Evolution of Antinuclear Antibodies and Clinical Patterns in Patients with Active Rheumatoid Arthritis with Longterm Infliximab Therapy

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ABSTRACT. ***Objective.*** To investigate the effect of longterm infliximab therapy on serum levels of fluorescent antinuclear and anti-double and single-stranded DNA antibodies (FANA, anti-dsDNA, anti-ssDNA) in patients with rheumatoid arthritis (RA), and their possible association with clinical evolution.

Methods. Sera from 58 RA patients, treated for one to 3 years with infliximab, were retrospectively analyzed. Matched control groups were RA patients treated with corticosteroids or methotrexate. FANA were tested using HEp-2 cells, and anti-dsDNA and anti-ssDNA IgG by ELISA. After 28 months of infliximab therapy, clinical status was evaluated in 43/58 patients with uninterrupted therapy and associations with autoantibody levels were investigated. Data were documented for patients who discontinued infliximab.

Results. Over the 3 year period, significant increases in FANA and anti-ssDNA IgG levels were observed in infliximab treated patients ($p < 0.001$ and $p < 0.01$, respectively). In 43 patients with an uninterrupted infliximab regimen, association was found between high FANA ($\geq$ 1/1280) and lower age ($p = 0.048$) and patient's assessment of infliximab's efficacy ($p = 0.014$). Three patients developed anti-dsDNA IgG, preceded by high anti-ssDNA IgG levels, and one of them developed a lupus-like syndrome. Neither the initial presence of high FANA levels nor their increase $\geq$ 1/1280 was significantly associated with discontinuation of infliximab. In contrast, at baseline ($p = 0.0012$) and at the time of infliximab discontinuation ($p = 0.0078$), anti-ssDNA IgG ($\geq$ 500 arbitrary units) were more frequent in 7 patients who stopped infliximab due to skin or systemic anaphylactoid reactions.

Conclusion. Monitoring of serum FANA, anti-dsDNA, and anti-ssDNA IgG antibodies provided predictors of lupus-like symptoms and/or anaphylactoid reactions in patients with RA. (J Rheumatol 2006;33:24–30)

Key Indexing Terms:
TUMOR NECROSIS FACTOR-α INFLIXIMAB RHEUMATOID ARTHRITIS
ANTINUCLEAR ANTIBODIES ANTI-DNA ANTIBODIES

Better understanding of immune mechanisms underlying rheumatoid arthritis (RA) has led to new strategies such as tumor necrosis factor-α (TNF-α) modulation that constitute a revolution in therapy[1-3]. Current information on longterm tolerance of anti-TNF-α treatments is limited, and concerns include the development of neoplasia and autoimmune manifestations[4,5]. Increased autoantibodies have been commonly reported in patients treated with anti-TNF-α agents, and more than 20 secondary cases of lupus syndromes have been reported[1,6-10]. Sporadic lupus cases were observed in patients with RA and Crohn's disease treated with infliximab, and in patients with RA and idiopathic juvenile arthritis treated with etanercept, while none has been reported so far in RA patients treated with adalimumab or in anti-TNF-α treated patients with ankylosing spondylitis (AS)[7-18]. Little is known about clinical significance of increased fluorescent antinuclear antibodies (FANA) and/or anti-DNA antibodies, particularly in the long term, and their possible relationships with the clinical response to therapy[19]. We retrospectively investigated the influence of longterm infliximab therapy (1–3 years) on the levels of FANA, anti-double-stranded DNA (anti-dsDNA), and anti-single-stranded DNA (anti-ssDNA) antibodies in a group of 58 patients with RA, and their possible associations with the clinical outcome.

MATERIALS AND METHODS

Patients. Table 1 summarizes characteristics of RA patients. All fulfilled

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Table 1. Characteristics of patients with RA.

Treatment	Group 1, Infliximab, n = 58	Group 2, CS, n = 37	Group 3, MTX, n = 56
Women, no. (%)	44 (75.8)	29 (78.4)	44 (78.6)
Men, no. (%)	14 (24.2)	8 (21.6)	12 (21.4)
Mean age, yrs ± SD	54 ± 12	57 ± 14	57 ± 14
Disease duration, yrs ± SD	13 ± 9	> 5	> 5

CS: corticosteroids, MTX: methotrexate.

the classification criteria of the American College of Rheumatology (ACR) and were recruited retrospectively to the study by one rheumatologist investigator[20]. Ethical issues were approved by the local ad hoc committee.

