Heterophile interference accounts for method-specific dsDNA antibodies in patients receiving anti-TNF treatment

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Abstract

Objective. To evaluate analytical explanations for the highly reported incidence of antibodies to dsDNA in patients receiving TNF antagonists.

Methods. Sixty serum samples from patients receiving biological anti-TNF medication were assessed for the presence of dsDNA antibodies using three standard diagnostic platforms [ELISA, IIF and multiplex bead array (MBA)], before and after treatment to block heterophile antibodies. Results were compared with those obtained using serum samples from patients with SLE.

Results. We identified significant method-specific discrepancies in the estimation of dsDNA antibodies in patients receiving TNF antagonists. dsDNA antibodies were frequent according to ELISA and IIF, but rare according to MBA. Blockade of heterophile antibodies resulted in a significant reduction in titres of dsDNA antibodies detected by IIF. In contrast, there was a much greater consistency for dsDNA antibody results in SLE, especially for those present in high titre, and blockade of heterophile antibodies did not result in a change between the two paired samples by IIF or MBA.

Conclusion. There is a significant method-specific variation in the detection of dsDNA antibodies in patients receiving TNF antagonists, due in part to the effects of heterophile antibodies.

Key words: Autoantibodies, Tumour necrosis factor, Heterophile.

Introduction

TNF is a key pro-inflammatory cytokine in the pathogenesis of many inflammatory systemic and dermatological diseases including RA, seronegative arthritides such as AS and PsA, and IBD [1–5]. As a result, various TNF antagonists are now in widespread clinical use for these and other disorders [4, 6]. Infliximab is a chimaeric human–murine anti-TNF antibody that inhibits both soluble monomeric or trimeric TNF and transmembrane TNF. Adalimumab is a fully humanized antibody with similar specificity to infliximab [7]. Etanercept is a fusion molecule made up of the extracellular domain of p55 TNF receptor and the hinge and Fc domain of human IgG1 that inhibits soluble TNF [3, 7, 8]. Overall, >1 million patients worldwide have received one of these agents.

One common complication of TNF antagonist therapy is development of autoantibodies, particularly ANAs and antibodies to dsDNA (dsDNA antibodies). ANAs have been reported in up to 82% and dsDNA antibodies in up to 49% of the patients with RA receiving infliximab [9, 10]. This does not appear to be peculiar to the combination of infliximab and HLA Class II-related autoimmunity since a similar frequency of autoantibodies has been observed in patients receiving infliximab for SpA (ANA: 61.8–88.6%; anti-dsDNA: 17.1–70.6%) [9, 10] and IBD [11, 12].

Understanding the pathogenic significance of dsDNA antibodies induced by anti-TNF treatment is important for making decisions about ongoing therapy. It might also contribute to our understanding of the mechanism of autoantibody formation. Various hypotheses have been proposed to explain this association between anti-TNF agents and autoantibodies. These include...
pro-apoptotic effects of anti-TNF, which could result in immunogenic exposure to self-antigens such as chromatin [13]. TNF antagonists could result in more frequent bacterial infections, which are also powerful stimulants of polyclonal B lymphocyte activation and therefore dsDNA production [14]. TNF blockade has also been suggested to bias T-cell responses towards production of Th2 cytokines, which might promote autoantibody production [15].

These pathogenic hypotheses are not well supported by empirical evidence. Indeed, it remains unclear whether autoantibodies that arise in the context of TNF blockade are pathogenic, since the high incidence of serological autoimmunity is not commonly associated with clinical features of drug-induced lupus [16]. Large surveys suggest that while autoantibodies are common in patients receiving biological TNF antagonists, drug-induced lupus is rare (∼0.2–1%) [16–18]. Furthermore, a lupus-inducing effect of TNF antagonists is at odds with their therapeutic effect in spontaneous lupus [19]. It has been suggested that the low rate of progression to clinical disease is due to the transience of the autoantibody response, and the predominance of dsDNA antibody isotypes other than IgG [10].

In the absence of strong evidence for a pathological effect or a biological mechanism, we investigated analytical rather than biological causes for the presence of autoantibodies in patients receiving biological TNF antagonists. We evaluated sera for the presence of dsDNA antibodies using three standard diagnostic platforms. We also evaluated each assay for sensitivity to the effects of heterophile interference. Heterophile antibodies are a well-known, though underestimated, cause of immunoassay interference. They include anti-animal antibodies that result from an immune responses to iatrogenic stimuli, such as chimaeric mAbs [20,21], especially when proteins from another species are part of the test reagent [22]. Heterophile antibodies are unpredictable, and vary with time in the same patient, and therefore their presence is consistent with satisfactory quality control assessments for an assay [21]. We demonstrated assay-dependent interference by heterophile antibodies in patients on TNF blockers, but not in patients with lupus and therefore bona fide dsDNA antibodies.

