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Pixantrone (BBR2778) Reduces the Severity of Experimental Autoimmune Myasthenia Gravis in Lewis Rats

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Pixantrone (BBR2778) (PIX) and mitoxantrone share the same mechanism of action because both drugs act as DNA intercalants and inhibitors of topoisomerase II. PIX is an interesting candidate immunosuppressant for the treatment of autoimmune diseases because of its reduced cardiotoxicity compared with mitoxantrone. The clinical response to conventional immunosuppressive treatments is poor in some patients affected by myasthenia gravis (MG), and new but well-tolerated drugs are needed for treatment-resistant MG. PIX was tested in vitro on rat T cell lines specific for the immunodominant peptide 97–116 derived from rat acetylcholine receptor (AChR), and showed strong antiproliferative activity in the nanomolar range. We demonstrate in this study that PIX administration reduced the severity of experimental autoimmune MG in Lewis rats. Biological and immunological analysis confirmed the effect of PIX, compared with vehicle-treated as well as mitoxantrone-treated experimental autoimmune MG rats. Anti-rat AChR Abs were significantly reduced in PIX-treated rats, and AChR content in muscles were found increased. Torpedo AChR-induced T cell proliferation tests were found reduced in both in vitro and ex vivo experiments. The effectiveness and the reduced cardiotoxicity make PIX a promising immunosuppressant agent suitable for clinical investigation in MG, although additional experiments are needed to confirm its safety profile in prolonged treatments. The Journal of Immunology, 2008, 180: 2696–2703.

The antineoplastic drug pixantrone (PIX), also known as BBR2778, is structurally related to mitoxantrone (MTX), which is devoid of toxic effects on cardiac tissue (1). PIX and MTX share the same mechanism of action because both drugs interact with DNA as intercalants and with topoisomerase II as inhibitors. Preclinical studies demonstrated that PIX, a cytostatic drug, reduced the severity of acute experimental allergic encephalomyelitis (EAE) and decreased the relapse rate of chronic EAE in rats (2, 3).

Experimental autoimmune myasthenia gravis (EAMG) is the animal model of human myasthenia gravis (MG) (4, 5); this model is characterized by the presence of Th cells and autoantibodies (IgG type) specific for the acetylcholine receptor (AChR). EAMG is routinely induced in susceptible rat and mouse strains by immunization with Torpedo AChR (TACr), and a proportion of anti-TACr Abs cross-react with self-AChR on muscle. EAMG mimics MG in its clinical and immunological manifestations and is considered a reliable model to investigate therapeutic strategies for treatment of the human disease.

In the present study we evaluated the immunomodulatory potential of PIX in EAMG. In particular, we studied the ability of PIX to modulate Ag-specific T cell responses in vitro vs peptide 97–116, an immunodominant and myasthenogenic T cell epitope of the rat AChR α subunit (6), and the effects on immune cell function in the spleen and lymph nodes of PIX-treated rats after TACr immunization. Finally, we tested the ability of PIX to modify the course of rat EAMG.

Our experimental data showed that PIX inhibited Ag-specific T cell responses in vitro and modified the course of EAMG in Lewis rats, making PIX a promising immunosuppressant agent suitable for investigation in the human disease.

Materials and Methods

Animals

Female Lewis rats, 6–8 wk of age, were purchased from Charles River Breeding Laboratories (Calco) and kept at the animal facility of the Neurological Institute Foundation “Carlo Besta” (Milan, Italy). The experimental plan was approved by the Ethical Committee of the Institute and was performed in accordance with the Principles of Laboratory Animal Care (European Communities Council Directive 86/609/EEC).

Ag production

AChR was purified from Torpedo californica electroplax tissue (Aquatic Research Consultants) by affinity chromatography on Naja-naja siamensis toxin (Sigma-Aldrich) coupled to Sepharose 4B (Amersham Biosciences) (7). The rat 97–116 peptide (DGDFAIKFTKVLDDYTGHI) and Torpedo 97–116 peptide (DGDFAVHMTKLDDLTYGTKI) were synthesized according to GenBank published sequences X74832 for rat AChR α subunit and J00963 for TACr α subunit. Peptides were synthesized using F-moc chemistry on a 431A automated peptide synthesizer (PE Applied Biosystems) and purified by reversed phase HPLC; their synthesis was confirmed by mass spectroscopy.

