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Autoantibody formation in patients with rheumatoid arthritis treated with anti-TNF-alpha therapy.

Catharina Eriksson*, Stefan Engstrand**, Karl-Gösta Sundqvist* and Solbritt Rantapää-Dahlqvist**

Department of *Clinical Immunology and **Rheumatology, University Hospital, S-901 85 Umeå, Sweden

Corresponding author: S.Rantapää Dahlqvist
Dept of Rheumatology
University Hospital, S-901 85 Umeå, Sweden

Keywords: TNF-α inhibitor, autoantibodies, anti-dsDNA, anti-nucleosome antibodies, rheumatoid arthritis
**Background:** Contradictory results have been presented on autoantibody formation in patients treated with TNF-α inhibitors.

**Objective:** To study the prevalence of autoantibodies in patients with rheumatoid arthritis (RA) treated with the TNF-α inhibitor, infliximab for 54 weeks.

**Material and methods:** Fifty-three patients (48 females and 11 males) with RA and treated with infliximab were followed for autoantibody production before treatment and after 14, 30 and 54 weeks. For comparison 6 patients treated with etanercept were included. The analyses included antibodies against nuclear antigens (ANA), extractable nuclear antigens (ENA), double stranded (ds)DNA (by ELISA, IIF on *Crithidia luciliae* for IgM and IgG, and Farr assay), nucleosomes, cardiolipin, smooth muscle, mitochondria, proteinase 3 and myeloperoxidase antigens.

**Results:** The number of patients treated with infliximab that developed antibodies against dsDNA both of IgG and IgM class (tested by IIF) increased significantly. The prevalence of positive patients for IgG class increased to 66% at 30 weeks and 45% at 54 weeks and of IgM class to 85% and 70%, respectively. Likewise, the titre and number of patients expressing antibodies against nucleosomes and ANA increased significantly. The number of RF or anti-cardiolipin positive patients was stable and nor was there any increase of antibodies against the other antigens. A lupus-like syndrome was seen in one patient. No patient treated with etanercept developed any of the autoantibodies.

**Conclusion:** A significant number of patients treated with infliximab developed anti-dsDNA antibodies, of both IgM and IgG class, anti-nucleosome antibodies and ANA with gradual increase until 30 weeks.
Introduction

Patients with rheumatoid arthritis (RA), spondylarthropathy and Crohn’s disease treated with selective tumor necrosis factor (TNF)-α inhibitors have been reported to develop autoantibodies, such as antinuclear antibodies (ANA), and anti-double stranded DNA antibodies (anti-dsDNA) (1-4). These antibodies are mostly of IgM class (1, 2). There have also been case reports of patients developing drug-induced lupus syndrome although the incidence has been fairly low (5). The development of autoantibodies and drug-induced lupus syndrome has been described during treatment with infliximab as well as with etanercept (1, 2, 5, 6). Etanercept inhibits binding of both TNF-α and TNF-β (lymphotoxin alpha) to cell surface TNF receptors, rendering TNF biologically inactive (7). Infliximab binds to both soluble and transmembrane forms of TNF-α in vitro but not to lymphotoxin. Binding to soluble TNF-α results in loss of bioactivity whereas binding to membrane-bound TNF-α leads to cytotoxicity by complement and/or antibody-dependent cellmediated mechanisms (8).

TNF-α is a proinflammatory cytokine, produced by multiple cell types including blood monocytes, macrophages, mast cells and endothelial cells, that interacts with receptors on a wide variety of cells (9). TNF-α has multiple complex functional roles within the immune system including pro-inflammatory properties, cytotoxic effect, regulation of cell adhesion, and induction of cachexia (10, 11, 12). Consequently there are several potential mechanisms by which anti-TNF-α treatment could exert its beneficial effect in RA and other diseases. These mechanisms include decreased expression of activation markers on circulating lymphocytes and down-regulation of T helper-1 cell production resulting in a net overall decrease in TNF-α expression (13, 14). Reports have suggested that TNF-α can induce not only apoptotic but also
anti-apoptotic signals (15, 16). Interestingly, TNF-α neutralisation ameliorates the severity of murine model of ileitis by abrogation of intestinal epithelial cell apoptosis whilst apoptosis was induced in lamina propria mononuclear cells (17). Therefore, interference with apoptosis is a possible mechanism by which anti-TNF-α therapy may exert its effect systematically albeit infliximab treatment did not alter the anti-apoptotic state of the synovium (18).

