# Pediatric rheumatology

# TNF and LT binding capacities in the plasma of arthritis patients: Effect of etanercept treatment in juvenile idiopathic arthritis

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## Abstract

### Background

*Etanercept (Enbrel®) induces a rapid and sustained decline in disease activity in the majority of patients with refractory juvenile idiopathic arthritis (JIA). For unknown reasons, however, a number of JIA patients fail to respond to this therapy. During this treatment neutralisation of tumour necrosis factor (TNF, previously termed TNFα) and lymphotoxin (LT, previously termed TNFβ) may be mediated by etanercept itself as well as by naturally occurring soluble TNF receptors. In light of this, it was of interest to study the total TNF neutralizing capacity in plasma before and during treatment with etanercept.*

### Results

*In initial experiments plasma samples from healthy individuals were incubated with etanercept, and spiked with TNF or LT to a final concentration of 1000 pg/mL. Detection of TNF and LT by ELISA was found to be reduced by approximately 50% and 80% respectively, at a concentration of etanercept of 5–500 ng/mL, which is close to the pharmacological plasma concentrations.*

*Plasma samples (n = 80) were then collected from 12 JIA patients (5 with pauciarticular, 5 with polyarticular and 2 with the systemic onset type) during treatment with etanercept (0.4 mg/kg twice weekly) for a period of 20.8 (15.6–23.9) months (median, range). The plasma samples were spiked with LT, and the inhibition of LT detection in ELISA was measured. In samples obtained 3 months after the start of etanercept, the inhibition of LT detection was augmented [72% (60-85)] compared with pre-treatment samples [16% (0-32)] (p = 0.0039). These findings were confirmed in binding assays using radiolabelled TNF. Among patients who responded insufficiently to therapy, reduced LT binding capacity, coinciding with flares of disease activity, was observed.*

### Conclusion

*We have developed an assay by which LT binding capacity, reflecting the level of free, pharmacologically active etanercept, may be monitored in the blood of patients treated with etanercept. This assay may prove to be useful in guiding dose adjustments in patients with an incomplete response to etanercept.*

**Key words**

Juvenile idiopathic arthritis, arthritis, etanercept, Enbrel, TNF, lymphotoxin.

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## Introduction

Tumour necrosis factor (TNF) is thought to play a major role in the perpetuation of the chronic inflammatory processes associated with rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA) (1-3). TNF induces the release of matrix metalloproteinases, induces the expression of endothelial adhesion molecules involved in the migration of leucocytes to extravascular sites of inflammation, and it stimulates the release of other proinflammatory cytokines. TNF concentrations are increased in plasma and synovial fluid in both RA and JIA, and TNF is expressed in the synovial tissue of inflamed joints (2).

Lymphotoxin (LT, previously termed TNFβ) is a closely related cytokine which binds to the same receptors as TNF. In contrast to TNF, the role of LT in JIA and RA is not well defined, but the cytokine has been found in the synovial tissue of diseased joints in JIA (4). The plasma levels of LT are generally low and frequently not measurable in arthritis patients (Müller *et al.*: unpublished data).

The effects of both TNF and LT are mediated through binding to two distinct cell-surface TNF receptors (TNFR), designated p55 (type I) and p75 (type II). Soluble, truncated versions of membrane TNFR (sTNFR), consisting of only the extracellular ligand-binding domains, are thought to function as natural inhibitors of both TNF and LT activity (5, 6). These sTNFR have been found at elevated levels in the plasma and joint fluid of JIA and RA patients with active disease, and in the culture supernatants of blood mononuclear cells from these patients and from patients with a number of other inflammatory diseases (7, 8).

A recombinant human TNFR p75-Fc IgG fusion protein, etanercept (Enbrel®, Immunex, Seattle, USA) has been developed for the therapeutic neutralisation of TNF. By binding excess TNF and LT, etanercept blocks the interaction between these mediators and the cell surface receptors, thereby inhibiting the biologic effects of both TNF and LT. The efficacy of etanercept in binding TNF is approximately 1000-fold greater than that of the p75 receptor (9). Etanercept induces a rapid and sustained decline in disease activity in the majority of JIA patients, including patients not responsive to other second-line drugs such as methotrexate. However, in some patients the response is variable, and a few patients are non-responders, particularly those with systemic onset JIA(10-12).