Immunization and treatment protocols

A total of 70 female Lewis rats (6–8 wk; Charles River Breeding Laboratories) was used in this study. EAMG was induced by immunization in the hind footpads with 50 μg of purified TACr in CFA (Difco Laboratories), further supplemented with 1 mg of H37Ra/rat (Difco Laboratories), as described (8).
For the studies on PIX efficacy on EAMG, TACRh-immunized rats were randomly assigned to different treatment groups: 1) preventive PIX group, starting 4 days after immunization, with 16.25 mg/kg PIX, administered i.v. via tail vein, once a week for three times; 2) therapeutic PIX group, starting 4 wk after immunization, with 16.25 mg/kg PIX, administered i.v. via tail vein, once a week for three times; 3) therapeutic MTX group (1.2 mg/kg, i.v. via tail vein, once a week for three times); and 4) vehicle group (sterile saline, i.v. via tail vein, once a week for three times). Treatments were given as reported for the doses of EAE model (2). The doses of PIX and MTX used in this study were in both cases equal to one-fourth of the LD₅₀, for single i.v. injection in rats. This correspondence was chosen to compare drug effect and the cardiotoxicity of the two compounds (9). Treatment assignment was performed at day 4 after TACRh immunization (preventive schedule) in coincidence of the acute phase of EAMG, or at onset of clinical signs (therapeutic schedule), which occurs after 4 wk. PIX was obtained from Cell Therapeutics Europe, while commercially available MTX (Segix Italia) was used for the animal treatment. Animals were sacrificed after deep anesthesia obtained by carbon dioxide; low-grade anesthesia with chloral hydrate administered i.p. was used for TACRh immunization and drug treatments. Cardiotoxicity of PIX was evaluated by histopathological examination as described (2). Briefly, hearts were quickly removed and fixed in 10% neutral-buffered formalin (pH 7). Tissue samples were processed, embedded in paraffin, sectioned (4μm thick), and stained with H&E. The 10 sections for each heart were evaluated for the presence of degenerative myocardiopathy, interstitial fibrosis, and interstitial lymphocytes. The histopathological evaluation of the hearts was performed using a scoring system for cardiomyopathy (score 1 or 2) and the extent of the damage (score from 0 to 5) (2). 10).

**Effects of PIX on AChR-specific T cell line proliferative responses in vitro**

T cell lines were grown from Lewis rats (n = 2) immunized with 50 μg of peptide 97–116 of the rat AChR α subunit in CFA. Lymph nodes were aseptically removed 10 days after immunization and processed into a single-cell suspension. Lymph node cells (LNCs) were cultured in RPMI 1640 medium plus 10% FCS, 1% pyruvate, 1% nonessential amino acids, 1% L-glutamine, 1% penicillin-streptomycin (Euroclone Celbio), and 2 × 10⁻³ M 2-ME (BDH) and stimulated with 5 μg/ml rat 97–116 peptide. T cell lines were maintained by restimulation with the appropriate peptide every 15 days, and expanded with IL-2 every 3–4 days thereafter. For proliferation assays, 3 × 10⁴ T cells were cocultured in triplicates with 2 × 10⁵ irradiated splenic cells per well plus rat 97–116 peptide (5 μg/ml) in RPMI 1640 medium with 1% normal rat serum. Con A (Sigma-Aldrich) was used at 2 μg/ml as positive control. Increasing concentrations of PIX (0.01–10 μM) were added to each culture well. After 72 h, 0.5 μCi [³H]thymidine (GE Healthcare Life Sciences) was added to each well. After a further 16 h, the plates were harvested and counted on a Wallac MicroBeta TriLux counter (PerkinElmer). Results were expressed as mean cpm of triplicate cultures.

**Effects of PIX on ex vivo proliferative responses to TACRh**

Popliteal and inguinal lymph nodes were aseptically removed from TACRh-immunized rats (n = 8) 28 days after immunization and processed into a single-cell suspension. T cells (2 × 10⁵) were plated in triplicate in 96-well culture plates (Costar) in RPMI 1640 medium (Euroclone Celbio) plus 1% normal rat serum with TACRh (1.25–0.25 μg/ml) and with Torpedo or rat 97–116 peptide (10 μg/ml); Con A (Sigma-Aldrich) was used at 2 μg/ml as positive control. After 72 h of culture 0.5 μCi of [³H]thymidine was added to each well and the plates were harvested after an additional 16–18 h. Results were expressed as cpm ± SE of triplicate cultures.