The inconsistencies between reports on the prevalence of production of various autoantibodies, and different isotypes expressed, in patients treated with TNF-α inhibitors encourage us to undertake a prospective long term follow-up study, including analyses of anti-nucleosome antibodies on patients treated with the selective TNF-α inhibitor infliximab. The development of autoantibodies was followed for 54 weeks and as comparison six patients with RA treated with etanercept were followed.
Material and methods

Patients. Fifty-nine patients (48 females and 11 males) with RA according to the criteria of American College of Rheumatology (19) were consecutively recruited into the study. Fifty-three patients were treated with infliximab (3 mg/kg body weight at inclusion, after 2 and 8 weeks and thereafter with 8 weeks intervall) and six patients with etanercept (25 mg twice weekly). Demographic data on the patients in the study are presented in Table I.

Methods. Blood samples were collected from patients before treatment and after 14, 30 and 54 weeks. The resulting sera stored at -80°C until analysed. The autoantibodies analysed included: rheumatoid factor (RF) by Waaler-Rose, antinuclear antibodies (ANA) by indirect immunofluorescence (IIF) on rat kidney, anti-smooth muscle antibodies and anti-mitochondrial antibodies by IIF on rat tissue (using an in-house protocol), anti-histone antibodies, anti-extractable-nuclear antigens (ENA: anti-Sm, RNP, SSA, SSB, Scl-70 and Jo-1), anti-proteinase 3, anti-myeloperoxidase (anti-MPO) and anti-cardiolipin (aCL, IgG) antibodies (Autozyme, Cambridge Life Science, Cambs, England) were assessed by ELISA. Antibodies against double stranded DNA (ds-DNA) were analysed by IIF on Crithidia luciliae coated slides for both IgM and IgG (Immu-noconcept), by ELISA (Varelisa: Pharmacia Diagnostics, Freiberg, Germany) and by the Farr assay (Ortho, Amersham, UK). Anti-nucleosome antibodies (IgG class) were analysed by ELISA (Euroimmun, Germany).

Strategy. All 53 patients treated with infliximab were assessed for RF, ANA, ds-DNA (IgG class) and anti-nucleosome antibodies. The first 27 patients included were also assessed for ds-DNA (IgM class) and aCL antibodies. Anti-DNA ELISA test and Farr assay were performed on
the first 20 patients recruited. At 54 weeks there were serum samples from only 45 patients because seven were prematurely withdrawn from treatment and for one individual there was no serum available for any analyses at 54 weeks and in another two for anti-nucleosome antibodies at 54 weeks. In three individuals anti-nucleosome antibodies were not analysed at any time point due to lack of sera. Furthermore, for some of the other analyses there was no serum available; this loss of sera was random. The six patients receiving etanercept were assessed for all autoantibodies except for anti-nucleosome antibodies.

Statistics. The statistic analyses were performed with the non-parametric tests: Kruskall-Wallis one-way analysis of variance for independent samples and Friedman two-way analysis of variance by ranks for related samples. The Chi-square test for trends were used for testing several categorical data.

RESULTS

Analyses of autoantibodies in patients treated with infliximab

The frequency of individuals with anti-dsDNA, anti-nucleosome and ANA antibodies increased significantly (Chi-square for trends) during treatment with infliximab (Table II). Two patients had either IgG and IgM antibodies against dsDNA, of a low titre, before treatment. The proportion of patients positive for IgG class anti-dsDNA antibodies assessed by the *Crithidia luciliae* -test increased to 66% at 30 weeks and 45% at 54 weeks and of IgM class to 85% and 70%, respectively (Table II and III). In the 27 patients tested for both IgG and IgM class anti-dsDNA antibodies, the concordance between the antibodies was high. Patients having IgM antibodies were also positive for IgG antibodies in 94% (at 14 weeks), 86% (at 30 weeks) and 81% (at 54 weeks), respectively. At inclusion five individuals were positive for anti-nucleosome antibodies and this number increased significantly to 13 at 30 weeks and remained at that level.
The concordance between positive anti-nucleosome antibodies and IgG class anti-ds DNA antibodies at 30 weeks was 85% and at 54 weeks 78% (Table II) and of IgM class it was 88% and 86% (Table III), respectively. The concordance between positive ANA and anti-dsDNA antibodies of IgM and IgG isotype or anti-nucleosome antibodies was high; 82-90% at 30 weeks and 88-100% at 54 weeks, respectively. The anti-DNA ELISA was only positive in one of the 20 patients tested after 30 weeks of treatment, whilst the Farr assay was positive in five patients all of whom were IgG positive in the *Crithidia luciliae test*. The numbers of RF positive and aCL positive patients were stable throughout the study (Table II and III).

