Rheumatoid arthritis (RA) is a common inflammatory and most often destructive polyarticular disease with a reported prevalence of around 1%. The model of collagen-induced arthritis (CIA) has been extensively used to elucidate the pathogenic mechanisms relevant to human RA and is widely used for the evaluation of potential anti-rheumatic agents. Etoposide and mitoxantrone are immunosuppressive drugs, both acting by inhibiting the topoisomerase II function. We have previously demonstrated an ameliorating effect of etoposide in CIA. The aims of this study were (1) to assess the optimal ameliorating dose of etoposide and (2) to ascertain that topoisomerase II inhibition, irrespective of the chemical composition of the drug, affects the course of autoimmunity.

Methods. Male DBA/1 mice were treated with 12.5 mg/kg body weight of etoposide five times, twice, once per week or once every second week. Mitoxantrone was administered as high dose (1 mg/kg body weight five times after immunization or after booster with collagen II) or low dose (3 μg/mouse, 5 days/week starting after collagen II immunization or after booster).

Results. Treatment with 12.5 mg/kg body weight five times or twice weekly with etoposide completely inhibited development of arthritis. Low-dose treatment with mitoxantrone after collagen II immunization or high-dose treatment after collagen II booster delayed the onset of arthritis. These results were observed clinically as well as histologically. In addition, serum levels of anti-collagen II antibodies were significantly lower in mice displaying less severe arthritis.

Conclusion. Treatment of collagen-induced arthritis with topoisomerase II inhibitors ameliorates the development of disease.

The results demonstrate a dose-dependent amelioration of clinical and histological arthritis in mice treated with etoposide. In addition, treatment with mitoxantrone affected in a dose- and time-dependent way the development of arthritis. Thus, our results indicate that the biological action of mitoxantrone and etoposide (i.e. topoisomerase II inhibition) rather than some other properties in these two chemically different molecules, lead to alleviation of chronic arthritis.

Materials and methods

Mice

DBA/1 male mice, 7 weeks old, were purchased from Taconic M&B A/S, (Ry, Denmark). The experiments were performed with the approval of the Ethical Committee of Göteborg University.

Induction of arthritis and treatment with etoposide and mitoxantrone

Chicken collagen II (CII) (Sigma Chemical Co., St Louis, MO) was dissolved at a concentration of 2 mg/ml in 0.1 M acetic acid and stored at 4°C. Arthritis was induced by intradermal injection at the base of the tail with 100 μl of 100 μg chick CII emulsified in an equal volume of incomplete Freund’s adjuvant (IFA; Sigma), supplemented with 0.5 mg/ml of Mycobacterium tuberculosis (Sigma). Booster immunization containing 100 μl CII in IFA was...
mitoxantrone 21 days after the priming. Mice were injected subcutaneously with etoposide (Bristol Meyers Squibb AB, Bromma, Sweden), 12.5 mg/kg body weight. The animals were divided into five groups with 10 mice in each experimental group and 15 mice in the control group. One group received etoposide 5 days per week starting 2 days prior to immunization with CII. A second group was treated with etoposide twice, a third group once per week and yet another group once every second week, starting on day 3 after CII immunization. Group A served as controls, also for the mice treated with mitoxantrone.

Mitoxantrone (Wyeth Lederle Nordiska AB, Solna, Sweden) was diluted in saline and administered intraperitoneally. The mice were divided into four groups with 10 mice in each group. Group F received 1 mg/kg body weight on five consecutive days starting on day 3 after collagen immunization. Group G received 1 mg/kg body weight on five consecutive days starting on day 3 after collagen booster. Group H was treated with 15 μg per week, divided into five daily injections, starting on day 3 after collagen immunization until the end of the experiment and group I received the same regimen starting on day 3 after collagen booster. Doses of mitoxantrone were chosen according to earlier studies [8, 9].