**Materials and methods**

**Serum samples**

Sixty consecutive serum samples referred for routine diagnostic assessment of autoantibodies in the context of anti-TNF treatment for IBD, RA and seronegative arthritis were evaluated using the three common immunoassays over a period of 1 year. We also tested 55 serum samples from SLE patients referred for routine autoantibody assessment during the same period using the same three immunoassays. The study was approved by the ACT Health Human Research Ethics Committee and conforms to local standards currently applied.

**dsDNA antibody assays**

Detection of anti-dsDNA was performed by three methods. ELISA (Organtec Diagnostika GmbH, Mainz, Germany) performed using an automated platform (Triturus, Diagnostic Grifols; Barcelona, Spain). IIF on *Crithidia luciliae* substrate (Immuconcepts, Sacramento, CA, USA), evaluated by two independent observers blinded to the clinical data. Slides were assigned a score according to the following scheme: 3+, maximal fluorescence; 2+, less brilliant yellow green; and 1+, if definite kinetoplast pattern was discernible by dim fluorescence, equivocal for very subdued fluorescence and negative. Samples were also analysed using a multiplex bead array analyser (FIDIS connective 10 kit; Biomedical Diagnostics, Marne-la-Vallée, France). Each assay was performed according to the manufacturer’s instructions. Indicative cut-offs for positive results on ELISA and multiplex bead array are those provided by the manufacturers. Untreated sample and heterophile binding tube (HBT)-treated samples were run in parallel as a pair on each of the three assays. The acceptable co-efficient of variation for ELISA and multiplex bead array (MBA) in our laboratory is ≤10%, whereas for IIF, a change of ±1 titre is still within our acceptable limit.

**Heterophile antibody testing**

Heterophile antibodies were removed from sera using the HBT kit (Scantibodies Laboratory, Santee, CA, USA). The method was modified from that described by the manufacturer as follows: 50 µl of phosphate-buffered saline (PBS) was pipetted into each tube of HBT reagent and mixed thoroughly. Diluted HBT was incubated with a serum sample in a ratio of 1:10 (HBT:serum). This modification was validated against the recommended method (which requires 500 µl of serum into one vial of HBT) using sera from groups to be run on the three assays and in each case results were identical with both volumes. Each serum sample was divided into two aliquots. The treated and paired untreated samples were then analysed by three different immunoassays for dsDNA: IIF on *C. luciliae* substrate, ELISA and MBA.

**Statistical analysis**

The results were analysed with MedCalc software (Mariakerke, Belgium) and Microsoft Excel (Washington, USA). The differences between the pre- and post-HBT-treated samples were analysed using Wilcoxon rank test (for non-parametric paired samples).

**Results**

**Frequency of dsDNA antibody detection among patients on anti-TNF**

We evaluated serum samples from 60 patients on anti-TNF therapy referred to the routine diagnostic laboratory to test for dsDNA antibodies. For 36 patients (60%), the indication for anti-TNF treatment was IBD, and the other 24 (40%) had either RA (n = 8, 13.3%) or seronegative arthritis (n = 16 with AS or PsA; 26.7%). The majority of
patients were on infliximab (n = 34, 94.4%, with arthritis and n = 21, 87.5%, with IBD). We also analysed 55 SLE patients. The anti-TNF group had a mean age of 43.4 years (19–74) and the male to female ratio was 0.4. The mean age of the SLE cohort was 43.45 (20–86), and the male to female ratio was 0.1.

Overall, 52/60 (86.7%) serum samples from patients receiving anti-TNF treatment were positive for dsDNA antibodies by at least one method. Thirty-one (86.1%) of 36 patients with IBD treated with anti-TNF were dsDNA antibody positive by at least one method and 21/24 (87.5%) patients with inflammatory arthritis were positive by at least one method (Table 1).

Discrepancy of dsDNA detection between the three methods

We identified significant discrepancies in both the magnitude of antibody titres and prevalence of dsDNA antibodies when assessed by different methods (Fig. 1). Overall, out of the 60 serum samples, 48 (80%) were positive by ELISA compared with 31 (51.7%) by IIF and only 2 (3.3%) by MBA. Twenty-one (35%) of 60 serum samples from patients receiving anti-TNF treatment were positive by ELISA only (IBD: 41.2%; RA: 42.9%; seronegative arthritis: 28.6%). In contrast, 6.7% were positive by IIF only (IBD: 5.9%; RA: 28.6%; seronegative arthritis: 0%). Only two serum samples were positive by MBA, and in both samples, the antibodies were present in only low titre and both were also positive by the other two methods (Table 1 and Fig. 1A).