To increase our understanding of the variability in the response to etanercept, we monitored the total TNF- and LT binding capacities (TNFBC and LTBC) in the blood of JIApatients during treatment with etanercept.

## Materials and methods

*Patients*

Twelve patients (1 male and 11 females) with JIAaccording to the Durban criteria (13) and treated with etanercept entered the study. Their mean age (range) was 11.8 years (4–16) and the mean (range) disease duration was 7.3 years (1–13). Seven had polyarticular onset disease, 2 had systemic onset JIA, and 3 had extended pauciarticular JIA. Previous medical treatments included methotrexate (MTX) (n=12), sulphosalazine (n=10), prednisolone (n=12), cyclosporine A (CyA) (n=11), hydroxychloroquine (n=7), myocrisin (n=8), dimethylcysteine (n=2), azathiophrine (n=4), and nonsteroidal anti-inflammatory drugs (n=12). Up until one month before commencement on etanercept 10 patients were treated with MTX at a mean (range) dosage of 13.6 mg/m$^2$/week (6.6 – 26.6), 1 was treated with sulphasalazine (1.5 g/day), 8 were treated with CyA at a mean (range) dosage of 103 mg/day (75 –200), and 12 with prednisolone at a mean (range) dosage of 0.17 mg/kg/day (0.06 – 0.6).

*Clinical parameters*

The patients were assessed before the start of etanercept and approximately every third month during treatment. The following clinical parameters were recorded: number of active joints (showing at least two of the following signs: pain on passive movement, tenderness, increased temperature, limited motion), childhood health assessment questionnaire (CHAQ); the pain score and the

patient's/parents' global assessment were graded on a visual analogue scale with the maximum at 100 mm. At the same time the erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and a full blood count were carried out (14).

*Plasma samples*
EDTA blood was centrifuged within 10 minutes after collection, and the plasma was frozen at – 80ºC.

*ELISA for TNF and LT*
TNF and LT were measured by a double sandwich ELISA using monospecific polyclonal rabbit antibodies to purified recombinant cytokines as detailed in reference 15. Briefly, immuno-Maxisorp plates (Nunc, Roskilde, Denmark) were coated with protein-A affinity-purified IgG. Non-attached sites were blocked with 1% human serum albumin in phosphate-buffered saline (PBS). The assays were calibrated with the international standards for the respective cytokines (National Institute for Biological Standards and Controls (NIBSC), Potters Bar, Hertfordshire, UK). Biotinylated rabbit antibodies were used as the detecting antibodies along with streptavidin-peroxidase (Kirkegaard and Perry La., Gaithersburg, MD, USA). Development was carried out with 1,2-phenylenediamine dihydrochloride and measured at 492 nm. The inter- and intra-assay coefficients of variation for the concentration range between 15 pg/mL and 1 ng/mL were less than 15%. The sensitivity limits of these ELISAs were 10 – 30 pg/mL.

*TNF and LT binding capacities (TNFBC and LTBC)*
Plasma samples from healthy individuals were incubated with serial dilutions of etanercept (0–500 ng/mL) in microtiter plates (Nunc), and spiked to a final concentration of 1000 pg/mL with native human TNF generated from a lipopolysaccharide stimulated whole blood culture, or recombinant LT (NIBSC). After incubation for 2h at 20ºC, the levels of TNF and LT were measured by ELISA.
Plasma samples from JIApatients were diluted serially in 0.1 M PBS, supplemented with 6% PEG, 0.05% Tween 20, 2% mannitol, 1% non-fat dry milk, 1% goat serum, 1% rabbit serum, and 1% sheep serum, and LT was added to a final concentration of 1000 pg/mL. After incubation for 2 hours at 20ºC, LT levels were measured by ELISA. LTBC was expressed as the percentage value of bound LT to added LT: [(1000 – LT measured)/1000] x 100. The inter-assay variation was 7%.