Clinical assessment of arthritis

Arthritis was defined as visible joint erythema and/or swelling of at least one joint. To evaluate the intensity of arthritis, a clinical scoring system of 0 to 3 points for each limb was used (1 point, mild swelling; 2 points, moderate swelling; 3 points, severe swelling of the entire paw) yielding a maximum score of 12 points per mouse. The mean arthritic score was determined by adding all points and dividing by the number of animals.

Histological examination

Forty days after immunization the mice were killed and all four paws from each animal in all groups were removed. Histological examination was performed after routine fixation, decalcification, paraffin embedding, and staining with haematoxylin and eosin. All slides were coded and the joints were studied with regard to synovial hypertrophy (defined as a synovial membrane thickness of more than two cell layers) and cartilage and bone destruction (loss of tissue integrity with resulting in growth of fibrotic tissue) [10]. The severity of synovial hypertrophy and cartilage/bone destruction was scored from 0 (intact synovial, cartilage/bone tissue) to 3 (intense synovitis with total destruction of cartilage and/or bone).

Detection of anti-CII-antibodies and assessment of interleukin-6 levels in sera

Quantification of serum anti-CII antibodies was performed as described earlier [11]. Interleukin-6 (IL-6) production was measured by a bioassay using cell line B13.29, subclone B9, which is dependent on IL-6 for growth [12].

Statistics

Statistical comparisons were made by the Mann–Whitney U-test and a χ² test with Yates correction. All values are reported as the mean ± standard error of the mean (SEM). A value below 0.05 was considered significant.

Results

Etoposide dose-dependently down-regulates arthritis

Treatment with etoposide significantly ameliorated the development of arthritis (Table 1). Animals treated with etoposide five times or twice per week still did not display any signs of clinical arthritis at the termination of the experiment 40 days after immunization with collagen II. The amelioration was dose dependent. Mice treated with etoposide once per week or every second week developed arthritis at a later time-point as compared with the controls. No animals died in any group and the changes in weight were similar in mice treated with etoposide once per week, twice per week or once every second week. These animals displayed a more beneficial weight progress than the mice treated either five times per week or control mice injected with phosphate-buffered saline (PBS).

Histological examination fully supported the clinical findings. All etoposide-treated groups displayed significantly less synovitis and destruction of cartilage/bone in the joints as compared with the controls (Table 2).

Impact of mitoxantrone on development of arthritis

Treatment with mitoxantrone at 1 mg/kg body weight on five consecutive days starting on day 3 after collagen immunization (group F) significantly prevented development of arthritis (Table 1). However, on day 40 after CII immunization three out of 10 mice in this group were dead and weight development revealed that these mice were severely ill. Treatment with 1 mg/kg body weight on five consecutive days starting on day 3 after

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence of arthritis (no. arthritic mice/total)</th>
<th>Mean arthritic scoresa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 28</td>
<td>Day 34</td>
</tr>
<tr>
<td>Controls</td>
<td>9/15</td>
<td>15/15</td>
</tr>
<tr>
<td>Etoposide 5 times/week</td>
<td>0/10a</td>
<td>0/10d</td>
</tr>
<tr>
<td>Etoposide twice/week</td>
<td>0/10d</td>
<td>0/10d</td>
</tr>
<tr>
<td>Etoposide once/week</td>
<td>0/10d</td>
<td>4/10d</td>
</tr>
<tr>
<td>Etoposide once/2 weeks</td>
<td>3/10</td>
<td>5/10e</td>
</tr>
<tr>
<td>Mitoxantrone F</td>
<td>0/9f</td>
<td>0/9g</td>
</tr>
<tr>
<td>Mitoxantrone G</td>
<td>4/10</td>
<td>5/10b</td>
</tr>
<tr>
<td>Mitoxantrone H</td>
<td>2/10</td>
<td>6/10</td>
</tr>
<tr>
<td>Mitoxantrone I</td>
<td>6/10</td>
<td>9/10</td>
</tr>
</tbody>
</table>

See Table 2 for further explanations regarding treatment groups.

aArthritic scores were determined on a scale of 0 to 3, as described in Materials and methods.

bP<0.05 as compared with controls.