The frequency of dsDNA antibodies was very high among patients receiving infliximab in all three disease groups (IBD: 88.2%; RA: 100%; seronegative arthritis: 85.7%). Only two patients were on etanercept of whom one had anti-dsDNA antibodies. Three patients were on adalimumab, of whom two had anti-dsDNA antibodies detected by at least one method (Table 1).

Method-specific detection of dsDNA antibodies in SLE sera

For comparison, we evaluated sera from 55 patients with SLE, where dsDNA antibodies are expected to be true
positives. In contrast to the discrepancy seen in dsDNA antibody results from different immunoassays among patients receiving anti-TNF therapy, in SLE sera there was much greater consistency of dsDNA antibody results across the three diagnostic platforms, especially in patients with high-titre antibodies (Table 1 and Fig. 1B). Only 12 (21.8%) were positive exclusively by ELISA, 2 (3.6%) were positive by IIF only and none of the sera were positive by MBA alone.

Effect of heterophile antibodies on anti-dsDNA antibody assays

Given the significant method-specific discrepancies for the dsDNA antibody results for patients receiving TNF antagonists, we decided to test whether this could be accounted for at least in part by the presence of heterophile antibodies in the sera of patients on TNF antagonists. Serum samples from the same patients were evaluated to determine the effect of pre-incubating the sample with heterophile blocking reagents. The median titre of dsDNA antibodies obtained by ELISA was 67.5 U/ml before and 56 U/ml after treatment with heterophile blocking reagent \( P = 0.0001 \) (Fig. 2A). By IIF, the titre of dsDNA antibodies also fell significantly \( P = 0.0005 \) (Fig. 2B). In contrast, almost all samples were negative even before heterophile blocking when evaluated by MBA, and they remained negative after treatment with HBT. Of the two positive samples on MBA, one became negative and the other remained low positive (data not shown).

In order to exclude the trivial explanation that a change in titre occurred simply due to serum dilution, representative serum samples were incubated 10:1 (vol.:vol.) with HBT or 10:1 (vol.:vol.) with PBS. As before, HBT resulted in a significant reduction in dsDNA antibodies (by IIF), whereas PBS dilution had no effect (data not shown).

To test whether the heterophile antibodies are targeted predominantly to mouse immunoglobulin, as infliximab contains murine epitopes, five samples from the anti-TNF cohort and five from the SLE cohort were either pre-incubated with normal mouse sera, or treated with HBT to remove heterophile antibodies, then each sample was assayed for dsDNA antibodies by IIF. Each test was performed in triplicate. Consistent with results obtained from the entire cohort, heterophile antibodies contributed to dsDNA antibodies detected in patients on anti-TNF treatment (and not SLE sera), but incubation with normal mouse sera had almost no effect on either type of the sample (Fig. 3).

Method-specific effects of heterophile antibodies in SLE sera

We also tested for possible effects of heterophile antibodies on assays of dsDNA antibodies in SLE sera. HBT treatment resulted in a reduction in dsDNA antibody titres measured by ELISA (82–68.4 U/ml, \( P = 0.0001 \); Fig. 4A). In comparison, paired pre- and post-treatment sera run on IIF were similar. Most samples showed no change, and none varied by more than one titre. Results obtained with MBA showed no significant change after treatment for heterophile antibodies (\( P > 0.5 \); Fig. 4B and C).

Fig. 3 Effect of heterophile antibody blockade on dsDNA titres by treatment with mouse serum.

Antibody titres were expressed on a 0–3+ scale as follows: 0 = negative, 1+ = equivocal, 2+ = low positive, 3+ = positive.
dsDNA antibodies are usually highly specific for SLE but are common in patients receiving TNF antagonists [11, 17]. If their presence in the context of anti-TNF therapy represents a true autoimmune response, their detection would be a significant contra-indication to ongoing but otherwise highly effective therapy [10]. We detected anti-dsDNA antibodies in 86.6% of the patients receiving anti-TNF across three different indications. This is higher than that reported previously. However, our study was not designed to assess autoantibody prevalence, but rather to explain their presence. We analysed samples referred for assessment for autoantibodies, rather than serum obtained from an unselected cohort of patients on TNF antagonists, which probably explains the high incidence. We found that most of these dsDNA autoantibodies are biological false positives. The majority of our patients had IBD, but the findings were similar in all patient groups. Our findings are significant, since they suggest that antibodies to dsDNA in patients receiving anti-TNF are not of the same biological significance as they are in the context of SLE, and therefore are very unlikely to represent a contra-indication to therapy.