**Ex vivo FACS analysis of T cell subpopulations following PIX treatment**

Spleen and LNC suspensions were prepared from aseptically removed organs collected from vehicle-treated (n = 4 rats) and PIX-treated (n = 4 rats) groups, at day 28 p.i. and from healthy control (n = 4 rats). Cell numbers were counted by trypan blue exclusion dye; total numbers of cells were calculated by correcting cell counts to the initial spleen (10 ml) and lymph node (2.5 ml) solution volumes. For FACS staining, cells were resuspended in PBS, 1% FCS, 0.1% sodium azide (FACS buffer). A total of 3 × 10⁵ cells in 50 μl were stained with FITC- or PE-conjugated monoclonal antibodies Abs to CD3 (clone G4.18; BD Biosciences Pharmingen), to CD4 (clone OX-35; BD Biosciences Pharmingen), and to CD8 (clone OX-8, BD Biosciences Pharmingen). After 30 min incubation on ice, the samples were pelleted (200 × g), washed three times with cold FACS buffer, and analyzed immediately in a FACSScan flow cytometer (BD Biosciences) equipped with CellQuest Pro software (BD Biosciences). At least 20,000 events were acquired for each sample. Nonviable cells were excluded from analysis. 

**FIGURE 1.** Effect of PIX on R97–116 T cell line. CD4⁺ T cell line specific for peptide 97–116 of the rat AChR α subunit was challenged in vitro with the specific peptide (5 μg/ml) (A) and with Con A as positive control (2 μg/ml) (B) for 3 days in culture medium or in the presence of an increasing amount of PIX (range 0.01–10 μM). The 0.5 μCi of [³H]thymidine was added for further 18 h. Data are expressed as mean cpm ± SE from triplicate wells on a y-axis logarithmic scale.

**FIGURE 2.** Efficacy of PIX treatment in modulating LNC responses from TACRh-immunized Lewis rats. Proliferative responses against TACRh and peptides 97–116 from TACRh and rat AChR α subunit were studied on LNCs from PIX-treated rats (16.25 mg/kg i.v. administered once weekly for three times, n = 4) (■) and vehicle-treated rats (n = 4) (□) at day 28 after a single TACRh immunization in CFA. Popliteal and inguinal LNCs were processed into a single-cell suspension and challenged in vitro with TACRh (1.25 and 0.25 μg/ml) and 97–116 peptides (10 μg/ml); Con A (2 μg/ml) was used as positive control. After 3 days of culture, 0.5 μCi of [³H]thymidine was added for a further 18 h. Data are expressed as mean cpm ± SE (from triplicate wells). The values were mean cpm ± SE for control wells (medium alone) (64.5 ± 8.4 PIX group and 1080.3 ± 183.0 vehicle group). * p < 0.05.
by physical gating. Aspecific staining was determined after incubation of cells with FITC plus PE-conjugated isotype control IgG1/IgG2a (BD Biosciences). Data are expressed as a percentage of positive cells on forward and side light scatter gated cell population.

EAMG clinical evaluation

Evaluation of disease manifestations in TACHR-immunized rats was performed by testing muscular weakness. Clinical scoring was based on the presence of tremor, hunched posture, muscle strength, and fatigability. Fatigability was assessed after exercise (repetitive paw grips on the cage grid) for 30 s. Disease severity was graded as follows: grade 0, normal strength and no fatigability; grade 1, mildly decreased activity and weak grip or cry; grade 2, clinical signs present at rest; grade 3, severe clinical signs at rest, no grip, moribund; and grade 4, dead. Each animal was weighed and evaluated at the beginning of each experiment and twice weekly; EAMG was confirmed by Edrophonium chloride test. Results are expressed as the mean of the evaluations for each animal at each time point.

AChR content in muscle

AChR content in muscles was assayed as previously described (11). Briefly, AChR was solubilized from muscle membranes with Tris-HCl buffer, 2% Triton X-100, overnight at 4°C, and the solutions containing solubilized AChR clarified by centrifugation at 100,000 x g for 30 min. AChR crude extracts (100 μl, duplicates) were incubated with [125I]-bungarotoxin (α-BTX; GE Healthcare Life Sciences) at room temperature, transferred on DE-81 DEAE disks (Whatman International) and washed with Tris-HCl buffer 0.5% Triton X-100. Radioactivity was determined by gamma counting. The aspecific binding was subtracted from each sample by parallel tubes preincubated with cold α-BTX (Sigma-Aldrich). The results were expressed as femtomoles of α-BTX binding sites per gram of muscle.