P<0.01 as compared with controls.

dP<0.001 as compared with controls.
Table 2. Microscopic evaluation of arthritis in mice treated with etoposide and mitoxantrone. Mice were treated with 12.5 mg/kg body weight with etoposide on indicated time points, starting 2 days prior to (five times/week) or 3 days after CII immunization. Group F and G received 1 mg/kg body weight of mitoxantrone on five consecutive days starting on day 3 after collagen immunization or booster, respectively. Group H was treated with 15 μg per week, administered on five consecutive days, starting on day 3 after collagen immunization or booster (group I) until the end of the experiment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Synovitis*</th>
<th>P</th>
<th>Destruction*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>15</td>
<td>20.5±1.8</td>
<td>14.0±1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Etoposide once/2 weeks</td>
<td>10</td>
<td>7.6±2.3</td>
<td>0.0010</td>
<td>2.7±1.2</td>
<td>0.0002</td>
</tr>
<tr>
<td>Etoposide once/week</td>
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<td>&lt;0.0001</td>
<td>3.4±1.0</td>
<td>0.0001</td>
</tr>
<tr>
<td>Etoposide twice/week</td>
<td>10</td>
<td>0.3±0.2</td>
<td>&lt;0.0001</td>
<td>0.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Etoposide 5 times/week</td>
<td>10</td>
<td>0</td>
<td>&lt;0.0001</td>
<td>0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Mitoxantrone F</td>
<td>7</td>
<td>2.0±0.8</td>
<td>0.0002</td>
<td>0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Mitoxantrone G</td>
<td>10</td>
<td>8.5±3.1</td>
<td>0.0084</td>
<td>4.5±2.2</td>
<td>0.0047</td>
</tr>
<tr>
<td>Mitoxantrone H</td>
<td>10</td>
<td>10.3±2.1</td>
<td>0.0043</td>
<td>5.7±1.8</td>
<td>0.0047</td>
</tr>
<tr>
<td>Mitoxantrone I</td>
<td>10</td>
<td>14.5±1.8</td>
<td>0.0350</td>
<td>11.5±1.8</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS, not significant.
*Synovitis and destruction scores were determined on a scale of 0 to 3, as described in Materials and methods.

collagen booster (group G) or 15 μg per week starting on day 3 after collagen immunization (group H) displayed a less frequent and less severe arthritis than the controls.

Consistent with the clinical data, histological investigation revealed that mice treated with a high dose of mitoxantrone after booster or a low dose after immunization with collagen II exhibited significantly lower synovial proliferation and bone/cartilage destruction in the joints as compared with control animals (Table 2).

Etoposide and mitoxantrone treatment suppresses collagen II antibody production

The groups of mice that exhibited fewer clinical and histological signs of arthritis displayed lower serum levels of CII antibodies (Table 1). Thus, etoposide dose-dependently down-regulated serum levels of anti-collagen II antibodies. In addition, all mitoxantrone-treated animals exhibited significantly lower circulating levels of anti-collagen II antibodies (Table 1).

Serum levels of IL-6 in etoposide- and mitoxantrone-treated animals

Serum levels of IL-6 were significantly lower in etoposide-treated mice displaying fewer signs of clinical or histological arthritis (Table 1). In contrast, mice treated with mitoxantrone at 15 μg per week starting on day 3 after collagen immunization (group H) or after collagen booster (group I) displayed significantly higher serum levels of IL-6 (Table 1).

Discussion

In the present study we demonstrate that treatment with etoposide completely prevented development of collagen-induced arthritis in mice, and that this effect was dose dependent. In addition, treatment with mitoxantrone, another topoisomerase II inhibitor, ameliorated the disease onset and outcome, depending on when treatment was commenced and what doses of the drug were administered.

