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CONCISE REPORT

Total abrogation of collagen II-induced arthritis and the B cell response to type II collagen using suboptimal doses of a topoisomerase II antagonist

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Background: Collagen-induced arthritis (CIA) is the most commonly used model of rheumatoid arthritis (RA). In both CIA and RA there is an increase in the cellular content of the synovium, this being dominated by macrophages.
Objective: To assess the impact of etoposide, a topoisomerase II antagonist known to induce monocyte apoptosis, on the development of CIA.
Methods: Mice were primed and booster immunised against collagen II (CII). One group of mice was treated with etoposide two days before CII immunisation and then on four consecutive days weekly until the end of the experiment. The second group of mice was injected with etoposide on four consecutive days a week starting 40 days after CII priming. The third group of mice were controls receiving phosphate buffered saline (PBS). The mice were examined for development of arthritis, numbers of circulating leucocytes, serum CII antibody, and cytokine concentrations.
Results: None of the mice given etoposide before CII immunisation developed arthritis. Serum concentrations of anti-CII antibodies were undetectable in these mice, whereas they displayed significantly increased concentrations of interferon γ and interleukin 6. In addition, the CII specific B cell responses in the draining lymph nodes were highly suppressed. Also, mice treated with etoposide at the onset of clinical arthritis showed reduced frequency of their disease by 50%.
Conclusion: There was a striking disease alleviating impact of topoisomerase II antagonist on the course of CII-induced arthritis.

The model of collagen-induced arthritis (CIA) has been extensively used to elucidate the pathogenic mechanisms relevant to human rheumatoid arthritis (RA) and is widely used for the evaluation of potential antirheumatic agents. Similarly to RA, CIA is characterised by massive infiltration of inflammatory cells in the synovium and hyperplasia of the synovial membranes in the joints.[1] The infiltrating cells in the inflamed synovium in RA and in CIA include T cells, B cells, and macrophages.[2 3]

Etoposide is a cytostatic drug, acting by inhibiting topoisomerase II function and thereby leading to apoptosis. It has been shown to selectively deplete the monocyte population in mice and in rabbits.[4 5] We have previously shown that mice pretreated with etoposide developed a significantly less severe septic arthritis.[6] However, in septic arthritis, the absence of bacterial clearance by monocytes and macrophages resulted in septicaemia and increased mortality in mice treated with etoposide. The aim of the present study was to assess the impact of etoposide treatment on the development of CIA.

MATERIALS AND METHODS

Female B10.QxDBA/1 age matched mice were used in all experiments. Arthritis was induced by intradermal injection at the base of the tail with 50 μl of 100 μg rat collagen II (CII) emulsified with an equal volume of complete Freund's adjuvant containing *Mycobacterium butyricum* (Difco, Detroit, MI, USA). Mice were boosted with the same volume and concentrations of CII emulsified in incomplete Freund's adjuvant (Difco) 21 days after the first immunisation.

Etoposide (Bristol Myers Squibb AB, Bromma, Sweden) was injected subcutaneously at a volume of 150–200 μl etoposide, corresponding to 12.5 mg/kg. The dose of etoposide was chosen according to earlier studies[4]. The mice were divided into three groups. One group (n=17) was treated with etoposide two days before immunisation with CII and then on four consecutive days weekly until the end of the experiment 61 days later. The second group (n=17) was similarly injected with etoposide starting 40 days after CII immunisation. The third group comprised controls (n=17), receiving treatment with phosphate buffered saline (PBS). Before the start of the experiment, six mice in each group were bled for haematological analyses.

The clinical and histopathological signs of arthritis were evaluated as previously described.[6]

Details of flow cytometry and haemocytometry determinations have been previously reported.[6] Serum concentrations of immunoglobulins and cytokines were measured as previously described.[6]

To test the effect of etoposide on the immune response of draining lymph nodes, mice were immunised with CII emulsified in complete Freund's adjuvant in both hindpaws and the base of the tail. One group of mice was treated with etoposide two days before immunisation and then on four consecutive days weekly until they were killed on day 10. The second group of mice comprised controls receiving treatment with PBS. On day 10, the mice were killed and draining (popliteal and inguinal) lymph nodes were taken for FACS analysis, immunohistochemical examination, and stimulation with CII in cell culture assays. Supernatants from lymph node cells incubated with CII in vitro were evaluated by enzyme linked immunosorbent assay (ELISA) for CII antibodies.