Anti-AChR Abs assay

Anti-rat AChR Abs were assayed in individual sera by conventional radioimmunoprecipitation (12). Briefly, rat AChR was extracted from denervated rat muscle as previously described (11). Briefly, rat AChR was solubilized from muscle membranes with Tris-HCl buffer, 2% Triton X-100, overnight at 4°C, and the solutions containing solubilized AChR clarified by centrifugation at 100,000 x g for 30 min. AChR crude extracts (100 μl, duplicates) were incubated with [125I]-α-bungarotoxin (α-BTX; GE Healthcare Life Sciences) at room temperature, transferred on DE-81 DEAE disks (Whatman International) and washed with Tris-HCl buffer 0.5% Triton X-100. Radioactivity was determined by gamma counting. The aspecific binding was subtracted from each sample by parallel tubes preincubated with cold α-BTX (Sigma-Aldrich). The results were expressed as femtomoles of α-BTX binding sites per gram of muscle.

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FIGURE 3. Flow cytometric analysis of T cell subpopulations in TACHR-immunized Lewis rats treated with PIX. Spleens and popliteal/inguinal lymph nodes were aseptically removed at day 28 postimmunization with TACHR and processed into single-cell suspensions; cells were counted by trypan blue staining. Total cell numbers of splenic cells (A) and LNCs (E) are reported as mean ± SE from healthy control (n = 4) (□), vehicle-treated animals (sterile saline i.v. once a week for three times, n = 4) (○), and PIX-treated animals (16.25 mg/kg i.v. once weekly for three times, n = 4) (●) groups. FACS analysis of T cell population (by CD3 staining) and of CD4+ and CD8+ subsets are expressed as mean percentage ± SE in B, C, and D for spleens and in F, G, and H for lymph nodes. *, p < 0.05; **, p < 0.001.
FIGURE 4. Comparison of the preventive and therapeutic PIX treatments on EAMG manifestation. Clinical score (A and C) and body weight (B and D) were recorded in EAMG rats twice weekly until day 64. For the preventive protocol, 4 days after TACrR immunization the animals (n = 18) were randomly assigned to PIX group (16.25 mg/kg i.v. once weekly for three times) or vehicle group (sterile saline i.v. once weekly for three times) (B in A and B). For the therapeutic protocol, rats were randomly assigned to PIX group (n = 6) (filled squares in C and D) or to vehicle group (sterile saline i.v. once weekly for three times) (open squares in C and D) 4 wk postimmunization with TACrR. Treatment schedule was identical as to the preventive protocol (sterile saline or PIX 16.25 mg/kg i.v. once weekly for three times). The arrow indicates the time for the first of the three injections for each protocol.

Statistical analysis

ANOVA was performed to assess statistical significance of results. Differences were considered significant when p < 0.05. Statview 5 for Macintosh (Abacus Concepts) and GraphPad Prism version 4.0 for Macintosh (GraphPad Software) programs were used for data elaboration.

Results

In vitro effect of PIX on rat-specific 97–116 T cell line proliferation

The 97–116 peptide of the rat AChR α subunit is a T cell immunodominant and myasthenogenic epitope (6). T cell lines grown from rat 97–116 peptide-immunized rats (n = 2) were challenged with 5 μg/ml specific peptide and 2 μg/ml Con A in a T cell proliferation test by [3H]thymidine incorporation in the presence of increased amounts of PIX (0.01–10 μM).

Results reported in Fig. 1A show that PIX was active in the nanomolar range, inducing 39.3% inhibition of rat 97–116 peptide-specific T cell proliferation at 0.01 μM (mean cpm ± SE 51981.0 ± 140.6 cpm with PIX 0.01 μM vs 85678.0 ± 8153.6 cpm for control culture without PIX); increased levels of PIX completely suppressed T cells proliferation. Not only Ag-specific responses but also Con A-induced T cell responses were drastically inhibited by PIX (Fig. 1B).

PIX treatment influenced LNC TACrR-specific ex vivo proliferation

We have then evaluated the ability of PIX treatment to modify ex vivo lymphocytes proliferation. Treatment protocol has been adapted from (2), with three drug administrations once weekly.

