoglycans, was decreased in vehicle-treated arthritic mice compared with treatment-naive (saline only) animals. This loss of GAGs was prevented in mice treated with RC-3095 (Figure 1C).

Effects of RC-3095 on the immune response in AIA. Levels of the proinflammatory cytokines IL-17, IL-1β, and TNFα and the antiinflammatory cytokine IL-10 in articular tissue were analyzed on day 8 after IA injection of mBSA and treatment with RC-3095. IL-17, IL-1β, and TNFα were systemically overproduced in the vehicle-treated mice with AIA. All cytokine levels were decreased in the RC-3095 treatment group, in correlation with the degree of amelioration of the inflammatory reaction as measured with the parameters utilized in this study (Table 1).

In order to determine whether RC-3095 alters the proliferation of lymphocytes from the DLNs (popliteal and inguinal) when mice were exposed in vitro to the immunization antigen, cells were isolated from mice 8 days after immunization. Treatment with RC-3095 in

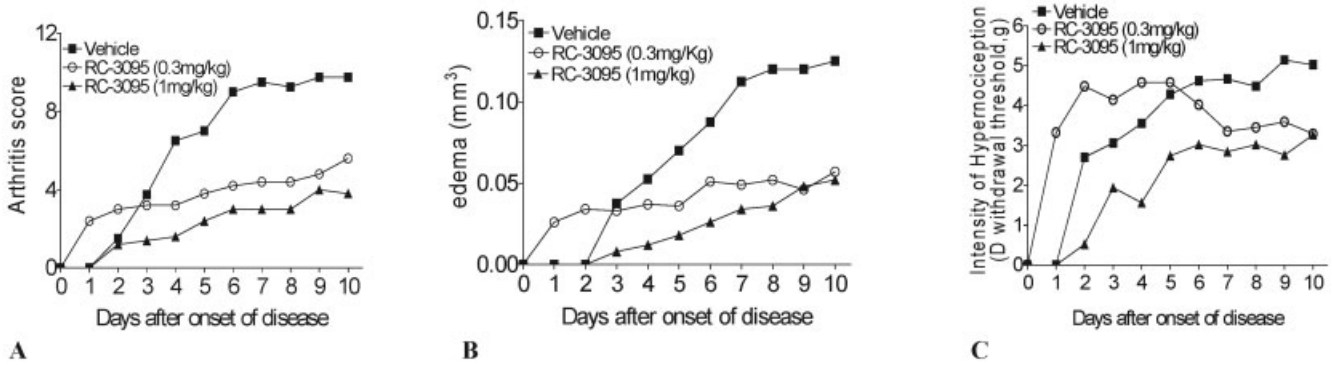

Figure 2. Effects of RC-3095 on the arthritis score, edema, and hypernociception in mice with collagen-induced arthritis (CIA). DBA/1J mice were sensitized to type II collagen, and the development of arthritis was evaluated clinically using a clinical arthritis score **(A)**, measurement of hind paw edema by plethysmometry **(B)**, and measurement of hypernociception using the paw pressure test **(C)**. $P < 0.01$ for both RC-3095 treatment groups versus vehicle-treated mice with CIA, by one-way analysis of variance (ANOVA) with Tukey's post hoc test for comparisons of clinical score and paw edema, and by two-way ANOVA with Tukey's post hoc test for comparisons of hypernociception. Results are the mean of 6 mice per group.