Group 1 consisted of 44 women and 14 men with refractory RA treated with infliximab. Data included age at the time of clinical evaluation and disease duration (baseline). They received 3 mg/kg infliximab intravenously at Weeks 0, 2, and 6 and every 8 weeks thereafter, in combination with methotrexate (MTX), corticosteroids (CS), or leflunomide in 25, 40, and 15 patients, respectively. According to the response to therapy, infliximab posology could thereafter have been increased or the perfusion advanced. Inflammatory status was documented by C-reactive protein (CRP) and erythrocyte sedimentation rate measurements. Sera obtained at baseline and during the 3 years of therapy were used for autoantibody studies. After 28 months of infliximab therapy, clinical evaluation was performed in the subgroup of 43/58 patients with uninterrupted infliximab treatment, and the context of infliximab discontinuation was documented in the subgroup of 15/43 patients who stopped. The occurrence of anti-TNF-α-induced lupus was considered in patients with at least 4 lupus criteria of the revised ACR classification[21]. Group 2 consisted of 37 control patients with RA of at least 5 years' duration diagnosed according to the ACR criteria as above, and treated with CS. Group 3 consisted of 56 control RA patients diagnosed as above for more than 5 years and treated with MTX.

Serum FANA measurement. Fluorescent antinuclear IgG antibodies were measured in an indirect immunofluorescence assay on fixed HEp-2 cells (ref. FA 1520-5010, Euroimmune, Bioadvance SA, Emerainville, France) using 1/80, 1/320, and 1/1280 serum dilutions. FANA levels were rated according to the last dilution with significant fluorescence, as low (1/80), moderate (1/320), or high (> 1/1280). Morphological fluorescence patterns were as described[22].

Anti-dsDNA and anti-ssDNA IgG antibodies were measured using an ELISA as described with modifications[23]. Briefly, protamine sulfate treated polystyrene microplates (ref. 269620, Nunc, VWR, Fontenay-sous-Bois, France) were coated with calf thymus DNA (ref. D-1501, Sigma-Aldrich) in phosphate buffered saline. In order to verify the native structure of DNA in the anti-dsDNA IgG assay, half of the DNA suspension was denatured by heating (100°C, 10 min) and quick cooling (in ice), and used to detect anti-ssDNA antibodies. In each microplate, specificity controls consisted of 3 previously characterized sera, i.e., one lacking detectable anti-ssDNA and anti-dsDNA antibodies, one containing anti-ssDNA without anti-dsDNA antibodies, and one exhibiting both specificities. Each serum dilution was tested in triplicate. After incubation and washing procedures, bound IgG antibodies were revealed with an alkaline phosphatase labeled anti-human-gamma chain goat antibody (ref. H10008, Caltag, TEBU, Le Perray-en-Yvelines, France) and paranitrophenyl phosphate as substrate. Results obtained in optical densities (OD) were expressed in arbitrary units (AU) using standard curves obtained on the same plates with serum previously characterized from a patient with lupus. The technical cutoffs, defined as the mean + 3 SD of values obtained in sera from 30 control blood donors, were 100 AU for anti-dsDNA and 200 AU for anti-ssDNA antibodies. Anti-ssDNA antibody levels were defined as absent (< 200 AU), moderate (200–499 AU), high (500–999 AU), or very high (≥ 1000 AU).

Statistical analysis. The number of patients for whom sera were examined is given for each period of time. Variance comparisons between groups were performed using the nonparametric Mann-Whitney test, chi-square test, or Fisher's exact test when suitable. Significance of linear correlation was estimated using r coefficients; p values < 0.05 were considered significant.

RESULTS

Evolution of FANA in infliximab treated patients. Sequential determinations of FANA during infliximab therapy are depicted in Figure 1A. At baseline, 10/58 patients had no significant FANA levels, 46 had low or moderate levels, and high levels were observed in 2 patients. At Month 6, all Group 1 patients exhibited FANA, with high levels in 26.3%. At Months 31–36, such high levels were observed in 9/17 patients. Over the 3 year period, most Group 1 patients exhibited a significant increase in FANA values ($p < 0.001$). In contrast, FANA stayed at low levels following longterm therapy with CS (Group 2) or MTX (Group 3) ($p > 0.05$). In Group 1 patients, immunofluorescent detection of FANA > 1/1280 resulted in nuclear homogenous and finely speckled HEp-2 cell patterns. Three infliximab treated patients exhibited significant anti-dsDNA IgG antibodies, while none was detected in patients treated with either CS or MTX. Anti-ssDNA IgG levels are presented in Figure 1B. In Group 1, at baseline, 44/58 patients had no significant anti-ssDNA IgG, 11/58 had low levels, and only 2/58 and 1/58 exhibited high and very high levels, respectively. At Month 6 of therapy, 8/19 patients exhibited high anti-ssDNA IgG. Over the 3 year period, most patients of Group 1 had significant increases of anti-ssDNA IgG ($p < 0.01$). In contrast, only 3/35 patients of Group 2, and none in Group 3, had high anti-ssDNA levels ($p > 0.05$). In Group 1 no correlation was found between FANA and anti-ssDNA IgG levels at each study time ($p > 0.05$).