Lymph nodes were aseptically removed from TACrR-immunized rats, treated with PIX (16.25 mg/kg, once a week for three times, n = 4 rats) or vehicle (n = 4 rats). LNCs were challenged with TACrR (1.25 and 0.25 μg/ml), Torpedo 97–116 peptide (10 μg/ml), and rat 97–116 peptide (10 μg/ml). Con A (2 μg/ml) was used as positive control. As shown in Fig. 2, PIX treatment in TACrR-immunized rats abrogated LNC TACrR-specific proliferation (TACrR concentration 1.25 μg/ml, mean cpm ± SE 1432.2 ± 148.4 cpm; TACrR concentration 0.25 μg/ml, mean cpm ± SE 243.2 ± 89.4 cpm) compared with the strong proliferative response observed in the vehicle-treated rat group (TACrR concentration 1.25 μg/ml, mean cpm ± SE 29381.5 ± 5603.7 cpm; TACrR concentration 0.25 μg/ml, mean cpm ± SE 15251.6 ± 3502.5 cpm). LNCs were also challenged with Torpedo 97–116 peptide and rat 97–116 peptide not used for immunization; recognition of both peptides in the PIX-treated group compared with the vehicle-treated group was again abrogated (Torpedo peptide: mean cpm ± SE 270.4 ± 61.5 cpm for PIX, 24802.9 ± 3101.3 cpm for vehicle; rat peptide: mean cpm ± SE 226.4 ± 52.1 cpm for PIX, 17955.7 ± 4088.4 cpm for vehicle). Also mitogen-induced proliferation was affected by PIX treatment (mean cpm ± SE 35800.8 ± 6288.6 cpm for PIX; 64187.6 ± 4295.8 cpm for vehicle). The pathological examination of hearts collected from PIX-treated and vehicle-treated rats did not show signs of cardiotoxic damage (data not shown).

FACS analysis of lymphocyte subpopulations

Previous experiments showed that PIX treatment modulated ex vivo proliferative responses of LNCs to AChR as well as to AChR-derived immunodominant peptides. We then evaluated PIX effect on spleen- and lymph node-derived immune cell subsets and compared with vehicle-treated rats and healthy rats. No differences were observed in splenic cell counts between healthy rats (n = 4) and TACrR-immunized rats (n = 4) (mean cell number ± SE 60.75 ± 1.49 × 10⁶ vs 61.75 ± 4.25 × 10⁶) (Fig. 3A). On the contrary, the total splenic cell count decreased in the PIX-treated group (n = 4 rats) (mean cell number ± SE 37.13 ± 9.07 × 10⁶;
FIGURE 5. Immunological evaluation of the preventive and therapeutic PIX treatments in EAMG rats. A, Total AChR content in skeletal muscles was measured in vehicle-treated EAMG rats (n = 6) ( ), in PIX-treated EAMG rats, preventive schedule (n = 6) ( ), and therapeutic schedule (n = 6) ( ). Muscle AChR content in normal healthy Lewis rats is also depicted (n = 4) ( ). AChR was evaluated by conventional radioimmunological assay with [125I]α-BTX from Torpedo X-100 solubilized muscle extract. Data are expressed as fmol/g muscle AChR contents (mean ± SE). B, Anti-rat AChR Abs were measured by conventional radioimmunoprecipitation assay in serum samples collected at the end of the experiment. Circulating anti-rat AChR Abs (IgG type) have been evaluated in individual samples and are expressed as mean ± SE anti-rat AChR Ab titers (pmol/ml) for treatment groups: vehicle-treated EAMG rats ( ); preventive PIX schedule in EAMG rats ( ); and therapeutic PIX schedule in EAMG rats ( ). Healthy normal rats were used as control ( ). C, Pooled and inguinal LNs from EAMG rats were aseptically removed at the end of the experiment and processed into a single-cell suspension. LNCs were challenged in vitro with TAChR (0.25 g/ml) or with Con A (2 µg/ml) for 3 days, followed by [3H]thymidine on overnight pulse. Data are expressed as mean cpm ± SE, from triplicate wells for each group: PIX preventive schedule ( ); PIX therapeutic schedule ( ); and sterile saline-treated EAMG rats ( ). Mean cpm ± SE for control wells (medium alone) were: 2581.3 ± 550.6 PIX preventive schedule; 5492.1 ± 4507.4 PIX therapeutic schedule; 3642.6 ± 380.6 sterile saline-treated EAMG.