*RIA for plasma TNFBC*
Undiluted plasma samples, 100 µl, were each incubated with 100 µl $^{125}$I-TNF (Amersham Pharamcia Biotech, Buckinghamshire, UK), 20,000 to 30,000 cpm, for 3 hours at 37°C. Bound and free tracer were separated on PD-10 columns (Amersham Pharmacia Biotech, Horsholm, Denmark), containing 1 ml Protein G Sepharose 4 Fast Flow (Amersham Pharmacia). The running buffer was 0.1 M PBS, pH 7.4 and bound material was eluted with 0.1 M glycine/HCl, pH 2.7. The TNF binding capacity (in percent) in plasma was calculated as ($cpm_{bound}$ / $cpm_{total}$) x 100.

*ELISA for sTNFR I*
sTNFR I was measured by ELISA(R & D) according to the instructions of the manufacturer. This assay measures the total amount of free receptor plus the total amount of receptor bound to TNF.

*Statistical analysis*
Differences between paired and unpaired samples were evaluated using the Wilcoxon and the Mann-Whitney tests, respectively. Correlations were tested using the Spearman correlation. The level of significance was chosen to be 5%.
This study was approved by the regional ethics committee.

**Results**
*Clinical data*
The patients were followed for a median of 20.8 (15.6–23.9) months (range) and generally responded well to etanercept as indicated by a steady, gradual decline in the CHAQ score, parent/ patient global assessment and active joint count (Fig. 1). Likewise, immediate and significant reductions in the ESR and CRP were observed, although both parameters tended to increase again about 9 months after start of the treatment (Fig.1). This increase was however isolated to the 9-month observation time point, as subsequent evaluations showed that these parameters continued to be low. One patient stopped treatment due to stable remission during the course of the study.
At the end of the observation period, 7 patients received prednisolone, mean dose (range) 0.09 mg/kg/day (0.04 – 0.2), and 4 patients received methotrexate, mean dose (range) 11.2 (7.1 – 19.2) mg/m$^2$/week.

*LTBC*
The ability of etanercept to interfere with the detection of TNF and LT was evaluated in plasma samples from healthy individuals incubated with etanercept and spiked with TNF and LT, respectively. The mean (range) level of inhibition induced by etanercept reached plateaus of 48% (34-60) (n=4) (for TNF) and 81% (69-96) (n=3) (for LT), respectively, at etanercept concentration ranges of 10-500 and 5-100 ng/mL, which is just below pharmacological plasma levels.
LTBC was measured in plasma samples from JIA patients obtained before and during treatment with etanercept. LT was added to the samples and the recovery of immunoreactive LT was measured by ELISA.
Figure 2Ashows the dilution curves for a single patient and Figure 2B shows data for all the patients at 40, 200 and 1000 times dilution of the plasma samples. Some degree of interference with LT detection was observed before the start of etanercept. However, a pronounced increase in the inhibition of LT was seen after the start of treatment with etanercept, reaching a plateau at 40–200 times dilution. Below this plateau (1000 times dilution), at the steep part of the curve, a considerable degree of variability in LTBC was observed with a mean intra-individual variation coefficient of 27% (range 8–102) in patients studied longitudinally and an inter-individual variation coefficient of 30% (11–44).
Measurement of TNFBC in plasma samples from JIA patients using RIA

showed a similar increase in TNFBC following treatment with etanercept (pre-etanercept: mean 8% (range 4 – 14); after 3 months of treatment: 70% (61-78) (p = 0.0156) (Fig. 3).

Figure 4 illustrates an example of a patient who responded well to treatment with etanercept; she stopped treatment after the induction of stable remission. The LTBC (1000 times dilution) reached fairly constant levels during treatment and then declined below measurable levels after the termination of etanercept therapy.

Some patients responded incompletely to etanercept, and among those decreasing LTBC values were found to coincide with flares (Figs. 5 and 6).

