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Involvement of the peripheral benzodiazepine receptor in the development of rheumatoid arthritis in Mrl/lpr mice

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Abstract

In this study, the effects of different peripheral benzodiazepine receptor ligands: PK 11195 [1-(2-chloro-phenyl)-*N*-methyl-*N*-(1-methylpropyl)-1-isoquinoline carboxamide], Ro5-4864 [7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one] and the newly described SSR 180575 (7-chloro-*N*,*N*,5-trimethyl-4-oxo-3-phenyl-3,5-dihydro-4*H*-pyridozine[4,5-b] indole-1-acetamide) were analysed on the progression and severity of rheumatoid arthritis in vivo in the Mrl/lpr mice model, following chronic treatment (at 3 mg/kg, i.p. for 30 days). We found that peripheral benzodiazepine receptor ligands have significant beneficial therapeutic action on the development of spontaneous rheumatoid arthritis-like signs. Concomitantly, we mapped immunoreactive peripheral benzodiazepine receptor in inflamed tissues, and we observed that in addition to the infiltrated leukocytes, peripheral benzodiazepine receptor was expressed in synovial membranes, at the cartilage pannus junction and in chondrocytes. Interestingly, we observed that peripheral benzodiazepine receptor expression in chondrocytes was reduced when Mrl/lpr mice developed the pathology and restored upon peripheral benzodiazepine receptor ligand treatment. Altogether, our data provide further evidence of a role played by peripheral benzodiazepine receptor in the regulation of inflammation processes and support new therapeutic applications for specific potent peripheral benzodiazepine receptor ligands.

Keywords: Benzodiazepine receptor, peripheral; SSR 180575; Rheumatoid arthritis; Mrl/lpr mouse; Chondrocyte

1. Introduction

Rheumatoid arthritis is a chronic, systemic, inflammatory disorder characterized by a symmetrical polyarthritis leading to progressive joint destruction. This has been attributed to complex cellular interactions among acute and chronic inflammatory cells, chondrocytes, macrophages and other monocytic modulatory cells. Joints affected by this disease show proliferation of synovial cells and continuous release of matrix-degrading enzymes that mediate attachment and invasion of synovial fibroblasts into adjacent cartilage and bone, thus contributing to joint disability and destruction (Firestein et al., 1990; Harris, 1984). Efforts to understand the etiology and pathogenic mechanisms of rheumatoid arthritis were long hindered by the lack of suitable animal models. Recently, several new spontaneous animal models, more representative of human rheumatoid arthritis and exhibiting many of its pathological features, have been reported in mice. The Mrl/lpr mice, first described by Murphy and Roths (1978), spontaneously develop a form of arthritis that is serologically and histologically similar to human rheumatoid arthritis (Theofilopoulos and Dixon, 1981; O'Sullivan et al., 1985; Pataki and Rodorf-Adam, 1985). Such a model has already been used to investigate anti-inflammatory agents targeting rheumatoid arthritis (Rodorf-Adam et al., 1986).

The mitochondrial benzodiazepine receptor, also commonly referred to as the peripheral benzodiazepine receptor, was first described as a diazepam binding site in rat peripheral tissues (Braestrup and Squires, 1977). The peripheral benzodiazepine receptor is a 18 kilodalton (kDa) protein primarily localised on the mitochondrial outer membrane (Anholt et al., 1986), where it is associated

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in a trimeric complex with the 32 kDa voltage-dependent anion channel and 30 kDa adenine nucleotide carrier. More recently, a novel cytoplasmic protein, termed PRAX-1 (peripheral benzodiazepine receptor associated protein-1), whose function has not yet been characterized, has been shown to interact with the peripheral benzodiazepine receptor (Galiegue et al., 1999). Peripheral benzodiazepine receptors are ubiquitously expressed throughout the body especially in steroid producing organs (Anholt et al., 1985), such as testis, adrenal gland and ovary, and are also widely expressed in the immune system. The Ro5-4864 [7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one] and the PK 11195 [1-(2-chloro-phenyl)-*N*-methyl-*N*-(1-methylpropyl)-1-isoquinolinecarboxamide] compound bind exclusively to peripheral benzodiazepine receptor with high affinity both in vitro and in vivo (Le Fur et al., 1983a,b). Considering peripheral benzodiazepine receptor functions, several studies point to a major role of this receptor in the regulation of immune functions and inflammatory responses (Ruff et al., 1985; Laird et al., 1989; Zavala et al., 1990a; Schreiber et al., 1993; Vowickel et al., 1997; Gehlert et al., 1997; Lacor et al., 1999; Scatton et al., 1990). The anti-inflammatory properties of Ro5-4864 and PK 11195 were recently shown in two mouse models of acute inflammation, where paw oedema formation and pleurisy were induced by carrageenan (Torres et al., 2000). In addition, PK 11195 was shown to inhibit and cure mouse arthritis in Mrl/lpr mice that spontaneously develop arthritis (Waterfield et al., 1999).

In the present study, we compared the effects of three different peripheral benzodiazepine receptor ligands on the development and severity of rheumatoid arthritis in Mrl/lpr mice: Ro5-4864, PK 11195, and the recently described SSR 180575 (7-chloro-*N*,*N*,5-trimethyl-4-oxo-3-phenyl-3,5-dihydro-4*H*-pyridozine[4,5-b] indole-1-acetamide). This compound is a new peripheral benzodiazepine receptor ligand that exhibits nanomolar affinity for this receptor. It shows potential in experimental models of peripheral neuropaties and motor neuron axonopathy as well as in experimental autoimmune encephalitis in the rodent (Ferzaz et al., 2002). We report that the new peripheral benzodiazepine receptor ligand SSR 180575 has a beneficial therapeutic action on rheumatoid arthritis-like syndrome as well as Ro5-4864 or PK 11195. Concomitantly, we explored the involvement of the receptor in the disease using a novel polyclonal antibody raised against the murine protein. This antibody revealed immunoreactive peripheral benzodiazepine receptor in synovial and inflammatory cells at the pannus junction, and for the first time, we highlighted the expression of the receptor in chondrocytes. To go further, we have also observed peripheral benzodiazepine receptor expression in human chondrocytes. Altogether, these data indicate that the peripheral benzodiazepine receptor may play a role in the development of rheumatoid arthritis and is of interest as a therapeutic target for modulating inflammatory responses.