In most patients who developed anti-dsDNA antibodies of both IgG (Figure 1A) and IgM types the titres were high and increased significantly both as a group (Kruskal-Wallis one-way analysis of variance P< 0.0001 and P < 0.05, respectively) and on an individual level (Friedmann two-way analysis of variance P<0.0001 and P< 0.0001, respectively) at 30 weeks and thereafter the titres declined although, not significantly so. This pattern was the same for the ANA titres in that they increased significantly (Kruskal-Wallis one-way analysis of variance, P< 0.001, Friedmann two-way analysis of variance, P < 0.001, Figure 1B) until 30 weeks and were, thereafter, stable (Table II). The titres of anti-nucleosome antibodies increased significantly (Kruskal-Wallis one-way analysis of variance, P< 0.01, Friedmann two-way analysis of variance, P < 0.0001) (Figure 1C) whilst the titres of RF decreased significantly over time (Kruskal-Wallis one-way analysis of variance, P< 0.001, Friedmann two-way analysis of variance, P< 0.05, Figure 1D). The anti-dsDNA titre, measured by ELISA, increased significantly, although all values below the cut-off value (data not shown). There was no increase in the number of individuals positive for antibodies against histones, ENA, proteinase3, MPO, smooth muscle and mitochondria.
Of the seven individuals who withdrew from the therapy prematurely \textit{i.e.}, before 54 weeks six did so because of side effects: three due to allergy, one due to anaphylaxis, and one due to infection. The other withdrawal was due to the development of a SLE-syndrome (with leucopenia, C3 and C4 consumption, myalgia and arthritis) with anti-histone antibodies. She recovered within a few weeks after stopping the treatment, but the anti-dsDNA antibodies of IgG isotype remained positive at a high titre 20 month later. Another of the patients had IgG class anti-dsDNA antibodies eight months after withdrawal. Five of the six individuals with side effects had anti-dsDNA antibodies of IgG class ($X^2 = 4.02$, $P< 0.05$). In two of the cases the withdrawal due to side effects was combined with a lack of efficacy another patient ceased purely because of lack of efficacy. Another patient with anti-dsDNA antibodies and anti-nucleosome antibodies was withdrawn just after 54 weeks due to development of a lupus-like syndrome (leucopenia, skin rash, arthralgia and vasculitis on finger tips).

There were no effects of methotrexate treatment or the dosage of the drug or of corticosteroid medication on the development of autoantibodies. Patients with ANA antibodies at 30 weeks had significantly higher DAS28 at 54 weeks ($p < 0.05$).

\textit{Analyses of autoantibodies in patients treated with etanercept}

No patients treated with etanercept developed any of the autoantibodies tested for.

\textbf{DISCUSSION}

In this study the frequency of autoantibodies against ANA, ds-DNA (of both IgG and IgM isotype) and anti-nucleosome increased significantly during treatment with infliximab in patients with RA. Anti-nucleosome antibodies have been suggested to be a useful marker for diagnosis
and disease activity assessment particularly in anti-dsDNA negative SLE patients (20, 21). This antibody has not previously been described in RA patients (21). In this study five of the 50 (10%) patients were positive for anti-nucleosome antibodies prior to treatment. Furthermore, anti-nucleosome antibodies were found to develop in both RA and ankylosing spondylitis patients treated with infliximab (2). These results are further supported by the recent findings of increased levels of nucleosomes in patients treated with infliximab (22).

The number of ANA-positive RA patients at inclusion into the study was as described for RA i.e., approximately 25%, however, the number increased, as did the titres, during infliximab treatment whilst the number of RF positive patients remained the same with decreasing titres. The increase of antibodies against ANA was in agreement with previously findings (2, 23) but the increase of anti-dsDNA and anti-nucleosome antibodies were higher in this study than in those reports. In previous studies on RA (24), or on Crohn’s disease (3) anti-dsDNA antibodies were only tested for in ANA-positive patients, which could contribute to the findings of lower frequencies. The development of autoantibodies during infliximab therapy in this study, and in those by others (2, 6), does not seem to have the same underlying mechanism(s) as the development of a lupus-like syndrome induced by other drugs since anti-histone antibodies were only found in one patient.

In this study, two patients treated with infliximab developed a lupus-like syndrome and, on the whole, patients with side-effects more frequently developed these autoantibodies. Anti-nucleosome antibodies, of IgG class, are considered to be a more sensitive marker for SLE than anti-dsDNA antibodies, as they occur at an earlier stage of disease progression than anti-dsDNA antibodies (25). Although there was a significant relationship between anti-dsDNA antibodies of IgG and IgM class and anti-nucleosome antibodies anti-dsDNA antibodies of IgG class were
most frequent and were not preceded by anti-nucleosome antibodies in all cases.