*sTNFR I*

The level of sTNFR I was determined by ELISA in samples obtained before the start of treatment with etanercept. The mean (range) level of sTNFR I: 2304 pg/mL (1385–3142) was higher in patients than in age-matched controls: 742 pg/mL (378–1125), p < 0.0001, n = 9. The LTBC correlated with borderline significance with that of sTNFR I: r= 0.57, p = 0.0883. sTNFR I levels were not reduced significantly during the first 6 months of etanercept treatment (data not shown).

**Discussion**

Etanercept has been introduced for therapeutic neutralisation of TNF/LT in patients with various immunoinflammatory diseases, including JIA, and it is routinely given subcutaneously twice weekly. This regimen usually results in rather stable plasma concentrations of

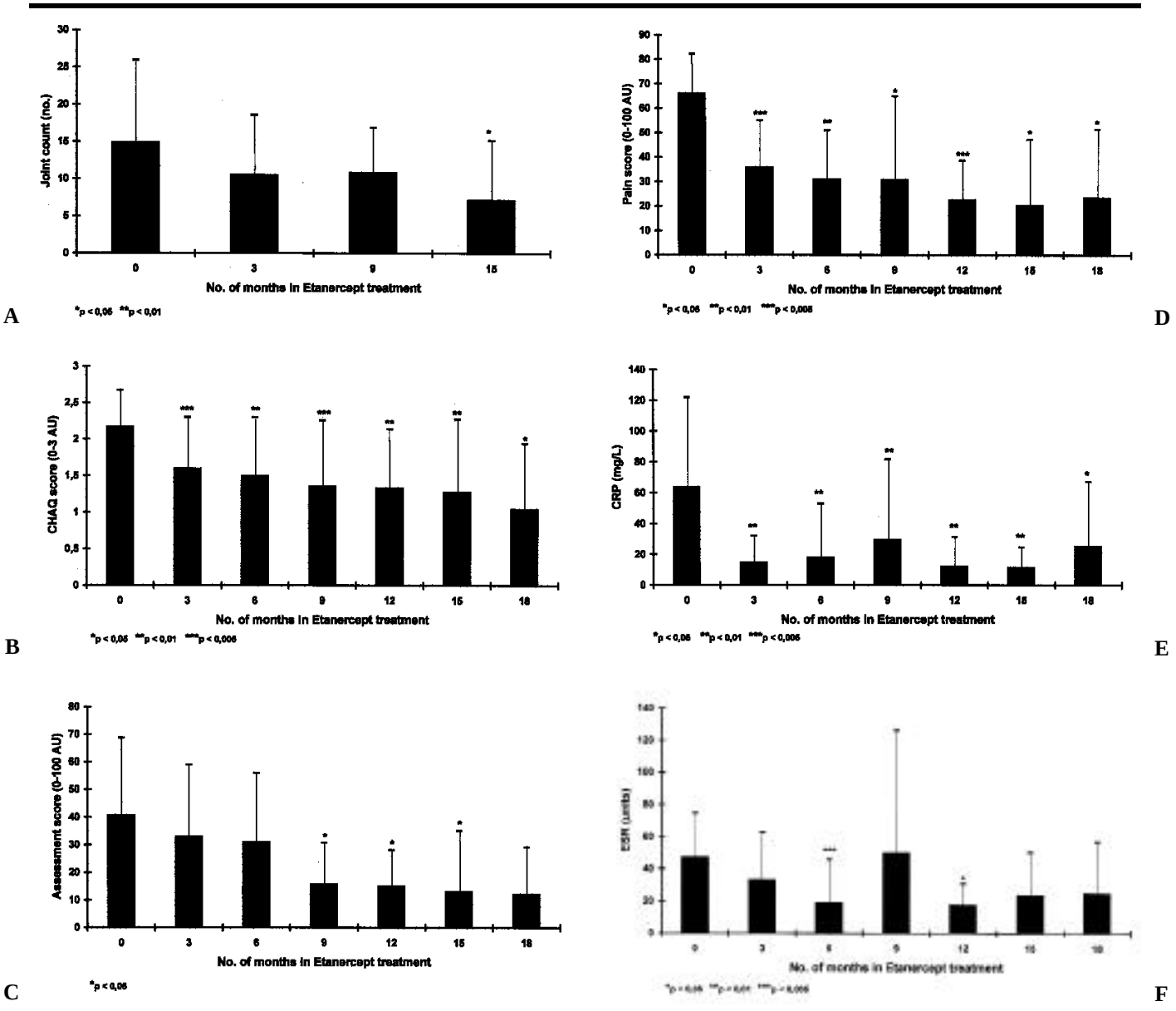