2. Material and methods

2.1. Animals, arthritis induction and treatment

The present animal experiments complied with the European and French laws and with the guiding principles for experimental procedures as set forth in the Declaration of Helsinki. Mrl/lpr mice were obtained from a breeding colony, which was established from stocks originally purchased from Harlan France. Thirteen-to-fourteen-week-old Mrl/lpr males ($n = 10$ for each group) were injected with complete Freund's adjuvant intradermally at two thoracic sites with 0.05 ml of complete Freund's adjuvant supplemented to 10 mg/ml with heat-inactived Mycobacterium tuberculosis H37 RA (Difco, Detroit, MI). The adjuvant was prepared as water in oil emulsion and administered with a 27-gauge needle. Mice were then intraperitoneally (i.p.) injected daily, with the different compounds PK 11195 (Sigma), Ro5-4864 (Fluka), and SSR 180575, or every 2 days with methotrexate (4-amino-10-methylfolic acid, Sigma). Each experimental group was injected with 0.1 ml of either PK 11195, Ro5-4864, SSR 180575, or methotrexate dissolved in 5% dimethylsulphoxide, 5% Tween 20 and 90% injection water. Final doses were 3 mg/kg for PK 11195, Ro5-4864 and SSR 180575, and 2 mg/kg for methotrexate. Control groups were injected with 0.1 ml of the vehicle solution. Injections were given i.p. once a day until killing on day 30.

2.2. Clinical examination of the hind paw

Signs of the disease were evaluated in all groups every 5 days and scored as positive if erythema and swelling on the hind paws were observed. The hind paw volume and ankle width were measured every 5 days (prior to drug injection) using a plethysmometer and a micrometer, respectively. The results were expressed in milliliters and in millimeters as the difference between the observed paw width and the paw width at the beginning of treatment.

2.3. Histological evaluation of the joints

Thirty days after the injection of adjuvant, animals were perfused transcardially with a solution of phosphate buffer saline, with heparin (CHOAY 5000 IU/ml) followed sequentially by a 10% formalin solution. Then the hind paw was removed and placed in buffered formalin. After decalcification in 10% formic acid for 48 h, tissues were processed or paraffin embedded. Serial sections of the tarso-metatarsal joints were cut to 5-μm thickness and stained with haematoxylin and eosin. Sections were examined and scored. A minimum of 14 sections/joint were assessed and scored to obtain a semiquantitative measurement of different criteria: subsynovial inflammation (0, normal; 1, focal inflammatory infiltrates; 2, inflammatory infiltrate that dominated the cellular histology); synovial hyperplasia (0, normal; 1, a

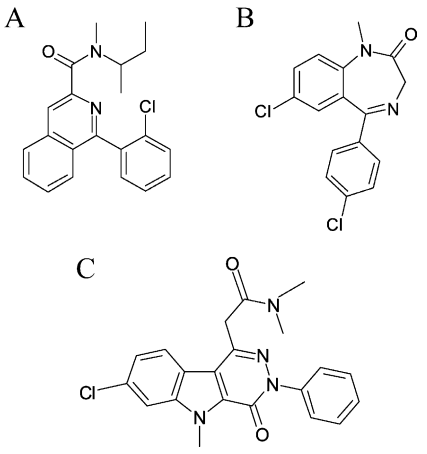

Fig. 1. Chemical structures of the peripheral benzodiazepine receptor ligands: PK 11195 (A); Ro5-4864 (B); SSR 180575A (C).

continuous minimum three-layer-thick, synovial lining seen in one joint; 2, minimum three-layer-thick, synovial lining detected in several joints); pannus formation and cartilage erosion (0, normal; 1, pannus partially covered cartilage surfaces without evident cartilage loss; 2, pannus connected to significant cartilage loss); bone destruction (0, normal; 1, detectable destruction of bone by pannus or osteoclast activity; 2, the pannus or osteoclast activity had destroyed a significant part of the bone); and finally, the global pathology status was the overall assessment derived by adding values for these criteria.

2.4. Radiological analysis

At the end of the experiment, ankle joints were removed and analysed radiologically to address joint destruction. X-ray photographs were carefully examined using a macroscopic table associated with a microscope, and joint destruction was graded on a 0–3 scale, where 0 denotes no damage, 1; minor joint destruction, or one enlightened spot; 2, moderate changes, 1–2 spots observed in one area; 3, marked changes, 2–3 spots observed in more areas, representing severe erosion affecting the joint. Articular surface destruction was scored on ankles and phalanx joints as previously described (Joosten et al., 1999).

2.5. Preparation and characterization of the anti-mouse peripheral benzodiazepine receptor antibody

A polyclonal antibody was produced against the C-terminal region of mouse peripheral benzodiazepine receptor (aa 156–169: RDNSGRRGGSRLPE). The immunizing peptidic sequence was obtained online from GenBank (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov:80/, accession number P50637). Immunosera were raised in rabbits using Gluta-Ova as carrier protein. Antibodies were purified by affinity chromatography against the synthetic peptide coupled to a sepharose matrix. Purified antibodies (concentration: 0.53 mg/ml) were stored at − 80 °C.

2.6. Western blot analysis

Homogenates from the Y1 cell line developed from mouse adrenal cells (ATCC CCL-79) were prepared in a

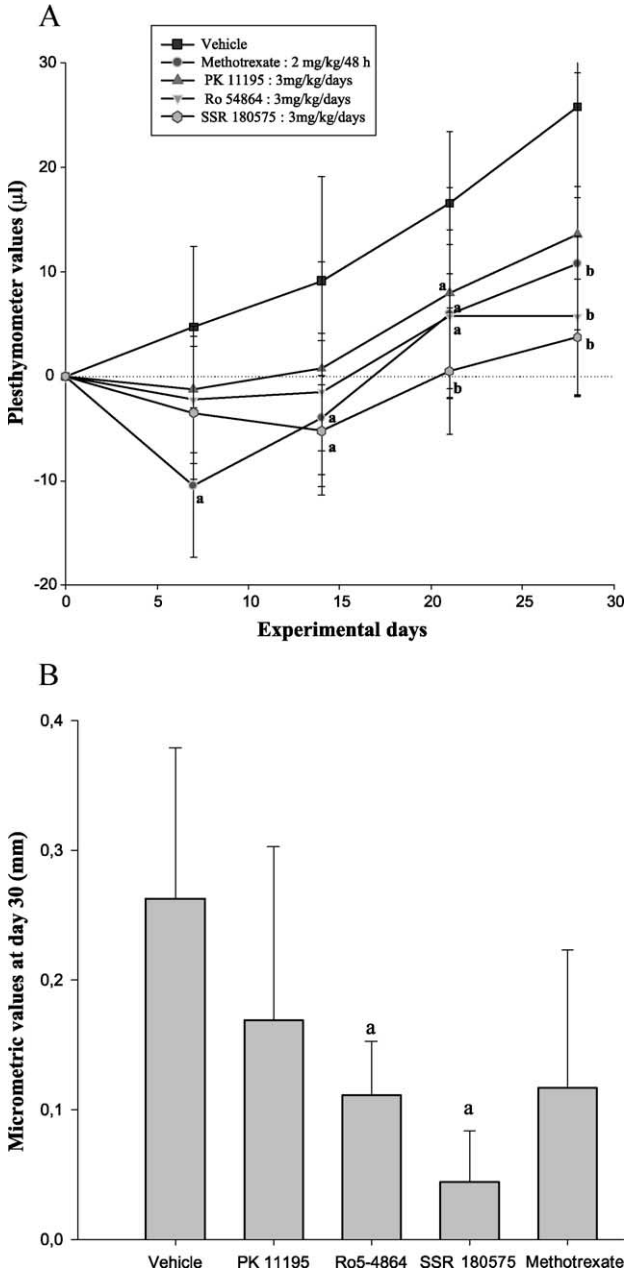

Fig. 2. Effect of PK 11195, Ro5-4864, SSR 180575, and methotrexate on the rheumatoid arthritis like syndrome in Mrl/lpr mice. Methotrexate (2 mg/kg, i.p.) was administered every 2 days. PK 11195, Ro5-4864, SSR 180575 (3 mg/kg, i.p.) were administered daily. Paw oedema (A) and ankle width (B) were assessed at day 30. Each point represents the mean for 10 animals and the vertical lines the S.E.M. a and b indicate statistically significant differences: [a]($P' < 0.05$) and [b]($P' < 0.01$) as compared with the respective control values.