Statistical comparisons were made by the Mann-Whitney U test and χ^2 test with Yates's correction. All values are reported as means (SEM).

RESULTS

Treatment with etoposide two days before immunisation with CII completely inhibited induction of the disease. Throughout

Abbreviations: CIA, collagen induced arthritis; IFNY, interferon γ; IL, interleukin; PBS, phosphate buffered saline; RA, rheumatoid arthritis; CII, type II collagen

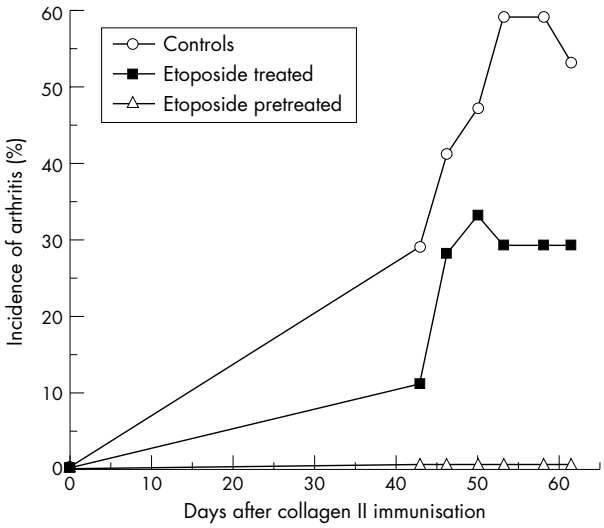

Figure 1 Effect of etoposide treatment on arthritis frequency in mice immunised against collagen II. Mice were treated with etoposide two days before immunisation and then four times a week (etoposide pretreated; n=19), or 40 days after immunisation and then four times a week (etoposide treated; n=17), or left untreated (controls; n=17).

the course of the experiment (almost nine weeks) none of 19 mice pretreated with etoposide (etoposide pretreated) displayed any clinical signs of arthritis (fig 1). Mice that received etoposide 40 days after immunisation with CII (etoposide treated) developed arthritis with a lower incidence than the controls. At the end of the experiment 29% (five of 17) of the etoposide treated mice exhibited arthritis versus 53% (nine of 17) in the control group (fig 1).

Histopathological analyses confirmed the clinical findings. None of the etoposide pretreated mice displayed synovial hypertrophy or bone or cartilage destruction. By contrast, there were no significant differences between the group of etoposide treated mice after immunisation and the controls (data not shown).

Concentrations of interleukin 6 (IL6) and interferon γ (IFNγ) in serum samples from mice treated with etoposide before or after CII immunisation were significantly higher than in control animals (table 1). Interestingly, etoposide pretreated mice did not produce CII antibodies (table 1). Both groups that received etoposide displayed decreased total concentrations of serum immunoglobulins compared with controls (table 1).

The mice immunised with CII were exsanguinated 61 days after immunisation—that is, three days after the last etoposide treatment. Surprisingly, mice treated with etoposide displayed significantly higher numbers of peripheral lymphocytes, granulocytes, and monocytes compared with the control animals (data not shown).

To investigate whether etoposide affected the draining lymph node response, known to precede the onset of CIA, the inguinal lymph nodes were isolated 10 days after CII immunisation. Surprisingly, no significant effects on numbers of B cells (CD19, B220, MHC class II), T cells (CD4, CD8), or macrophages (CD11b, F4/80), or formation of germinal centres were seen using immunohistochemical staining of inguinal lymph nodes. In addition, flow cytometric analysis showed no effect on T or B cell populations (table 2). However, challenge of lymph node cells in vitro with CII disclosed a dramatic decrease of B cell functions as antibodies to CII were not produced at all in cell cultures from etoposide treated mice compared with PBS controls (table 2). In contrast, no differences in T cell function, such as proliferation or IFNγ secretion, were found (data not shown).