Lower age and positive personal assessment of infliximab therapy were associated with elevated FANA levels in patients with uninterrupted therapy. The clinical response to infliximab therapy was evaluated in 43 patients with uninterrupted treatment: 35 women and 8 men, mean age 54 ± 12 SD years, mean disease duration 15 ± 9 SD years at the time of clinical evaluation, mean duration of infliximab treatment 28 ± 11 SD months; there was association of infliximab with MTX, leflunomide, and disease modifying antirheumatic drugs in 26, 10, and 38 patients, respectively. As shown in

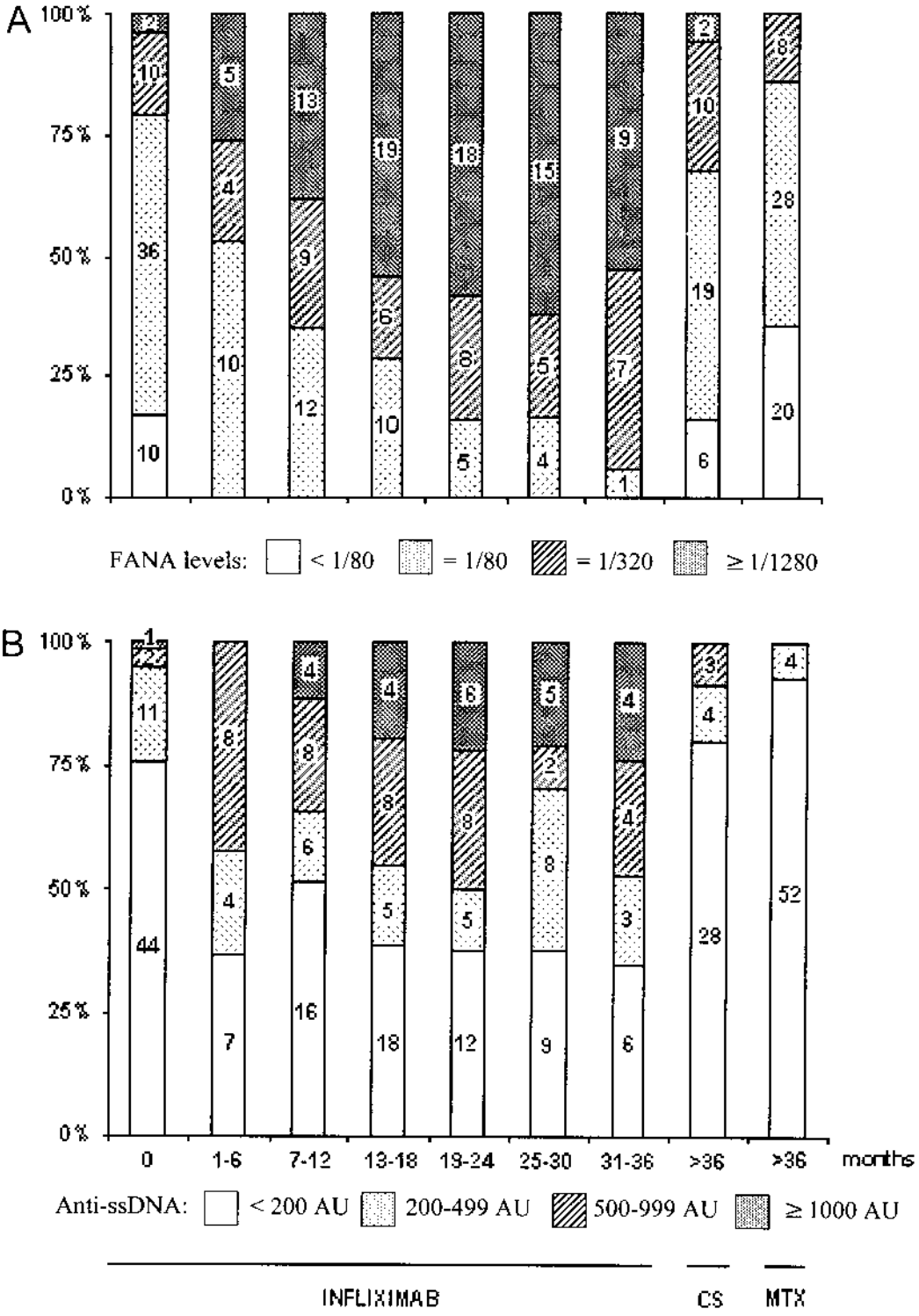

Figure 1. Serum FANA and anti-ssDNA IgG antibodies in RA patients treated with infliximab and in control RA patients treated with corticosteroids (CS) and methotrexate (MTX). Results are expressed in numbers and percentages of patients for each period of time.