buffer containing 2 mM Hepes (pH 7.4), 70 mM sucrose, 200 mM mannitol, 1 mM ethylenediaminetetraacetic acid (EDTA), and a protein inhibitor cocktail (Boehringer Mannheim, Germany), and centrifuged at 100 000 × *g* (20 min, 4 °C). Pellets were suspended in 20 mM Tris–HCl buffer (pH 7.4). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 4–20% polyacrylamide), and electroblotted onto nitrocellulose. Membranes were soaked for 60 min in tween-phosphate buffer saline (TPBS; 10 mM sodium phosphate, pH 7.6, 150 mM NaCl, 0.1% Tween 20), containing 5% dried milk. Blots were then incubated for 3 h in TPBS containing 1 μg/ml anti-peripheral benzodiazepine receptor antibody. Blots were incubated with anti-rabbit immunoglobulin-horseradish peroxidase-conjugated antibody (Dako; 60 min), and proteins were visualized using an enhanced chemiluminescent detection system (Super Signal; Pierce Chemical, Rockford, IL).

2.7. Confocal microscopy

Anti-peripheral benzodiazepine receptor antibody specificity was assessed at the subcellular level in the Y1 cell line. Cells were grown until confluence (3 days, 37 °C) in Ham's F10 medium (82.5%) containing 15% horse serum, and 2.5% fetal bovine serum (Gibco, Grand Island, NY). Cells were

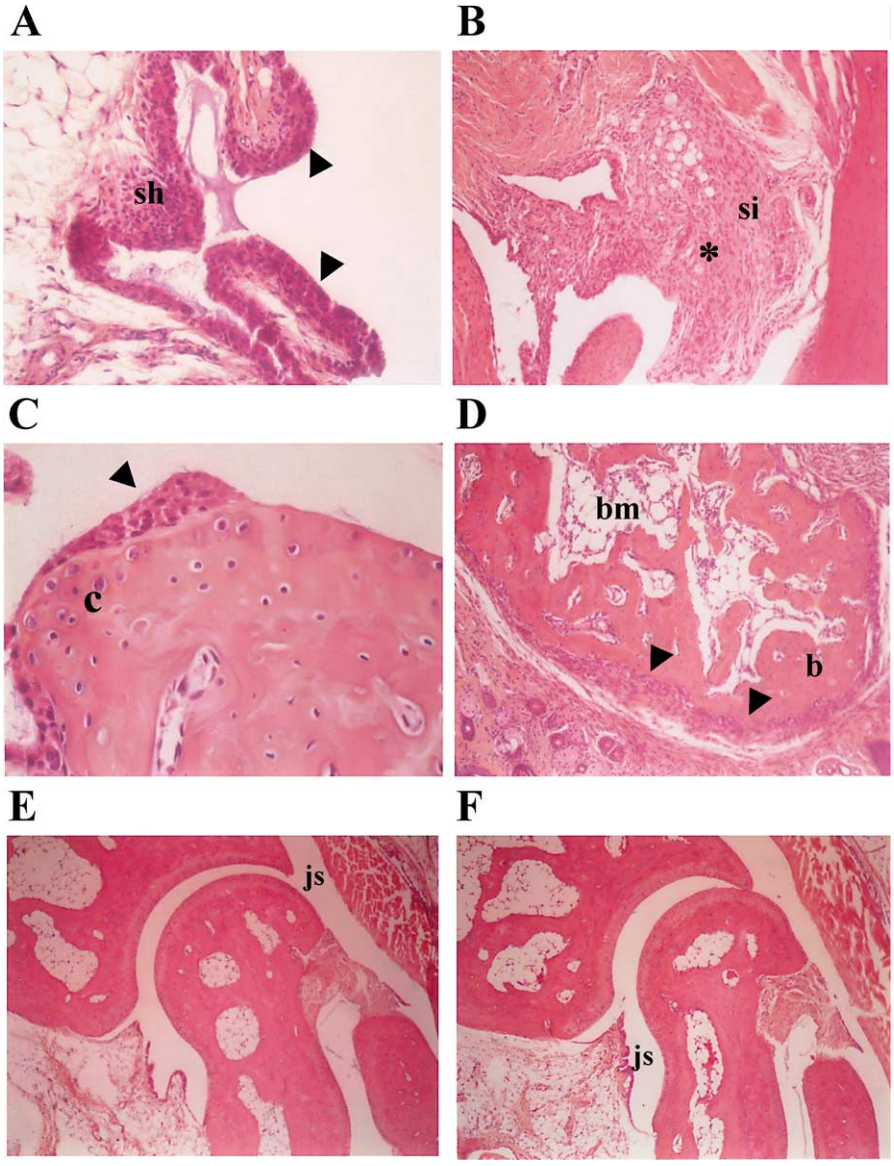

Fig. 3. Effects of the peripheral benzodiazepine receptor ligands and methotrexate on the joint histopathology. Representative photomicrographs of the joints are shown. For the control group: (A) synovial hyperplasia (× 200 objective); (B) marked subsynovial infiltration by mononuclear cells (× 100 objective); (C) pannus formation; (D) bone erosion (arrow × 200 objective). For the peripheral benzodiazepine receptor ligand SSR 180575 (3 mg/kg, i.p.-treated group): (E) indicates some stromal thickening of the subsynovium, but absence of any infiltrating cells or joint destruction (× 50). (F) Actual ankle joint of a mouse treated with methotrexate (× 50). c, cartilage; b, bone; bm, bone marrow; js, joint space; p, pannus; sh, synovial hyperplasia; si, subsynovial inflammation.