Comparison of preventive and therapeutic effect of PIX in EAMG rats

EAMG was induced in Lewis rats (n = 18) by a single immunization with purified TAChR from Torpedo electricplax (50 µg) in CFA, evaluated by assessing body weight and clinical score, and confirmed by Edrophonium chloride test. The onset and progression of the disease are shown in Fig. 4. EAMG manifestations became evident at 4–6 wk after immunization. Two different treatment protocols were compared: 1) preventive treatment, starting 4 days after immunization (n = 6 rats) (Fig. 4, A and B, arrow) and 2) therapeutic treatment, starting 4 wk after immunization (n = 6 rats) (Fig. 4, C and D, arrow). Six animals received vehicle. Both treatment schedules modified the course of the disease in rats treated (Fig. 4, filled squares), as compared with vehicle-treated EAMG rats (Fig. 4, open squares). The positive effect of both treatment protocols on the progression of the clinical score in vehicle- and PIX-treated EAMG rats is shown in Fig. 4, A and C (mean clinical score ± SE at the end of the experiment: vehicle group, 2.4 ± 0.7; preventive group, 1.1 ± 0.1; therapeutic group, 0.9 ± 0.1). Mean weight ± SE (gram) of rats in the different treatment groups were: vehicle group, 172.4 ± 7.6 g; preventive group, 193.8 ± 2.4 g; therapeutic group 194.3 ± 4.5 g, showing that PIX-treated rats were able to gain more weight than the EAMG group.

To further confirm the efficacy of PIX, we evaluated skeletal muscles AChR content (Fig. 5A), circulating pathogenic Abs against rat AChR (Fig. 5B) and proliferative LNCs responses to TAChR (Fig. 5C). Vehicle-treated EAMG rats had significantly reduced muscle AChR content compared with normal rats (fmol/g muscle ± SE 57.5 ± 21.7 vehicle group vs 186.7 ± 20.3 normal rats), whereas both preventive and therapeutic PIX-treated EAMG rats showed an increased muscle AChR content (fmol/g muscle ± SE 112.0 ± 5.8 preventive group vs 98.3 ± 17.6 therapeutic group). Mean anti-rat AChR Abs titers, measured in the sera collected at the end of the experiment, were significantly different among experimental groups (normal rats 0.07 ± 0.01; vehicle-treated EAMG rats 25.74 ± 7.75; preventive PIX group 0.46 ± 0.17; therapeutic PIX group 5.81 ± 3.93; all values are

p < 0.05) compared with the vehicle group (39.8% reduction). In draining lymph nodes, PIX effect was more dramatic, whereas TAChR/CFA immunization (in the hind footpads) induced an increase of the total number of inguinal and popliteal LNCs compared with nonimmunized rats (mean cell number ± SE 72.75 ± 9.20 × 10⁶ vs 5.67 ± 0.54 × 10⁶) (Fig. 3E). In PIX-treated TAChR/CFA-immunized rats, we observed a significant reduction of total LNC counts (mean cell number ± SE 16.50 ± 5.32 × 10⁶, p < 0.001 vs vehicle group) in comparison with vehicle-treated rats (77.3% reduction). We then analyzed the expression of CD3, CD4, and CD8 T cell markers by FACS analysis on both spleen cell population (Fig. 3, B–D) and LNC population (Fig. 3, F–H), and the analysis showed no significant differences in the percentage of expression for these T cell subsets among living (gated) cells between vehicle- and PIX-treated groups.
expressed as pmol/ml serum ± SE) (Fig. 5B). Proliferative LNC responses to TAChR (0.25 μg/ml) were affected by both PIX treatments (Fig. 5C), and a significant reduction was observed with the therapeutic schedule (mean cpm ± SE in vehicle-treated, 135943 ± 21444; preventive PIX, 110760 ± 13972; therapeutic PIX 72892 ± 10450, p < 0.05 vs vehicle-treated group). PIX did
not affect Con A-induced LNC response. The effect of therapeutic PIX treatment was then repeated; EAMG rats (n = 14) were randomly treated 4 wk after immunization with PIX (n = 7) or with placebo (n = 7), and EAMG score and weight were monitored. At the end of the experiments, rats were sacrificed, and muscle AChR content, anti-AChR Ab levels in the blood, and proliferative LNC responses were tested. These results further confirm our observations on the therapeutic use of PIX in modulating clinical manifestations of EAMG.