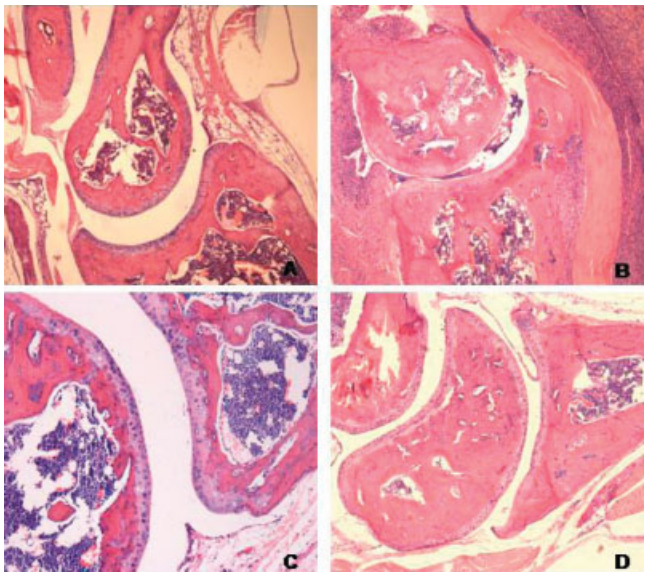

Figure 3. Histopathologic features of representative ankle joints from male DBA/1J mice, as assessed by hematoxylin and eosin staining. **A,** Nonimmunized mice showed normal articular cartilage, absence of infiltrates in the synovium, and open joint space. **B,** Vehicle-treated mice with collagen-induced arthritis (CIA) showed marked infiltration of inflammatory cells and synovial hyperplasia with pannus formation in the joint space, as well as extensive cartilage and bone erosions. **C,** Mice with CIA treated with 0.3 mg/kg RC-3095 showed moderate synovial inflammation and extension of pannus similar to that in the vehicle-treated group. **D,** Mice with CIA treated with 1 mg/kg RC-3095 showed only mild inflammatory cell infiltration and nearly normal joint architecture. Original magnification × 20.

vitro dramatically inhibited the proliferation of lymphocytes ($P < 0.0001$) when compared to that in vehicle-treated mice and that in mice with GRP exposure (results not shown).

Proportions of the Treg cell population are known to change with AIA immunization, and therefore we sought to investigate the effect of RC-3095 on the ratio of positive expression of Treg cells, by assessing differences in surface marker expression ($CD25^{high}$ FoxP3+GITR+) in peripheral DLN-derived cells. Flow cytometry analysis indicated that RC-3095 markedly increased the proportion of $CD25^{high}$FoxP3+GITR+ cells ($P < 0.05$) when compared with that in the vehicle-treated group (results not shown).

Inhibition of the development of CIA by RC-3095. The CIA model is characterized by aggressive synovitis, extensive pannus formation, cartilage degradation, and focal bone erosion. After 21 days of CII induction of disease, all animals had developed a severe arthritis in the paws, whereas there were no changes in the nonimmunized control group. Progression of disease was indicated by an increase in edema and erythema of one or both ankle joints, followed by involvement of the metatarsal and interphalangeal joints. Fully developed arthritis, including red and swollen paws, was observed 3–6 days after the onset of inflammation.

There was a clear difference in the arthritis score, extent of edema, and mechanical hypernociception between the vehicle-treated and RC-3095 (0.3 mg/kg or 1 mg/kg twice per day)–treated animals (Figures 2A–C). Both doses markedly improved all 3 of these parameters, with differences in edema and the arthritis score seen from day 3 of disease onset.

Reduction in the histopathologic changes of the joint by RC-3095 in mice with CIA. Representative histologic findings for each experimental group are shown in Figure 3, and the histologic scores are detailed in Table 2. The nonimmunized animals exhibited a normal architecture of the ankle joints, with normal

Table 2. Synovial histologic scoring of the ankles of mice with collagen-induced arthritis treated with RC-3095*

	RC-3095		
	0.3 mg/kg	1 mg/kg	Vehicle
Synovial inflammation	2 (0–3)	0 (0–3)‡	3 (2–3)
Synovial hyperplasia	0 (0–1)†	0 (0–1)‡	1 (1–2)
Extension of pannus	1.5 (0–3)	0 (0–2)‡	2 (1–3)
Cartilage erosions	1 (0–2)†	0 (0–2)‡	2 (1–3)
Bone erosions	0 (0–1)†	0 (0–2)‡	2 (1–3)

* Values are the median (25th–75th percentile) histologic scores (scale 0–3 for all measures) of the ankles from mice with collagen-induced arthritis (n = 10 animals per group) treated with RC-3095 at 0.3 or 1 mg/kg, or saline as vehicle control, for 10 days after the onset of experimental arthritis. All comparisons of the RC-3095 treatment groups with the vehicle-treated group were made using the Kruskal-Wallis test with Dunn's test for multiple comparisons.
† $P < 0.05$ versus vehicle-treated and RC-3095 (1 mg/kg) groups.
‡ $P < 0.05$ versus vehicle-treated and RC-3095 (0.3 mg/kg) groups.