Table 1
Preventative effects of peripheral benzodiazepine receptor ligands on Mrl/lpr arthritis

	Vehicle	PK 11195 (3 mg/kg)	Ro5-4864 (3 mg/kg)	SSR 180575 (3 mg/kg)	Methotrexate (2 mg/kg)
Number of hind paws	18	18	20	18	18
Incidence of arthritis, %	100	20	0	0	0
Synovial hyperplasia	1.7 ± 0.42	0.66 ± 0.7[a]	1 ± 0.7[a]	0.55 ± 0.6[a]	1.22 ± 0.5[b]
Synovial inflammation	1.38 ± 0.69	0.83 ± 0.7	0.66 ± 0.7[b]	0.38 ± 0.6[a]	0.44 ± 0.6[a]
Cartilage destruction and pannus formation	1.5 ± 0.5	0.88 ± 0.6	1 ± 0.5	0.55 ± 0.6[a]	0.72 ± 0.5[a]
Bone destruction	1.33 ± 0.48	0.55 ± 0.7[b]	0.55 ± 0.7[b]	0.33 ± ± 0.5[a]	0.66 ± 0.6[b]

The histopathological score is given as the mean ± S.E.M. for each index.

[a] A significant difference from vehicle-treated mice, as determined by the results of a Kruskal–Wallis test and Wilcoxon multiple comparisons test with Bonferroni adjustment ($P' < 0.01$).

[b] A significant difference from vehicle-treated mice, as determined by the results of a Kruskal–Wallis test and Wilcoxon multiple comparisons test with Bonferroni adjustment ($P' < 0.05$).

incubated with a fluorescein isothiocyanate-conjugated mitochondrial probe (MitoTracker, Molecular Probes, Eugene, OR) as specified by the manufacturer, fixed with 1% paraformaldehyde (Sigma), and permeabilized with 0.1% saponin. Cells were exposed to anti-peripheral benzodiazepine receptor antibody (1:200, 60 min, room temperature), washed in phosphate buffer saline and exposed to indocicarbocyanine-5-conjugated goat anti-rabbit immunoglobulin G (IgG) (1:200, Jackson Immunoresearch/Interchim, Paris, France). Analyses of cell distribution, colocalization of immunoreactive peripheral benzodiazepine receptor and mitochondrial probe (MitoTracker, Molecular Probes) were performed with 650/670 and 492/520 nm filter sets, respectively. Experiments were performed with a laser scanning confocal microscope (LSM 410, Zeiss, Oberkochen, Germany), equipped with a 63× Zeiss planapo oil immersion objective (N.A. 1.4), using LSM 410 image analysis software, as previously described (Bribes et al., 2002). Specificity controls were carried out by preincubation of anti-peripheral benzodiazepine receptor antibody overnight (1:200, 4 °C) with the immunizing peptide (10 μg/ml).

2.8. Immunohistochemistry

2.8.1. In the articular joint

Immunochemical analysis of the specific peripheral benzodiazepine receptor antibody was carried out on deparaffinized tissue sections. Sections were exposed to anti-peripheral benzodiazepine receptor antibody (dilution 1:300) for 25 min at room temperature. Immunoreactions were visualized by incubation with a biotinylated secondary antibody against rabbit IgG for 25 min at 37 °C followed by an indirect streptavidin–biotin method, using H_2O_2/3-amino-9-ethylcarbazole as chromogenic substrate (red label, Dako ChemMate™ Detection kit, peroxidase/AEC, rabbit/mouse, Dako, Glostrup, Denmark). Specificity was demonstrated by the absence of staining after preadsorbing anti-peripheral benzodiazepine receptor antibody overnight at 4 °C with the immunizing peptide. Negative controls were also obtained by the absence of staining after omission of the primary antibody.

Sections were then counterstained with hematoxylin. Sections were mounted on faramount-covered slides (Dako), air-dried and analysed using a Leica DMLB microscopy.

2.8.2. In a human chondrocyte cell line

Immunohistochemical analysis of the human chondrocyte cell line (HCH; Promo Cell) was carried out using the 8D7 antibody (anti-human peripheral benzodiazepine receptor) (Dussosoy et al., 1996). The cells were exposed to anti-peripheral benzodiazepine receptor antibody (dilution 1:350) for 25 min at room temperature. Immunoreactions were visualized by incubation with a biotinylated secondary antibody against mouse IgG for 25 min at 37 °C followed by an indirect streptavidin-*R*-phycoerythrin immunofluorescence method (red label, Dako). Specificity was demonstrated by the absence of staining after preadsorbing anti-peripheral benzodiazepine receptor antibody overnight at 4 °C with the immunizing peptide. Negative controls were also obtained by the absence of staining after omission of the primary antibody.

2.9. Statistical analysis

The statistical analysis was realized with the Kruskal–Wallis test and Wilcoxon multiple comparisons test with Bonferroni adjustment, with [a]$P' < 0.05$ and [b]$P' < 0.01$.

Table 2
Preventative effects of peripheral benzodiazepine receptor ligands on radiological features in Mrl/lpr arthritis

	Vehicle	PK 11195 (3 mg/kg)	Ro5-4864 (3 mg/kg)	SSR 180575 (3 mg/kg)	Methotrexate (2 mg/kg)
Number of hind paws	18	18	20	18	18
Reduction of the articular space	1.45 ± 0.68	0.54 ± 0.52[a]	0.66 ± 0.5	0.4 ± 0.51[a]	0.45 ± 0.52[a]

The radiological score is given as the mean ± S.E.M. for each index.

[a] A significant difference from vehicle-treated mice, as determined by the results of a Kruskal–Wallis test and Wilcoxon multiple comparisons test with Bonferroni adjustment ($P' < 0.05$).

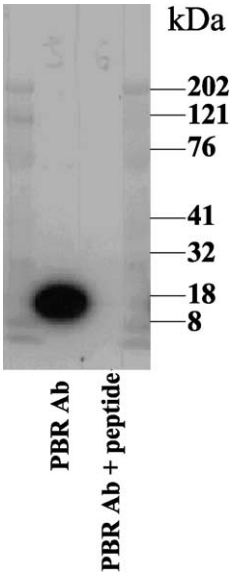

Fig. 4. Western blot analysis of peripheral benzodiazepine receptor expression in mitochondrial Y-1 cell extracts. A single 18 kDa band appeared, as indicated by the arrow on the left lane. In the right lane (peripheral benzodiazepine receptor + peptide), the anti-peripheral benzodiazepine receptor antibody was preadsorbed with the immunizing peptide.

3. Results

3.1. The newly described peripheral benzodiazepine receptor ligand

SSR 180575 is a recently discovered peripheral benzodiazepine receptor ligand that binds with high affinity to peripheral benzodiazepine receptor (Ferzaz et al., 2002). This study was aimed at investigating its effect on rheumatoid arthritis progression and severity in the Mrl/lpr mouse model and to compare its efficacy with two well-characterized peripheral benzodiazepine receptor ligands: PK 11195 and Ro5-4864 (Fig. 1).