We were unable to identify a suppressive effect on autoantibody production by methotrexate therapy as has been suggested by Boehm et al., (26). However, the number of individuals was low when stratified for methotrexate treatment. Patients with ANA antibodies had significantly higher Disease Activity Score (DAS28) activity at 54 weeks. This could suggest that they also produced autoantibodies that blocked the infliximab therapeutic effects.

An increased frequency of aCL antibodies has been reported in patients treated with infliximab or etanercept by others (5, 27). In this study we did not find any increase in the prevalence of these antibodies. However, there is great variability in the methods of analysis of aCL which could contribute to the contradictory findings (28, 29).

Only six individuals were treated with etanercept and none of them developed autoantibodies or any side effects. There have been reports of lupus-like syndrome as a side-effect also in patients receiving etanercept (5, 6). Increased production of autoantibodies during therapy with etanercept has not been reported at a high frequency (30). Only 5% were reported to develop anti-dsDNA antibodies measured by radioimmunoassay (30). However, 67% of the individuals developing these antibodies were ANA negative.

In vivo neutralisation of TNF-α in RA, as demonstrated here, has a profound stimulatory effect on humoral immunity to DNA and other nuclear antigens. This effect may be accounted for by several possible mechanisms including abrogation of apoptosis and down-regulation of T-helper
cell activity. The induction of autoimmunity to nuclear antigens may also reflect inhibition of cytotoxic cells with potential to suppress autoreactive B-cells as has been proposed as an explanation for SLE-like autoimmunity in chronic graft-versus-host disease (31). Another possibility is that induction of autoimmunity to nuclear antigens reflects interference by anti-TNF-α with the capacity of TNF-α to terminate lymphocyte responses by promoting activation-induced cell death in CD8+ T cells (32). It is also possible that TNF-α may directly modulate the immunogenicity of DNA via effects on serum amyloid P (SAP), complement factors, C1q and C4b which bind chromatin DNA in apoptotic bodies and account for the clearance of DNA. Absence of these mediators in mice leads to the development of anti-nuclear autoimmunity and lupus-like disease (33, 34). It is interesting in this context that TNF-α and IL-6 deficiency does not allow for the release of acute phase proteins such as SAP pointing to a pathway by which reduced TNF-α may aid in the development of lupus (35). Although there are several possible mechanisms underlying anti-dsDNA, anti-nucleosome antibodies and ANA formation in RA patients treated with TNF-α inhibitors it is apparent that these autoantibodies reflect a selective development of antibodies to one particular type of intracellular antigens, i.e., from the nucleus. Thus, it also remains to explain why the altered immune reactivity provoked by anti-TNF-α therapy does not induce autoantibodies to cytoplasmic antigens.

In summary, in this study we have shown that patients treated with infliximab therapy significantly developed autoantibodies such as ANA, anti-dsDNA of IgG and IgM class measured by Crithidia luciliae and anti-nucleosome antibodies. The increase was most evident in patients with side-effects. Methotrexate treatment did not suppress the autoantibody production.
Acknowledgement

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Figure legends.

1A. The titres of anti-dsDNA antibodies of IgG class in 53 infliximab treated RA patients. (Kruskal-Wallis one-way analysis of variance, P< 0.0001, Friedmann two-way analysis of variance, P < 0.0001)

1B. The titres of ANA antibodies in 53 infliximab treated RA patients. (Kruskal-Wallis one-way analysis of variance, P< 0.001, Friedmann two-way analysis of variance, P < 0.001)

1C. The titres of anti-nucleosome antibodies of IgG class in 50 infliximab treated RA patients. (Kruskal-Wallis one-way analysis of variance, P< 0.01, Friedmann two-way analysis of variance, P < 0.0001).

1D. The titres of RF in 53 infliximab treated RA patients. (Kruskal-Wallis one-way analysis of variance, P< 0.001, Friedmann two-way analysis of variance, P < 0.05).
Table 1. Demographic data of the patients in the study. The figures refer to numbers of patients. The age and disease duration are presented with mean values.