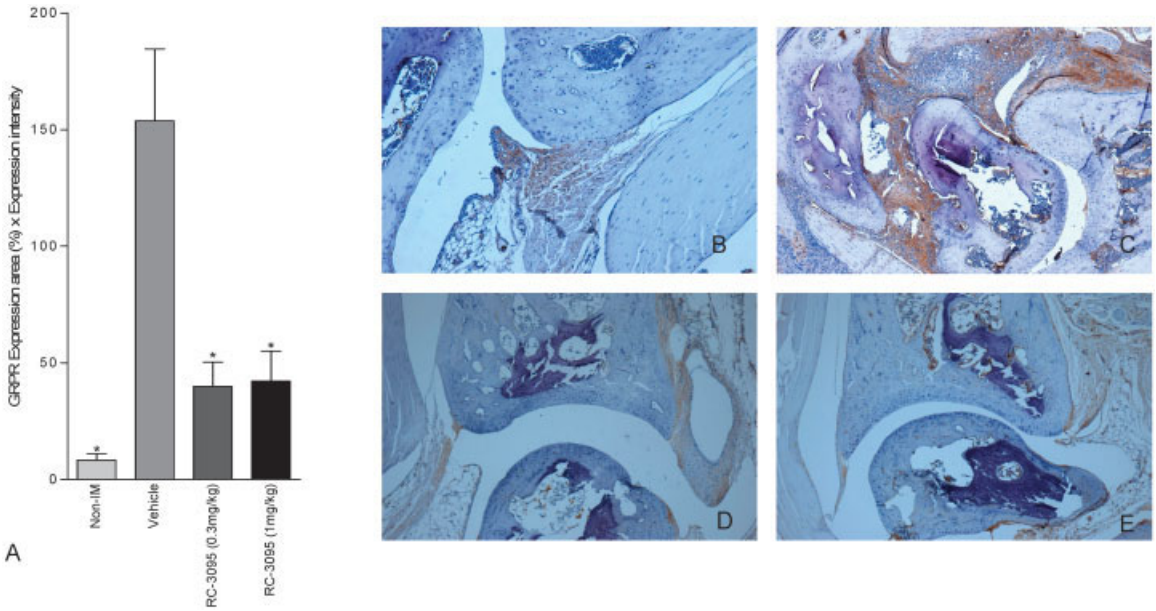

Figure 4. Evaluation of gastrin-releasing peptide receptor (GRPR) expression in the ankle joints of mice with collagen-induced arthritis (CIA), as assessed by immunohistochemistry. **A,** Immunostaining for GRPR was significantly less intense in the RC-3095–treated groups (both doses) than in the vehicle-treated group of mice with CIA. Nonimmunized (non-IM) animals were used as a control. Bars show the mean ± SEM of 6 mice per group. $* = P < 0.01$ versus vehicle-treated mice, by one-way analysis of variance followed by Tukey's post hoc test. **B–E,** Results of immunohistochemical analyses of representative ankle joint tissue sections reveal mild immunostaining for GRPR in nonimmunized animals **(B)**, strong immunostaining on inflammatory infiltrates in cartilage of vehicle-treated mice with CIA **(C)**, and mild immunostaining with less intensity and a smaller area of expression, indicative of a lower expression of GRPR, in RC-3095–treated animals with CIA receiving a dose of 0.3 mg/kg **(D)** or 1 mg/kg **(E)** twice per day. Original magnification × 20.