3.2. Peripheral benzodiazepine receptor ligands delay the onset of arthritis symptoms

Thirteen-to-fourteen-week-old Mrl/lpr mice injected with complete Freund's adjuvant rapidly developed severe rheumatoid arthritis-like syndrome. Ninety percent of the mice exhibited marked hind paw swelling assessed by plethysmometer and micrometer analysis (Fig. 2). Swelling onset was significant at day 14 post-complete Freund's

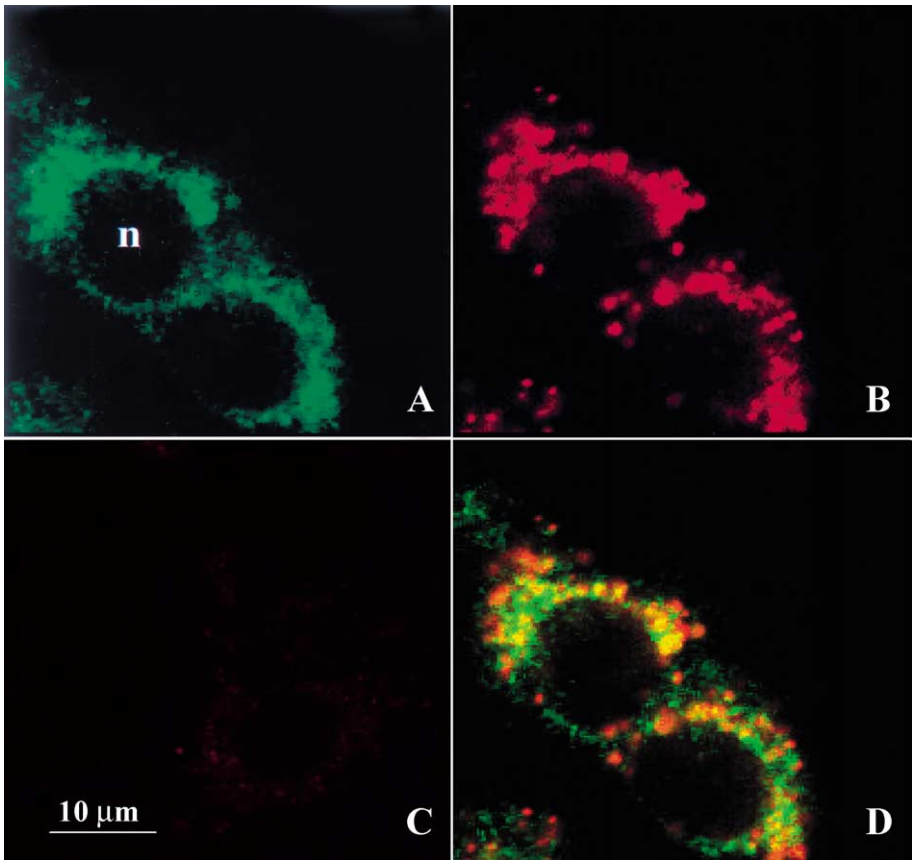

Fig. 5. Assessment of the subcellular distribution of immunoreactive peripheral benzodiazepine receptors in cultured Y-1 adrenocortical cell lines by confocal microscopy. In (A), fluorescence distribution of the mitochondrial probe (green). In (B), fluorescence distribution of immunoreactive peripheral benzodiazepine receptor (red). In (D), merging A and B demonstrates almost complete colocalization of both probes. In (C), preadsorbing anti-peripheral benzodiazepine receptor antibody with immunizing peptide eliminated the immunoreactive peripheral benzodiazepine receptor signal. n, cell nucleus.

adjuvant administration (Fig. 2A). Treatment with methotrexate (2 mg/kg/48 h, i.p.), as a reference compound, dramatically reduced these arthritic signs because no significant swelling was observed during the first 21 days of the experiment. Only moderate swelling was detected on day 30. Treatment with PK 11195, Ro5-4864 or SSR 180575 was also very efficient during the first 21 days. Following SSR 180575 treatment, both the ankle width and the paw oedema were dramatically reduced at the end of the experiment as compared to either Ro5-4864 or PK 11195 (Fig. 2B).

3.3. Effect of peripheral benzodiazepine receptor ligands on the joint histopathology

Histological analysis of ankle joints was performed at day 30 after complete Freund's adjuvant injection. This arthritic model predominantly affects the hind paw joints, while the upper ones are rarely affected. In vehicle-treated mice, we observed some of the hyperplasic synovial assumed villous configurations. We also noticed perivascular infiltration by lymphohistiocytic cells, plasma cells, and polymorphonuclear neutrophils in the subsynovial and