DISCUSSION

Treatment of mice with etoposide before CII immunisation had a most striking effect on the development of arthritis. Indeed, none of these mice displayed any signs of arthritis, either clinically or histopathologically. In parallel with this finding antibodies to CII were not detectable. A strong antigen specific B cell response is operational in CIA.[7] The B cells seem to be required for the induction of disease as B cell deficient mice do not develop CIA.[8] More importantly, CII specific antibodies are important effector molecules in CIA, as shown by passive transfer experiments.[9–11] Thus, one of the major reasons for the beneficial results obtained in the present study is the total absence of CII specific B cell responses. This outcome might be due to either a general suppression of immune responses or to an effect more specifically directed towards B cells. Thus it is important to study the general B cell reactivity during treatment with etoposide. Having in mind the relatively modest decrease in total immunoglobulin

Table 1 Serum concentrations of collagen II specific antibodies, immunoglobulins, IFNγ, and IL6 in mice receiving etoposide†

Manipulation	Mice (n)	Anticollagen II antibody (μg/ml)	Total immunoglobulin concentration (mg/ml)	IFNγ (U/ml)	IL6 (pg/ml)
Etoposide pretreated	18–19	8 (1)***	9.2 (0.5)***	1848 (248)*	117 (21)***
Etoposide treated	17	289 (47)*	8.5 (0.8)***	2094 (199)**	179 (21)***
Controls	16–17	424 (47)	13.2 (0.8)	1202 (175)	53 (7)

*p<0.05; **p<0.01; ***p<0.001 *v* controls. †Data are expressed as mean (SEM). Mice were treated with 12.5 mg/kg of etoposide two days before (etoposide pretreated) or 40 days after (etoposide treated) collagen immunisation.

Table 2 Flow cytometry and in vitro analysis of lymph node cells from mice treated with etoposide*

Treatment	FACS CD19+B220+ cells (% of gated lymphocytes)	ELISA Anti-CII antibodies (OD value)	Immunohistochemical scoring					
			F4/80+ cells	CD11b+ cells	CD19+ cells	B220+ cells	CD4+ cells	CD8+ cells
Etoposide (n=3)	59.2 (1.2)	0 (0)	2 (0)	2.7 (2.1)	1.7 (2.1)	5.3 (0.6)	3.7 (0.6)	2 (0)
PBS (n=3)	63.7 (4.2)	1.2 (0.1)	2.3 (2.3)	1.7 (1.2)	5 (1.7)	6.3 (2.1)	3 (1.7)	2.7 (0.6)

*The lymphocytes were stained with CD19 and B220. Also, supernatant from lymph node cells challenged with CII in vitro was tested in ELISA for CII antibodies. Immunohistochemical scoring (using a scale from 0 (0%) to 8 (88%–100%)) was on sections taken from lymph nodes of mice treated with etoposide or PBS and stained with F4/80, CD11b, CD19, B220, CD4, or CD8.

production after long term etoposide treatment it is suggested that the drug is a highly potent inhibitor of newly activated B lymphocytes.

Earlier findings have implicated macrophages as the main target for etoposide in modulating effects on inflammatory diseases.[6] In the present experiments we could not confirm a depletion of monocytes or macrophages in blood or in draining lymph nodes. Also, serum concentrations of cytokines originating from monocytes were higher in mice treated with etoposide than in controls. In this context, both proinflammatory and anti-inflammatory properties have been ascribed to IL6 and IFNγ.[12–16]

The most striking effect on immune functions in our experiments is the dramatic effect on the B cell response to CII. Together with the fact that CIA is largely mediated by CII antibodies binding to cartilage and thereby triggering an immune complex mediated arthritis it is likely that the main effect of etoposide on CIA is mediated by directly or indirectly suppressing B cell function. Overall, our study shows a beneficial impact of etoposide treatment on the course of CIA. Based on the above findings we think that clinical trials of etoposide treatment in patients with RA would be of interest.

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