**Fig. 1.** Means of disease activity parameters in patients with juvenile idiopathic arthritis before and during treatment with etanercept. Bars illustrate standard deviations. (**A**) number of active joints; (**B**) CHAQ; (**C**) Childrens/parents global assessment; (**D**) pain score; (**E**) CRP; (**F**) ESR. The p values refer to the comparison of pre-treatment values of disease activity with the values obtained at each time point after the initiation of treatment with etanercept.

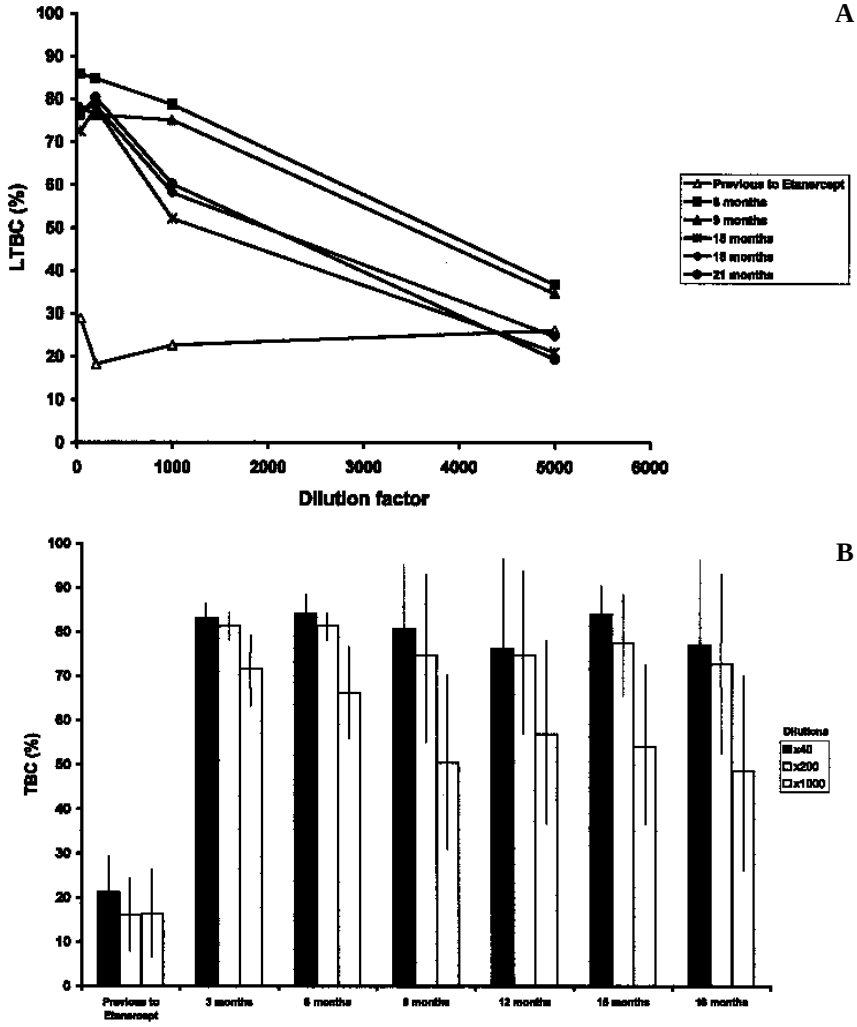


**Fig. 2.** Plasma samples from patients with JIAtaken before and during treatment with etanercept were serially diluted and incubated with LT to a final concentration of 1000 pg/mL. LT was detected by ELISA(mean of triplicate runs). (**A**) Dilution curves from a single patient. (**B**) Data from all included patients (n = 12). Mean of interference with detection of LT expressed in percentages at 40, 200 and 1000 times dilution (see text).