appearance of the cartilage lining, the joint surface, and the underlying bones, without any inflammatory infiltrates in the synovial tissue (Figure 3A). Vehicle-treated animals presented a highly abnormal histologic appearance of the joint, with pronounced synovial inflammation, synovial hyperplasia, pannus formation, and extensive erosive changes in the cartilage and bone (Figure 3B). In contrast, the animals treated with RC-3095 at a dose of 1 mg/kg showed a remarkable inhibition in all histologic findings of arthritis, displaying only mild inflammatory cell infiltration and nearly normal joint architecture of the anterior paws (Figure 3D) when compared with vehicle-treated mice. In contrast, the 0.3 mg/kg dose of RC-3095 was shown to be less effective in all scores of histologic features; in particular, the mice treated with 0.3 mg/kg RC-3095 exhibited synovial inflammation and extension of pannus similar to that in the vehicle-treated group (Figure 3C).

Effect of treatment with RC-3095 on GRPR expression in articular tissue in the CIA model. Immunohistochemical analysis for GRPR was performed in the articular tissue of mice with CIA (Figure 4). Staining for GRPR was detected both in the cytoplasm and on the cell membrane, and all ankle tissue sections expressed the receptor. No membrane staining was seen. The staining was often multifocal and sometimes diffuse.

GRPR was significantly less intensely expressed in the RC-3095–treated group than in the vehicle-treated group (Figure 4A). The nonimmunized animals showed light staining for GRPR (Figure 4B). An increase in GRPR expression could be observed in the synovial cells (fibroblasts and interstitial cells) and sometimes in the cartilage (chondrocytes lining the articular surface) in the animals of the vehicle-treated group, in conjunction with severe articular inflammation, characterized by inflammatory infiltration into the vessels, synovial membrane, and cartilage (Figure 4C). The RC-3095–treated groups (0.3 mg/kg and 1 mg/kg twice per day) showed a smaller expression of GRPR, in terms of both area and intensity (Figures 4D and E).

Alteration of the release of proinflammatory cytokines by RC-3095 in mice with chronic CIA. The levels of the proinflammatory cytokines IL-17, IL-1β, and TNFα in the articular tissue were analyzed using

ELISA on day 10 after the onset of disease. Treatment with RC-3095 (1 mg/kg twice per day) significantly reduced the levels of all 3 proinflammatory cytokines in the joint tissue of mice with CIA, when compared with that in the vehicle-treated group, the latter of which displayed a systemic overproduction of all 3 cytokines in the articular tissue (Table 1).

DISCUSSION

In the present study, we evaluated the effects of a selective GRPR antagonist, RC-3095, on 2 different models of RA, comprising mice with CIA and mice with AIA. In both models, the histopathologic findings are similar to those observed in RA patients. Our findings demonstrate that RC-3095 treatment significantly attenuates the development of arthritis in both AIA and CIA. This was evidenced by the observed reduction in clinical scores and synovial inflammation in the treated animals from both experimental models. In the AIA model, reduction of neutrophil influx into the joint cavity, hypernociception, damage to the articular cartilage, lymphocyte proliferation, and an increase in Treg cell numbers were observed, while in the CIA model, there was significant reduction in the hypernociception, number of inflamed paws, visually determined inflammation score, and proinflammatory cytokine levels. Virtual abrogation of the chronic synovitis, seen on histopathologic analysis, and prevention of damage to the joint are indicative of a protective effect of RC-3095, also at the structural level. Demonstration of efficacy in different species and models is a crucial proof-of-concept step to establish the therapeutic value of a compound.

Despite the known effects of GRP on tissue growth (16) and on the gastrointestinal system (37,38), respiratory tract (39), and nervous system (40), little is known about the its physiologic effects on immune function. GRP seems to modulate, as either a stimulator or inhibitor, the function of lymphocytes, phagocytes, and natural killer cells. Lymphocytes, neutrophils, eosinophils, macrophages, mast cells, and endothelial cells all express the receptor for GRP, indicating a possible role of this peptide in inflammatory conditions (11,12). The expression of GRPRs depends on cell differentiation, and GRP can stimulate or inhibit responses, depending on the maturity and state of activation of the target cell (41).