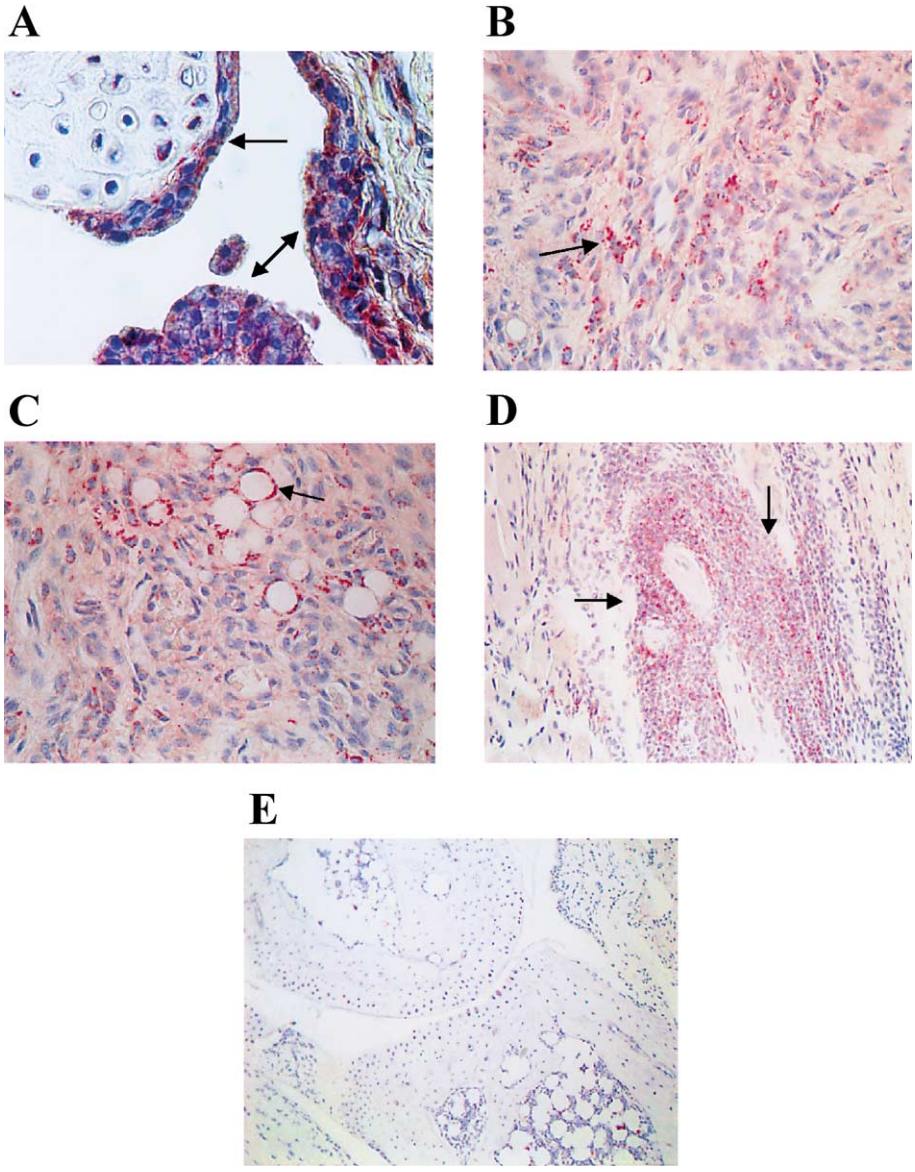

Fig. 6. Distribution of peripheral benzodiazepine receptor-positive cells in inflamed joints from Mrl/lpr mice suffering from rheumatoid arthritis. Using a polyclonal anti-mouse peripheral benzodiazepine receptor, peripheral benzodiazepine receptor-containing cells were found in: (A) the lining synovial cell layer (double arrow) and pannus junction (arrow); (B) a perivascular distribution of inflammatory cell aggregates (arrow); (C) with some endothelial cell staining (arrow); (D) inflammatory cells around muscles; control joint where the primary antibody is omitted is shown in (E). Original magnification $\times 400$ for A, $\times 200$ for B, C; $\times 100$ for D; $\times 50$ for E.

periarticular regions, involving muscles, tendons, and nerves. Similar inflammatory nodules were present in subcutaneous regions (data not shown). Photomicrographs representative of peripheral benzodiazepine receptor ligand-treated and control animals are shown in Fig. 3. Vehicle-treated mice showed synovial hyperplasia (Fig. 3A), infiltration of mononuclear cells into subsynovial tissue (Fig. 3B), pannus formation (Fig. 3C) and bone erosion (Fig. 3D). These scored arthritic signs are given in Table 1. By contrast, mice receiving peripheral benzodiazepine ligand ligands at 3 mg/kg exhibited significantly lower subsynovial inflammation, synovial hyperplasia, pannus formation, and bone destruction. Mononuclear cell infiltration, bone and cartilage pathologies were absent in the peripheral benzodiazepine receptor ligand-treated animals, except for minor thickening of the synovium seen when mice were treated with SSR 180575 (Fig. 3E) (as well as with PK 11195 and Ro5-4864, data not shown). Similar signs were apparent for methotrexate-treated mice (Fig. 3F). The groups that received peripheral benzodiazepine receptor ligands or methotrexate had a significantly lower histopathological score and the incidence of the disease is strongly reduced (Table 1).

3.4. Peripheral benzodiazepine receptor ligands protect against joint destruction

Table 2 shows clear protection against joint destruction, determined by the reduction of the articular surface, when mice were treated with PK 11195, Ro5-4864 and SSR 180575 (3 mg/kg). Cartilage destruction was also markedly reduced following methotrexate treatment (2 mg/kg) (Table 2).

3.5. Characterization of the anti-mouse peripheral benzodiazepine receptor antibody

To investigate peripheral benzodiazepine receptor expression in articular joints, we developed a specific antibody raised against the mouse peripheral benzodiazepine receptor. As shown in Fig. 4, the anti-mouse peripheral benzodiazepine receptor antibody was specific to the immunizing peptide and recognized a single 18 kDa band in mouse Y-1 cell extracts, in complete agreement with the peripheral benzodiazepine receptor molecular weight predicted from its complete cDNA sequence. In addition, the subcellular specificity of the anti-peripheral benzodiazepine receptor antibody was also demonstrated, as illustrated in Fig. 5. Peripheral benzodiazepine receptor-associated immunofluorescence was confined to mitochondria, as demonstrated by a complete overlap with the mitochondrial probe. When the antibody was preincubated with the immunizing peptide,

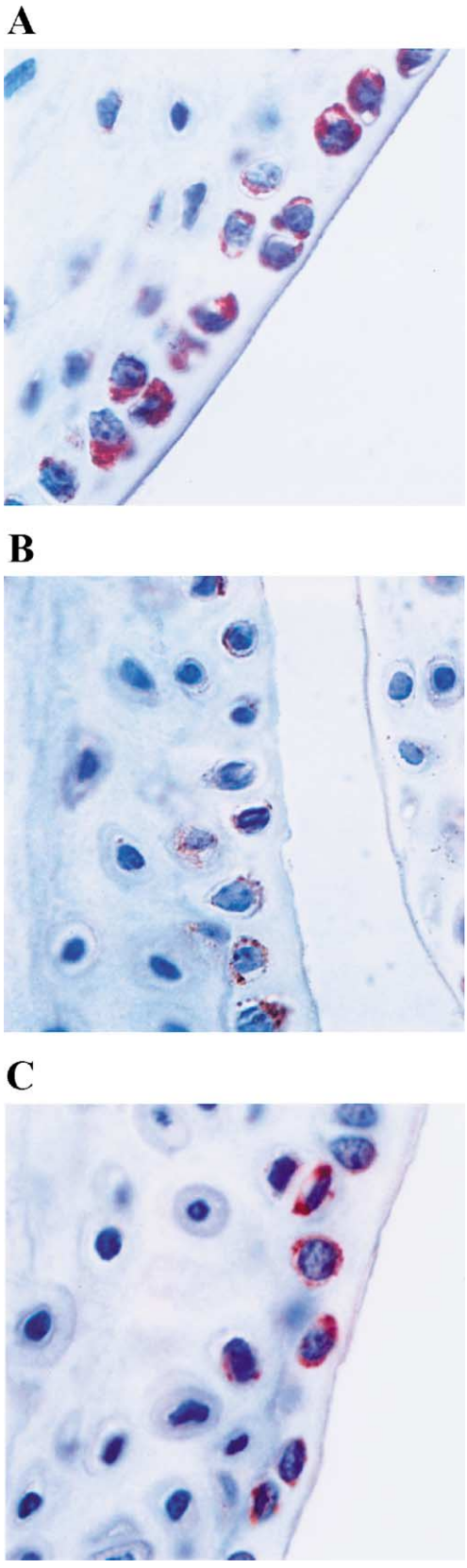

Fig. 7. Peripheral benzodiazepine receptor expression in articular cartilage. The polyclonal anti-mouse peripheral benzodiazepine receptor revealed immunoreactive peripheral benzodiazepine receptor in chondrocytes in 8-week-old Mrl/lpr mice (A). Peripheral benzodiazepine receptor expression was markedly diminished in Mrl/lpr vehicle-treated mice (B). Decreased expression compared with young Mrl/lpr mice was recovered in SSR 180575-treated mice (C).