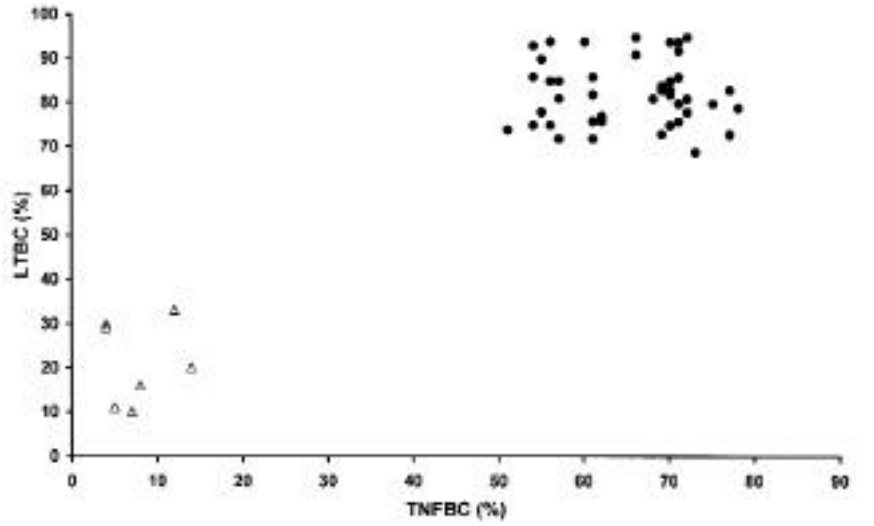


**Fig. 3.** TNFBC and LTBC in plasma before and during treatment with etanercept. Plasma samples from patients with JIA, taken before (Δ) and during (●) treatment with etanercept, and LTBC and TNFBC were detected by ELISAand RIA, respectively.

200–500 ng/mL(16). However, inflammation induced and maintained by the TNF/LT system of cytokines and cytokine receptors is likely to be dynamic, with a highly variable production/ release of the agonists TNF and LT, and of the two known antagonists sTNFR I and sTNFR II, reflecting at least in part the clinical state of inflammatory activity (2, 5, 6). As a consequence the level of free, pharmacologically active etanercept may be expected to fluctuate in parallel with the fluctuations in the production of endogenous TNF modulators, despite a stable molar concentration of the drug. Since the clinical response to etanercept varies considerably, and since little is known about the factors influencing the pharmacological potential of etanercept in individual patients, we found it of interest to gather information of the TNFBC and LTBC before and during treatment with etanercept.

In the initial experiments, we found that etanercept interfered with the detection by ELISA of both LT and TNF. However a plateau of inhibition was reached beyond which no further inhibition could be obtained. This may indicate that our ELISAs are capable of partially detecting LT/TNF molecules bound by etanercept. Since the inhibition of TNF was less pronounced than that of LT, and since only TNF has been found at significant levels in the plasma of patients with JIA, we chose to base our interference-ELISA assay on the use of LT for spiking experiments.

At baseline, before the start of etanercept, we observed some reduction in the detection of exogenous LT in the majority of patients. This finding most likely represents the presence of interfering substances in the plasma, including sTNFR I, which was elevated in the patients and tended to correlate with the LTBC, although this did not reach significance.

After the start of treatment with etanercept, a pronounced increase in the LTBC was observed, consistent with the observation of a clear reduction in several parameters of disease activity. The mean LTBC was remarkably high, with a plateau at the 40–200 times dilution of the plasma sample. Due to the