The main clue to a significant difference between the nature of dsDNA antibodies detected in patients receiving TNF antagonists and those in lupus was a major method-specific discrepancy in their detection in patients on TNF antagonists. In the latter group, positive results were obtained for many samples by ELISA, fewer samples by IIF and only rarely by MBA. In contrast, in lupus patients, there was a far greater consistency of results obtained by all three methods (Fig. 1). Even when taking into account the variability of the assay (intra-assay co-efficient of variation of 10% between paired samples), we observed a significant effect of heterophile antibodies on ELISA (Fig. 2A). There was a statistically significant reduction in antibody titre measured by ELISA after removal of heterophile antibodies. Similarly, removal of heterophile antibodies resulted in a significant reduction in antibody titres determined by IIF ($P < 0.0001$; Fig. 2B). IIF is generally considered to be more specific for dsDNA antibodies than ELISA, with less interference by heterophile antibodies. Our data are consistent with this conclusion in sera from patients with SLE, but the same does not appear to hold true for patients on anti-TNF agents. In contrast, assessment of dsDNA antibodies by MBA immunoassay appears to be relatively unaffected by heterophile antibodies.

Removal of heterophile antibodies also resulted in an overall reduction in titres of dsDNA antibodies in lupus sera measured by ELISA. One interpretation of this finding is that because ELISA detects antibodies with a range of affinities, and heterophile antibodies also contribute to the pool of lower affinity anti-dsDNA antibodies in lupus sera, which are detectable by ELISA, whereas they account for the higher affinity antibody pool in patients on anti-TNF treatment. Therefore, blocking heterophile antibody activity by HBT in both groups would be expected to cause a reduction in the dsDNA titre detected by ELISA. In contrast, the pre- and post-treated lupus sera contained similar titres of dsDNA antibodies when measured by more specific immunoassays such as IIF and MBA ($P > 0.5$, MBA), consistent with the expectation that these tests detect predominantly true autoantibodies.

Of the TNF antagonists in widespread clinical use, infliximab has been most strongly associated with dsDNA antibodies and a risk of drug-induced lupus. Infliximab is a
chimaeric human–murine fusion and therefore is expected to induce human–mouse antibodies. Indeed, counteracting the anti-mouse response is necessary to maintain the therapeutic efficacy of infliximab [23]. Although the majority of patients included in this report were receiving infliximab, anti-dsDNA antibodies were also observed in the small number of individuals receiving fully humanized antibodies (adalimumab). Indeed, one out of two patients receiving etanercept and two of three patients on adalimumab were dsDNA antibody positive. These results are consistent with other evidence that all forms of TNF blockade induce dsDNA antibodies [17, 24]. This raises the possibility that TNF antagonism in addition to the immunogenicity of a heterologous fusion protein might somehow facilitate this heterophile response. On the other hand, the absence of heterophile antibodies in sera from lupus patients suggests that immunosuppression per se is unlikely to account for their formation.

Irrespective of the mechanism of heterophile antibody induction, we have shown a substantial method-specific difference in sensitivity for detecting dsDNA antibodies in patients on TNF antagonists. Consistent with the previous reports, the effect of heterophile antibodies varies from assay to assay [20, 21]. To our knowledge this is the first report on heterophile interference on dsDNA antibody detection, especially by IIF. Heterophile interference of immunoassays is known to be method specific, and MBA seems to be less perturbed by such interference. On the other hand, simple ELISA suffers from low specificity rather than heterophile interference.

While further longitudinal studies will be necessary to confirm that the majority of dsDNA antibodies arising in the context of anti-TNF therapy are biological false positives, the results presented here provide significant reassurance. On the strength of these results, we suggest measurement of dsDNA antibodies by more than one method and consideration of heterophile antibody blockade when testing for serological autoimmunity in patients on TNF antagonists before deciding on their correlation with drug-induced lupus.

**Rheumatology key messages**

- There is substantial method-to-method variation in the detection of anti-dsDNA antibodies in patients receiving anti-TNF treatments.
- dsDNA antibodies are common by ELISA and IIF, but rare with multi-addressable bead assays.
- Heterophile antibodies are partially responsible for this analytical error.

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