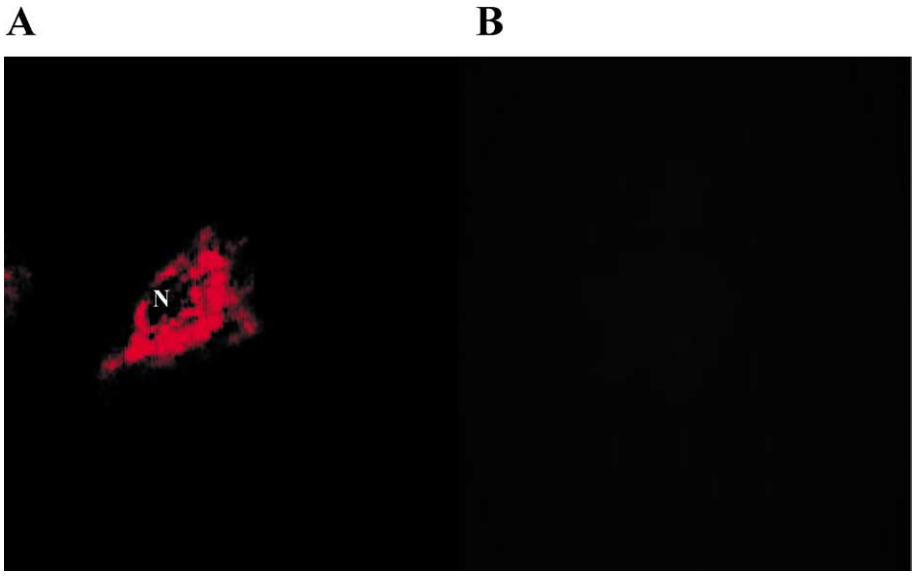

Fig. 8. Peripheral benzodiazepine receptor expression in cultured human chondrocyte cells. In (A), immunoreactive peripheral benzodiazepine receptor. In (B), control where immunizing peptide has been added before anti peripheral benzodiazepine receptor antibody labeling. N: cell nucleus.

no signal was detected in either Western blot or confocal experiments.

3.6. Localization of peripheral benzodiazepine receptor-containing cells in articular joints

The ankle joint of Mrl/lpr mice contained cells positively stained with anti-peripheral benzodiazepine receptor antibodies (Fig. 6). Peripheral benzodiazepine receptor-containing cells were found in the synovial membranes (mainly in the synovial lining layer; Fig. 6A). Most peripheral benzodiazepine receptor-positive cells in the synovial tissue were elongated and spindle-shaped, characteristics of a fibroblast, macrophage or dendritic cell-like morphology. In the subsynovial space composed of lymphocytes, a paucity of plasmocytes, histiocytes and polymorphonuclear neutrophils, we detected peripheral benzodiazepine receptor expression in the cell aggregates (Fig. 6B) and in the perivascular zone. Furthermore, endothelial cells around blood vessels were also stained with anti-peripheral benzodiazepine receptor antibody (Fig. 6C), their peripheral benzodiazepine receptor expression level being particularly high. Inflammatory peripheral benzodiazepine receptor-containing cells were also found around muscle (Fig. 6D), tendons, nerves and in the inflammatory nodules in subcutaneous areas (data not shown). Peripheral benzodiazepine receptor expression was also observed in the pannus at the articular surface (Fig. 6A). Specificity of the labeling was demonstrated by the absence of staining when the primary antibody is omitted. Interestingly, we observed for the first time that chondrocytes were positively stained for peripheral benzodiazepine receptor either in articular chondrocytes in Mrl/lpr mice (Fig. 7) or in human chondrocyte cell line (Fig. 8), and this staining was cytoplasmic. In addition, when comparing the level of peripheral benzodiazepine receptor expression in chondrocytes from non-arthritic young mice and vehicle-treated animals, we observed that the level of peripheral benzodiazepine receptor expression in viable chondrocytes was reduced in mice developing the arthritic pathology compared with non-arthritic mice (Fig. 7). By contrast, when Mrl/lpr mice were treated with SSR 180575, immunoreactive peripheral benzodiazepine receptor levels are restored to a level similar to that observed in young non-arthritis mice (Fig. 7). Similar trends were observed when Mrl/lpr were treated with either Ro5-4864 or PK 11195 but not with methotrexate (data not shown).

4. Discussion

In the present work, we evaluated the effects of three different peripheral benzodiazepine receptor ligands on rheumatoid arthritis. We thus performed clinical, histological and radiological examination of the joints of complete Freund's adjuvant-primed Mrl/lpr treated with either PK 11195, Ro5-4864, SSR 180575 or methotrexate as reference. Our data show that all peripheral benzodiazepine receptor ligands at a dose of 3 mg/kg/day dramatically limited the disease progression, as demonstrated at three different levels: (i) peripheral benzodiazepine receptor ligands delayed and reduced by 50% to 85% the formation of paw oedema and increase in ankle width as assessed using a plethysmometer and a micrometer, respectively; (ii) peripheral benzodiazepine receptor ligands significantly reduced the total histological score to a level similar to that obtained following methotrexate treatment; (iii) radiological analysis of the ankle joint revealed that treatment with peripheral benzodiazepine receptor ligands prevented joint destruction and

limited the reduction of the articular surface, a typical feature of this pathology. Altogether, these results support the findings of previous studies, indicating that PK 11195 was able to inhibit arthritis in the Mrl/lpr (Waterfield et al., 1999), and also demonstrate that SSR 180575 is highly potent at limiting arthritis progression in this mouse model.

Considering the impact of peripheral benzodiazepine receptor ligands on rheumatoid arthritis development in this mouse model, an important question to consider is whether the observed effects are receptor-mediated. Several pieces of evidence argue for a peripheral benzodiazepine receptor-mediated process. First, three ligands whose structures differ but which can all bind the peripheral benzodiazepine receptor are shown here to have similar preventative effects. Secondly, considering the affinity of the three ligands for the peripheral benzodiazepine receptor, the doses we used are within the pharmacological range. For instance, the dose of SSR 180575 used in this study was found to occupy 83% of peripheral benzodiazepine receptors in a peripheral organ like spleen in rat (Ferzaz et al., 2002). Lastly, using a specific polyclonal antibody targeting the mouse peripheral benzodiazepine receptor, we found that the protein was highly expressed in inflamed tissues, strongly indicating that it may play a role in the disease.