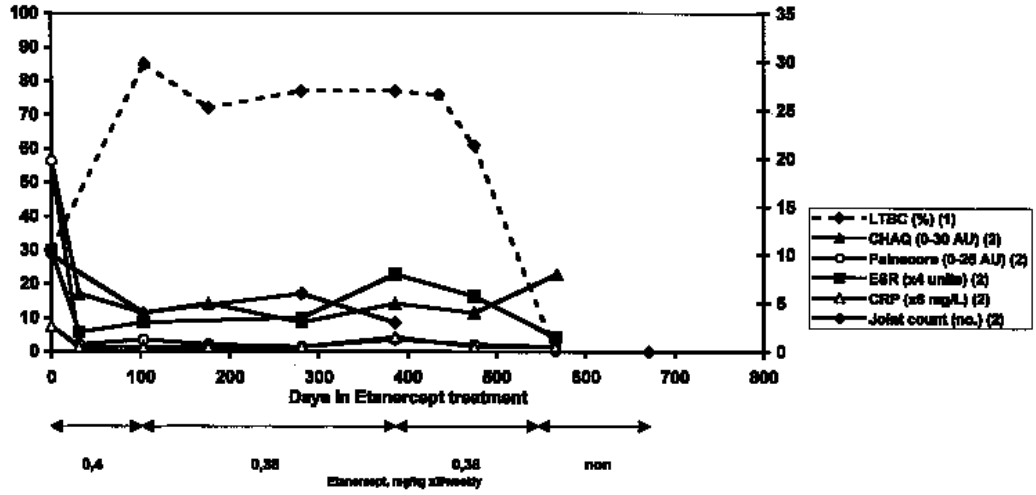


**Fig. 4.** Clinical and laboratory data on a girl with polyarticular onset JIA. This patient responded well to the treatment, as indicated by declines in several parameters of disease activity. Stable LTBC were noted during treatment with etanercept. Due to a stable remission, treatment was terminated and this was followed by a decline in LT binding to below the detection limit. Interference with the detection of LT is expressed in percentages (see text). AU: arbitrary units. LTBC is shown along the left x-axis, and the other parameters along the right.

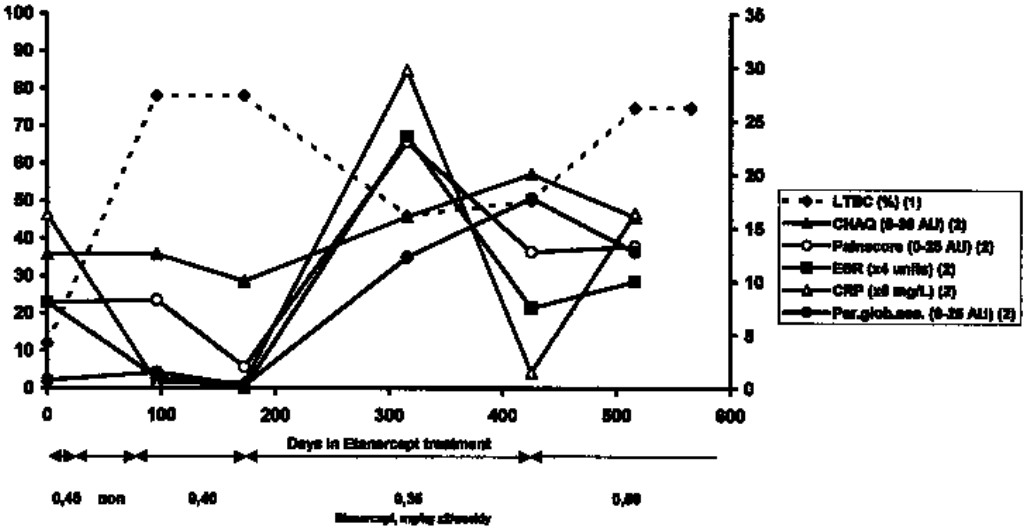


**Fig. 5.** Data on a girl with systemic onset JIA. Initially, she responded well to etanercept and as a consequence the concomitant therapy with prednisolone was tapered from 0.57 mg/kg/day to 0.25 mg/kg/day. During this period, the LTBC reached levels of about 80%. At about day 300 after the start of etanercept she had a flare with spiking fever up to 40ºC, joint pain and general malaise. Prednisolone was increased to 0.5 mg/kg/day and MTX 10 mg/m$^2$/week was administered together with prednisolone pulse treatment. There was a marked decrease in LTBC during this period of flare. Interference with the detection of LT is expressed in percentages.