In RA, proliferation of synovial cells contributes to hyperplasia of the synovium and to the formation of inflammatory pannus tissue, which exhibits tumour-like proliferation and invades the articular cartilage and surrounding tissues. One explanation for the synovial proliferation is an imbalance between cell proliferation and apoptosis. For this reason, the induction of apoptosis has been proposed as a potential therapeutic approach [13]. Etoposide and mitoxantrone are topoisomerase II inhibitors that induce apoptosis by activation of nuclear factor kappa-B (NF-κB) [14, 15]. Mitoxantrone belongs to the intercalating anthracine group and etoposide to the non-intercalating epipodophyllotoxin group of the so-called topoisomerase II poisons [3]. The activity of etoposide and mitoxantrone depends on the cellular amount of topoisomerase II, a proliferation-dependent enzyme, which is highly increased in rapidly dividing cells. Treatment with mitoxantrone was more effective when the drug was administered after collagen II immunization as compared with after CII booster. The reason for this could be that fibroblast proliferation is prominent early in the disease and the proliferative status of the cell influences the efficacy of these drugs [16].

IL-6 is found in large quantities in synovial fluid and serum of RA patients [17]. Both pro- and anti-inflammatory properties have been ascribed to IL-6, complicating the establishment of its role in RA. IL-6 induces expression of IL-1 receptor antagonist, soluble tumour necrosis factor (TNF) receptor, and tissue inhibitor of metalloproteinases, all of which down-regulate inflammation and reduce connective tissue damage in the inflamed joint. On the other hand, IL-6-deficient mice display reduced severity and delayed onset of arthritis as compared with wild-type mice in CIA [18]. In accordance with this, mice treated with etoposide five times or twice per week and not exhibiting signs of arthritis displayed significantly lower levels of serum IL-6 than mice treated only once per week, once each second week, or the controls. In contrast, treatment with low doses of mitoxantrone gave rise to significantly higher levels of serum IL-6. Despite that fact the severity of arthritis was significantly diminished. The reasons for these discrepant results relating to IL-6 are currently unknown. We hypothesize that they may have to do with the dual physiological role of this cytokine as depicted above.

Treatment of inflammatory brain disease with mitoxantrone has previously been demonstrated by Lublin et al. [19]. Onset of relapsing experimental allergic encephalomyelitis (EAE) in the mouse was delayed and development of acute EAE was highly suppressed by treating mice with 0.5 mg/kg body weight of mitoxantrone. In the present study mice treated with 1 mg/kg body weight of mitoxantrone after CII immunization did not develop arthritis but were in a bad physical shape and died.

B cells play a crucial role in the induction of CIA, since B-cell deficient mice do not develop arthritis [20]. Collagen II specific antibodies are important effector molecules in CIA, as shown by passive transfer experiments [21]. Both etoposide and mitoxantrone suppress B-lymphocyte function [7, 22]. Consequently, levels of anti-CII antibodies in sera from all groups of mice treated with etoposide or mitoxantrone were significantly lower as compared with the control animals. Our results demonstrate that treatment with etoposide inhibits the development of collagen-induced arthritis dose dependently, and that treatment with mitoxantrone, another topoisomerase II inhibitor, alleviates the disease, depending on dose and time regimen. Taken together, our data demonstrate that inhibition of topoisomerase II is an attractive target in treatment of chronic arthritis.

**Table 1.** Key messages

<table>
<thead>
<tr>
<th><strong>Rheumatology</strong></th>
<th><strong>Key messages</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment of CIA with topoisomerase II inhibitors ameliorates the development of disease.</td>
<td></td>
</tr>
<tr>
<td>The action of both topoisomerase inhibitors tested is mediated through inhibition of collagen II-specific B-cell response.</td>
<td></td>
</tr>
</tbody>
</table>
Acknowledgements

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The authors have declared no conflicts of interest.

References

17. Uson J, Balsa A, Pascual-Salcedo D et al. Soluble interleukin 6 (IL-6) receptor and IL-6 levels in serum and synovial fluid of patients with different arthropathies. J Rheumatol 1997;24:2069–75.