Comparison of PIX vs MTX treatment in EAMG rats

Next, we evaluated the efficacy of PIX compared with MTX; 24 Lewis rats were immunized with TAChR/CFA and treatments with both drugs (PIX group n = 8 animals, MTX group n = 8 animals) started 4 wk after TAChR immunization (Fig. 6). Eight animals received placebo. The doses of PIX and MTX used in this study were in both cases equal to one-fourth of the LD50 for single i.v. injection in rats and this correspondence was chosen to compare drug effect and the cardiotoxicity of the two compounds (9). During the observation period we found that both PIX and MTX treatments improved EAMG symptoms as compared with vehicle-treated EAMG rats. Fig. 6 shows the progression of the disease expressed as mean clinical score (Fig. 6, A and C) and body weight loss (Fig. 6, B and D). PIX and MTX reduced body weight loss (mean weight ± SE at the end of experiment 205.4 ± 6.1 PIX group; 189.0 ± 10.4 MTX group; 173.6 ± 12.1 vehicle group) and improved clinical scores in treated vs EAMG untreated rats (mean clinical score ± SE at the end of experiment 0.94 ± 0.11 PIX group; 1.75 ± 0.49 MTX group; 2.50 ± 0.57 vehicle group).

The mean muscle AChR content (fmol/g muscle ± SE) measured in the three treatment groups is reported in Fig. 7A. AChR content was significantly reduced in EAMG rats compared with PIX- and MTX-treated animals (178.6 ± 20.2 PIX; 136.3 ± 10.7 MTX; 101.3 ± 14.3 vehicle; 330.0 ± 20.8 normal rats). Anti-rat AChR Ab titers (pmol/ml serum ± SE) measured at the end of the experiment paralleled our previous findings (Fig. 7B), being more elevated in untreated rats (26.10 ± 3.55) compared with treated animals (10.91 ± 2.30 PIX; 4.63 ± 1.32 MTX; 0.07 ± 0.01 normal rats).

To further investigate the effect of PIX vs MTX treatment, we analyzed the ex vivo proliferative response of LNCs to TAChR and Con A as polyclonal stimulator. Lymphocytes were isolated from lymph nodes of EAMG animals, treated with vehicle (saline solution), PIX, or MTX. As shown in Fig. 7C, TAChR-specific proliferation was statistically reduced in treated groups (particularly with MTX) (mean cpm ± SE 34132.9 ± 8403.3 for PIX; 4175.3 ± 1313.9 for MTX; 66231.2 ± 20737.9 for vehicle). In contrast, no differences were observed in Con A-stimulated cells (mean cpm ± SE 32047.0 ± 6404.1 PIX; 32195.7 ± 6979.4 MTX; 25126.7 ± 5569.4 vehicle).

Discussion

Therapeutic options for MG include corticosteroids and immuno-suppressive drugs, pharmacological agents exerting a nonspecific generalized immune dysfunction (5, 13–15). These drugs are effective in a large proportion of patients. However, the clinical response to conventional treatments is poor in some patients, or the severity of side effects is a limiting factor to their prolonged administration. In this regard, new potent but well-tolerated immunosuppressants are needed for treatment-resistant MG, for patients with intolerable side effects or with major contraindications to high-dose corticosteroid treatment.

MTX is a synthetic anthracenedione antineoplastic compound, initially used for the treatment of adult acute myeloid leukemia, more recently approved by FDA for use in specific forms of multiple sclerosis (16–20). MTX has a broad range of effects on the immune system because it is able to suppress proliferation of T cells, B cells, and macrophages and impairs Ag presentation mainly by the induction of apoptosis in APCs. Moreover, experimental studies showed that MTX suppresses B cell function and Ab production and reduces the release of proinflammatory cytokines. Such a wide range of effects might be useful to control the autoimmune attack in patients with MG, a T cell-dependent, B cell-mediated disease. However, the major limitation to the use of MTX in MG as well as in other autoimmune disorders is cardiotoxicity resulting in congestive heart failure, with a risk increasing with cumulative doses above 100 mg/m² (21–23). The clinical experience with MTX from clinical trials in MS suggested that the incidence of symptomatic heart failure is low, but asymptomatic reduction of left ventricular ejection fraction might be more common (20, 22, 23). Moreover, little data are yet available regarding the potential late cardiac toxicity as observed in cancer series, as well as the risk of developing leukemia (24).