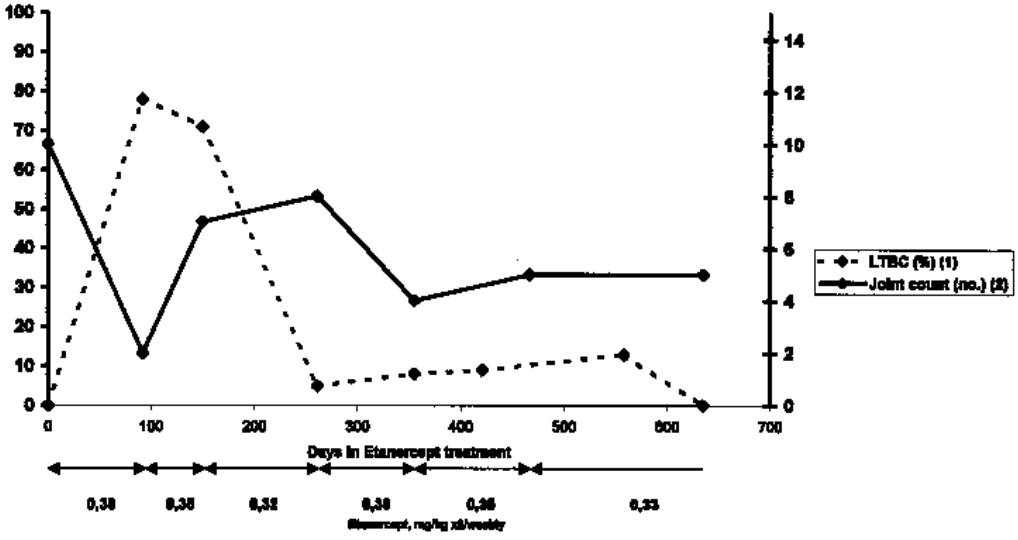


**Fig. 6.** Data on a girl with polyarticular onset JIAwho was treated with a combination of MTX, CyA and prednisolone 0.06 mg/kg/day until the start of etanercept. She responded initially with less pain, an increasing functional level and less severe arthritis. Consequently, prednisolone was tapered off within the first 3 months of etanercept treatment. Following this, however, an increased number of active joints and progression in erosions were seen in several joints. After an initial increase in LTBC, the levels decreased to very low levels for the rest of the observation period; this coincided with an increased number of active joints. Interference with the detection of LTis expressed in percentages.

plateau, this part of the dilution curve did not allow a correct evaluation of the variations in LT binding. However, at the steep part of the dilution curve, located at 1000 times dilution, considerable intra- and inter-individual variations were observed.

Studies of LTBC in the synovial fluid of arthritic joints are needed for obvious reasons. However, LTBC in inflamed tissues may be significantly lower than that observed in plasma, and

the variability in LTBC seen at the 1,000 times dilution of the plasma samples may therefore be of pharmacological relevance. In accordance with this thesis, among patients who responded less well to the treatment, we observed reduced LTBC in the 1000 times diluted sample, and this coincided with periods of increased disease activity. Whether the decline in LTBC during flares is the cause or a result of the increased disease activity is, however, unclear. Increased production of TNF and LT may reduce the level of unbound, biologically active etanercept. Alternatively, an altered distribution or metabolism of etanercept may lead to lower plasma LTBC, causing insufficient TNF antagonism and thereby increased disease activity.

The possibility of the induction of specific antibodies to etanercept must also be considered. This explanation is, however, less likely since these antibodies are observed in very few patients (approximately 1-2%) and they do not appear to affect either the binding activity of etanercept or its therapeutic effect (17). The possibility of insufficient patient compliance cannot be ruled out. In any case, however, a decline in LTBC seen during a flare would indicate that the dosage of etanercept should be re-evaluated and possibly increased.

About 70% of RA patients have been found to respond to a standard dose of etanercept, and a number of the remaining patients have been shown to respond to an increased dosage of etanercept (10,18). Studies in JIA and in RA patients are in progress to evaluate to what degree monitoring LTBC during treatment with etanercept may help to guide dosage adjustments.

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