The antibody we used was raised against the C terminal end of mouse peripheral benzodiazepine receptor and specifically recognized a single 18 kDa protein in Y-1 cell extracts that showed an exclusive mitochondrial localization. In inflamed tissues, we observed a global increase in peripheral benzodiazepine receptor expression and evidenced that peripheral benzodiazepine receptor-positive cells were located in synovial membranes, in the inflammatory infiltrate and in the cartilage pannus junction. The increase in peripheral benzodiazepine receptor expression in inflamed tissues was mainly due to a florid arrival of inflammatory cells in the articular cavity. Such upregulation of this receptor expression has already been shown in tissue sections and in homogenized membrane binding studies in a variety of immune pathological conditions including multiple sclerosis (Benavides et al., 1988; Vowickel et al., 1997) and arthritis (Scatton et al., 1990). Interestingly, using the new specific antibody, we obtained original findings on peripheral benzodiazepine receptor expression in tissues involved when rheumatoid arthritis develops. Indeed, we show for the first time that the peripheral benzodiazepine receptor is also expressed in chondrocytes. In addition, while a decrease in receptor expression was observed when Mrl/lpr mice developed arthritic symptoms compared to young unaffected control mice, we also showed that when Mrl/lpr mice were treated with peripheral benzodiazepine receptor ligands, their peripheral benzodiazepine receptor expression level in chondrocytes was restored. Such a modulating effect was not observed when mice were treated with methotrexate, further arguing for peripheral benzodiazepine receptor ligand effects being receptor-mediated.

As rheumatoid arthritis results from the interplay of many different effectors, peripheral benzodiazepine receptor ligand preventative effects could possibly be explained by the modulation of various pathways. This modulation can be considered at five levels. First level, as the stimulation of steroid synthesis is one of the best characterized functions of peripheral benzodiazepine receptors, the anti-inflammatory properties exhibited by peripheral benzodiazepine receptor ligands may be steroid mediated. Peripheral benzodiazepine receptor ligands stimulate steroid synthesis in adrenal medulla, placenta, testis, ovaries and nervous cells by enhancing the translocation of cholesterol from the outer to inner mitochondrial membranes (Krueger, 1995). However, if steroid synthesis induction were the only mechanism of action, the anti-inflammatory response of ligands should have not been effective in adrenalectomized mice in which paw oedema formation was induced by carrageenan (Torres et al., 2000). Thus, the anti-inflammatory effects of peripheral benzodiazepine receptor ligands cannot be exclusively explained by the modulation of steroid synthesis.

Second level, we showed that peripheral benzodiazepine receptor expression is downregulated in chondrocytes when rheumatoid arthritis developed. An ultrastructural study in young, disease-free Mrl/lpr mice has shown that chondrocytes contained several mitochondria with well-developed crystae. In older Mrl/lpr mice presenting with rheumatoid arthritis signs, chondrocytes of the articular cartilage diminish in size, their organelle content changes, the numbers of mitochondria per cell decrease, and the mitochondria grow larger (Bartlett et al., 1986). Such an alteration in the mitochondrial size may be indicative of changes in the apoptotic pathway as mitochondria are known to play a critical role in this process. As it has been shown that the peripheral benzodiazepine receptor may modulate apoptosis (Bono et al., 1999), it could be assumed that the increase in peripheral benzodiazepine receptor expression observed in chondrocytes following its ligand treatment may partially account for the preventative action of peripheral benzodiazepine receptor ligands observed on the progressive erosion of cartilage and bone destruction through a peripheral benzodiazepine receptor-mediated modulation of apoptosis in chondrocytes.

Thirdly, the peripheral benzodiazepine receptor anti-inflammatory effect may also be explained by modifications of cytokine levels. Indeed, cartilage destruction is a major characteristic of rheumatoid arthritis and is also linked to aberrant cytokine and growth factor expression in affected tissues (Van der Berg, 1999). Interleukin-1, tumor necrosis factor (TNF-α) and interferon-gamma are known to affect chondrocytes function (Dingle et al., 1987; Ikebe et al., 1988; Jahn et al., 1987), and interleukin-6 has been shown to boost progression from an initial inflammation to a chronic state (De Hoodge et al., 2000). Peripheral benzodiazepine receptor ligands are known to reduce macrophage secretion of interleukin-1, interleukin-6 and TNF-α (Zavala et al., 1990b). Furthermore, Ro5-4864 and PK 11195 dra-

matically reduce both interleukin-6 and interleukin-13 expression in pleural exudation of mice injected with carrageenan (Torres et al., 2000). The presence of peripheral benzodiazepine receptors in chondrocytes, T cells, macrophages, and mesenchymal cells suggest that peripheral benzodiazepine receptor ligands may interfere with the cytokine network and thus modulate inflammatory responses.

Fourth level, peripheral benzodiazepine receptor ligand effects may be explained considering the general immune dysregulation found in the Mrl/lpr mouse. In those mice, the lpr gene which is a deletion mutant of the Fas antigen (Watanabe-Fukunaga et al., 1992) is responsible for the disease onset. The disease phenotype includes formation of multiple autoantibodies and lymphoproliferation with accumulation of large numbers of CD4−CD8 T lymphocytes in the lymph nodes and spleen (Cohen and Eisenberg, 1991). Dysfunctions of these T cells lead to reduced immune responses which presumably underlie the establishment of the disease. In that context, one can assume that peripheral benzodiazepine receptor ligands may affect the disease progression and severity by limiting the overall dysfunction in T cell and antibody responses. The peripheral benzodiazepine receptor ligands have been observed to modulate the humoral response of mice to T-cell-dependent antigens (Zavala et al., 1984; Lenfant et al., 1986). However, at the doses used in this study, no activity of SSR 180575, PK 11195 and Ro5-4864 could be evidenced either in the T-cell-dependent graft vs. host disease model in the F1 hybrid B6D2F1 mice or in an antibody response against immunization of mice with sheep red blood cells, a T-cell-dependent antigen (data not shown). From these experiments, it appears that the three peripheral benzodiazepine receptor ligands do not significantly affect the main immunologic parameters in those mice (data not shown). Further studies are needed to specifically address this issue in the Mrl/lpr mouse model.

Finally, as the peripheral benzodiazepine receptor is highly expressed in monocytes and polymorphonuclear cells, peripheral benzodiazepine receptor ligand effects may also be mediated through modulation of the activity of these cells. In rheumatoid arthritis, large numbers of polymorphonuclear neutrophils are found in the synovial fluid of inflamed joints. As peripheral benzodiazepine receptor ligands have been shown to modulate chemotaxis, the diminished cellular infiltration observed following peripheral benzodiazepine receptor ligand treatment may be due to their inhibitory effect on the attraction, rolling or extravasation of neutrophils in joint tissue. Considering these hypotheses, further studies are warranted to clarify what is the mode of action of these ligand effects in that model.

In summary, we showed for the first time that when rheumatoid arthritis developed, peripheral benzodiazepine receptor was expressed in inflammatory, synovial, and pannus cells and chondrocytes. We also demonstrated that treatment with peripheral benzodiazepine receptor ligands is protected against the massive invasion of inflammatory cells in the joint space and against cartilage and bone destruction, thus limiting rheumatoid arthritis progression and severity. While further arguing for a role of the peripheral benzodiazepine receptor in rheumatoid arthritis development, these data suggest a new therapeutic approach of rheumatoid arthritis.

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