Previous work has shown that GRP can exert a significant effect on different aspects of immune regulation in arthritic joints. Bombesin/GRP-like peptide is clearly detectable in the synovial fluid of patients with RA, but not that of control subjects, in correlation with inflammatory cytokine levels and the erythrocyte sedimentation rate (7). Grimsholm et al (13,14) have clearly demonstrated the presence of GRPR in both the inflammatory infiltrate and on the articular chondrocytes of arthritic mice. They also observed frequent bombesin/GRP-immunoreactive varicose nerve fibers in association with the inflammatory infiltrate, and the presence of GRPR in some cells in the infiltrate (13,14). The present study thus shows that bombesin/GRP can be added to the list of neuropeptides present in joint innervations, especially in association with inflammatory infiltrates.

Many antagonists for the bombesin/GRPR have been synthesized and tested for their ability to inhibit the effects of this peptide (37,38). RC-3095 has been tested in an animal model of sepsis, in which the treatment was shown to decrease the production of proinflammatory cytokines, such as TNFα and IL-1β, without changing the production of the antiinflammatory cytokine IL-10, leading to improved survival of the treated animals (17). In an animal model of uveitis, RC-3095 elicited important action against oxidative damage in the irides, and also exhibited antiinflammatory actions by reducing the activity of myeloperoxidase and decreasing the levels of TNFα and MCP-1 to a higher extent than that with dexamethasone (18). In an animal model of gastric ulcer, RC-3095 treatment, administered alone or in combination with omeprazole, was effective for the inhibition of acid secretion, in addition to exerting protective effects by reducing gastric oxidative injury (19). Finally, in an animal model of ulcerative colitis, RC-3095 reduced the severity of inflammatory bowel disease, and its antiinflammatory activity was associated with a reduction in the colonic expression of TNFα (39).

Recently, our group demonstrated the effect of RC-3095 in a model of arthritis induced by CFA. The results demonstrated that treatment with RC-3095 led to improvement of arthritis, by reducing the ankle thickness in the affected paw, reducing the joint damage, and decreasing cytokine serum levels. This prior study was the first to evaluate the protective effect of an antagonist of bombesin/GRP in these conditions (20).

New therapies for RA have recently been developed, in which the aim has been to inhibit inflammation mediators such as TNFα (etanercept, adalimumab, and infliximab), IL-1 (anakinra), and IL-6 (tocilizumab). However, there is evidence that although these anticytokine therapies are effective, a single cytokine target will likely benefit a specific subset of patients with RA.

Other proinflammatory mediators, such as neuropeptides, are also involved, but the relationship of neuropeptides with cytokines in RA has received little attention. A large number of agonists and antagonists of neuropeptide receptors have been produced and could be tested alone or in combination with preexisting therapies (40,42).

These studies of the GRPR antagonist RC-3095 in experimental models of arthritis may provide relevant information on the development of the disease and increase the knowledge of new treatments for RA. The promising results that we have observed in the model of arthritis induced by CFA point to the need to better assess the efficacy and potential mechanisms of action in other models that are most similar to human RA. Such knowledge would be essential for the subsequent progression to the stage of clinical trials with the drug.

It is likely that multiple mechanisms are involved in the protection conferred by RC-3095 in arthritis. The reduction in the acute influx of neutrophils in AIA, leading to decreased hypernociception and cartilage degradation, contributes to the joint protection. The extent of joint damage observed in AIA is closely related to an increase in the production of proinflammatory cytokines, but, most prominently, that of IL-17 (43). RC-3095 decreased the release of TNFα, IL-1β, and IL-17 and increased the Treg cell population. There was also a significant reduction in the rate of lymphocyte proliferation in RC-3095–treated animals.

In conclusion, the present results support the view that interference of the neuropeptide GRP pathway by RC-3095 has immunomodulatory and antiinflammatory effects and was able to attenuate damage and progression of disease in experimental models of arthritis. Such observations support the future development of clinical trials in RA with this potential new therapeutic strategy.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Drs. Cunha and Xavier had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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