<table>
<thead>
<tr>
<th></th>
<th>Infliximab (N=53)</th>
<th>Etanercept (N=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>F/M</strong></td>
<td>43/10</td>
<td>5/1</td>
</tr>
<tr>
<td><strong>Age, years</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>range</td>
<td>54.8 (26 - 79)</td>
<td>48.3 (36-65)</td>
</tr>
<tr>
<td><strong>Disease duration, years</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>range</td>
<td>13.8 (2 - 37)</td>
<td>9.1 (1.3 – 15)</td>
</tr>
<tr>
<td><strong>Corticosteroids,</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(median dose 7.5 mg/day)</td>
<td>30 (57%)</td>
<td>3 (50%)</td>
</tr>
<tr>
<td><strong>Methotrexate,</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5 - 20 mg/week)</td>
<td>43 (81%)</td>
<td>2 (33%)</td>
</tr>
<tr>
<td><strong>other DMARDs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>19 (36%)</td>
<td>2 (33%)</td>
</tr>
<tr>
<td><strong>DAS28² at onset</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mean ± SEM)</td>
<td>5.9 ± 0.14</td>
<td>6.6 ± 0.52</td>
</tr>
<tr>
<td><strong>DAS28² at 54 weeks</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mean ± SEM)</td>
<td>3.7 ± 0.2</td>
<td>3.2 ± 0.33</td>
</tr>
</tbody>
</table>

¹ anti-malarials (3/1), minocycline (1/1), cyclosporin A (4), azathioprine (8), sulphasalazine (2), leflunomide (2) and injectable gold (1); ² DAS28=Disease activity score for 28 joints
Table II. Frequency of antinuclear antibodies, anti-ds-DNA of IgG subclass, anti-nucleosome antibodies and rheumatoid factor (RF) in 53 patients with RA treated with infliximab.

<table>
<thead>
<tr>
<th></th>
<th>At inclusion</th>
<th>14 weeks</th>
<th>30 weeks</th>
<th>54 weeks</th>
<th>p-value(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (%)</td>
<td>N (%)</td>
<td>N (%)</td>
<td>N (%)</td>
<td></td>
</tr>
<tr>
<td>ANA(IF) (ref. &lt;1:100)</td>
<td>12/50 (24)</td>
<td>22/51 (43)</td>
<td>41/53 (77)</td>
<td>31/45 (69)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ds-DNA, IgG (IIF) (ref. &lt;1:10)</td>
<td>1/50 (2)</td>
<td>23/51 (45)</td>
<td>35/53 (66)</td>
<td>20/44 (45)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>anti-nucleosome antibodies, ELISA (ref. &lt; 20RU/mL)</td>
<td>5/50 (10)</td>
<td>nt</td>
<td>13/50 (26)</td>
<td>10/40 (25)</td>
<td>&lt;0.033</td>
</tr>
<tr>
<td>RF, IgM (ref. &lt;1:80)</td>
<td>44/49 (90)</td>
<td>46/51 (90)</td>
<td>nt</td>
<td>36/44 (82)</td>
<td>ns</td>
</tr>
</tbody>
</table>

\(^1\) calculated by Chi-square test for trend. nt=not tested; ns = not significant
Table III. Frequency of anti-ds-DNA of IgM subclass analysed by immunofluorescence, anti-DNA analysed by ELISA and Farr assay and anti-cardiolipid antibodies (aCL) of IgG class in patients with RA treated with infliximab.

<table>
<thead>
<tr>
<th></th>
<th>At inclusion</th>
<th>14 weeks</th>
<th>30 weeks</th>
<th>54 weeks</th>
<th>P-value¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
<td>N</td>
</tr>
<tr>
<td>ds-DNA, IgM (IF)</td>
<td>1/27</td>
<td>(4)</td>
<td>16/27</td>
<td>(59)</td>
<td>23/27</td>
</tr>
<tr>
<td>(ref. &lt;1:10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-DNA-ELISA (ref. &lt;55 IU/ml)</td>
<td>0/20</td>
<td>(0)</td>
<td>0/20</td>
<td>(0)</td>
<td>1/20</td>
</tr>
<tr>
<td>Farr assay (ref. &lt;7 IU/ml)</td>
<td>0/20</td>
<td>(0)</td>
<td>8/20</td>
<td>(40)</td>
<td>5/20</td>
</tr>
<tr>
<td>aCL, IgG (ELISA) (ref. &lt;13.0GPLU/ml)</td>
<td>2/27</td>
<td>(7)</td>
<td>1/20</td>
<td>(5)</td>
<td>1/15</td>
</tr>
</tbody>
</table>

¹ calculated by Chi-square test for trend, nt= not tested; ns= not significant
1A.
Autoantibody formation in patients with rheumatoid arthritis treated with anti-TNF-alpha therapy

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