PIX, structurally related to MTX, has similar mechanism of actions, similar antineoplastic activity but less cardiotoxicity. The effect of PIX has been evaluated in the animal model of multiple sclerosis (EAE). Experimental studies showed that PIX can prevent the onset of EAE in SJL mice but is also able to modify the course of the disease in rats once the disease is established (2, 3). These effects have been attributed to the long-lasting effect of PIX on several lymphocyte subpopulations and on Ag presentation. The observed effect was dose-dependent, and no signs of cardiotoxicity compared with MTX were observed, at least in the reported experimental settings. Interestingly, the B cell response to myelin basic protein was also inhibited, providing a further mechanism of action to explain the reduced demyelination.

The effect of PIX has never been investigated in EAMG, the animal model of MG. EAMG can be induced in Lewis rats by immunization with purified TAChR or with the immunodominant epitope 97–116 of the rat AChR α subunit (6, 25). In the present study we investigated the potential immunomodulatory effect of PIX in TAChR-induced model of EAMG. We first addressed the issue of PIX effect on the in vitro proliferative responses of a T cell line specific for peptide 97–116 (6). PIX inhibited T cell responses in a dose-dependent fashion up to the complete suppression of the proliferation (Fig. 1). Next, we studied the ex vivo response of LNCs from PIX-treated TAChR-immunized rats, showing that not only TAChR-specific T cell proliferation was markedly inhibited, but also LNC response to immunodominant epitope 97–116, not used for rat immunization (Fig. 2). The total number of spleen cells and peripheral LNCs was markedly reduced by PIX, without a differential effect on CD3⁺, CD4⁺, and CD8⁺ subsets as shown by FACS analysis (Fig. 3). This finding is similar to that reported in EAE on the effect of PIX on circulating lymphocytes. Circulating CD45RA⁺ B cells were also markedly reduced in the EAE model treated with PIX. As far as cardiotoxicity is concerned, we performed a limited histopathological assessment on hearts from PIX- and vehicle-treated animals, without detecting any signs of damage to cardiomyocytes. We did not analyze cardiotoxicity in PIX- vs MTX-treated rats; however, the lack of cardiotoxicity of PIX has been already reported (2, 9, 10).

The inhibitory effect of PIX on immunological parameters in vitro was paralleled by improvement of EAMG clinical findings. The effect of PIX was investigated both on a preventive or a therapeutic schedule: in both conditions, PIX modified the course of the disease as shown by the positive trend of clinical scores in treated rats, as well as by the serial assessment of body weight (Fig. 4). The clinical effect was associated with a marked reduction...
of specific anti-rat AChR Abs in animals treated with both protocols, together with a protective effect on neuromuscular end-plates as indicated by the assays of rat muscle AChR content (Fig. 5); proliferative responses to TACrH were also reduced in PIX-treated EAMG rats. Even though we did not analyze the effect of PIX on CD45RA+ subset of cells, our data on specific Abs and muscle AChR content confirm the effect of PIX at the B cell level. These findings were replicated in a further experiment in which the effect of PIX was compared with that of MTX, given according to the therapeutic protocol. Both drugs improved the clinical manifestations of the disease, with a more pronounced effect of PIX compared with MTX (Fig. 6), and the clinical effect was associated with a reduction of circulating IgG anti-rat AChR, together with an increase of rat muscle AChR content (Fig. 7). MTX treatment was more effective than PIX on ex vivo responses to TACrH (Fig. 7C).

Further studies will address whether PIX treatment in vivo will modify cytokine/chemokine production by T cells in response to AChr or to polyclonal stimuli ex vivo or in vitro.

On the basis of our results we think that PIX can be a promising immunomodulatory agent suitable for clinical investigation in MG due to its combined effect at both T cell and B cell level. Patients with severe forms of the disease or with major side effects or contraindications to corticosteroids are the principal candidates. A further important feature of PIX is its administration schedule every 3–4 wk, thus limiting the severity of side effects ensuing with daily administration and increasing tolerability. Clinical trials are awaited to evaluate PIX efficacy for the treatment of MG, especially in case of failure of conventional immunosuppressive treatments.

Disclosures

Gabriella Pezzoni is employed by Cell Therapeutics Europe (Bresso, Milan, Italy), which supplied Pixantrone for this study. All other authors have no financial conflict of interest.

References