Table 2, an association of high FANA levels was found with age ($p = 0.048$) and with positive personal assessment of efficacy of infliximab ($p = 0.014$). A concomitant treatment with MTX was not associated with lower FANA level ($p > 0.05$). No clinical association was found with high anti-ssDNA levels ($p > 0.05$).

Higher anti-ssDNA IgG antibody levels were present in RA patients who discontinued infliximab due to anaphylactoid reactions. In 15 Group 1 patients, infliximab was stopped due to therapeutic inefficacy or manifestations possibly due to therapy (Table 3). In these patients, baseline FANA levels did not differ from patients with an uninterrupted therapy regimen ($p = 0.42$). In contrast, anti-ssDNA IgG levels > 500 AU were more frequent at baseline in patients who stopped infliximab than in those who did not ($p = 0.012$). Three patients stopped because of inefficacy, as defined by the referring rheumatologist. Four patients experienced severe infections, including 3 with bacterial pneumonia (*Streptococcus pneumoniae*) and one with septic shock due to *Staphylococcus aureus*. After an 18 month period of improvement, one male patient developed a lupus-like syndrome with 3 ACR lupus criteria, with intense atypical migratory arthralgia of unusual topography and severe asthenia, which increased despite higher dose therapy. He had no cutaneous or other systemic symptoms. Kidney and liver functions were normal. He had high FANA levels (> 1/1280), very high anti-ssDNA IgG antibody levels (> 1400 AU), and low but significant anti-dsDNA IgG antibody levels (250 AU). Clinical symptoms lasting one month in spite of interruption of infliximab were controlled by 4 months' CS therapy, with unmodified FANA levels. Subsequent etanercept therapy for 6 months was not associated with clinical symptoms or significant decreases in ANA levels (FANA > 1/1280, anti-ssDNA > 1400 AU, anti-dsDNA = 150 AU).

Seven patients, including 6 women, had to stop infliximab because of reactions during or within minutes after the infliximab infusion (rashes, arm edema and myalgia, or systemic anaphylactoid reaction). Compared with other patients treated with infliximab, their FANA were not increased at baseline or at the time of stopping infliximab ($p > 0.05$; Table 3). In contrast, high anti-ssDNA IgG levels (≥ 500 AU) were more frequent in this group than in other infliximab treated patients, both at baseline and at the time of interruption ($p = 0.0012$ and $p = 0.0078$, respectively; Table 3). One woman with anaphylactoid reaction had very high anti-ssDNA IgG levels (> 1200 AU) and low but significant anti-dsDNA IgG antibodies (100 AU) at baseline. After 11 months' treatment, anti-dsDNA IgG reached 440 AU, and after 16 months she experienced arm edema and myalgia attributed to infliximab, which was discontinued. Two years later, this patient still has anti-dsDNA IgG antibodies at 300 AU while under therapy with CS and MTX.

DISCUSSION

In this study, FANA and anti-DNA antibody levels and their

Table 2. Clinical evaluation in 43 RA patients with uninterrupted infliximab therapy and high FANA and/or high anti-ssDNA IgG antibody levels.

	Patients with FANA > 1/1280	Patients with Anti-ssDNA > 500 UA
No. of patients	27	17
Women/men	21/6	14/3
Age of patients at time of clinical evaluation, mean yrs ± SD	52 ± 11*	55 ± 13
Disease duration at time of clinical evaluation, mean yrs ± SD	14 ± 9	16 ± 9
Duration of infliximab treatment, mean mo ± SD	27 ± 10	28 ± 12
Personal assessment of infliximab efficiency (VAS), median (range)	2.2 (1–5.1)**	2.7 (0–5.1)
Doctor's assessment of disease activity (VAS) median (range)	4.1 (1.2–8.4)	4.1 (0.5–8.4)
Pain intensity at day of perfusion (VAS), median (range)	4.4 (0–8)	4.4 (0–8)
Morning stiffness, min, median (range)	30 (0–240)	30 (0–240)
Nocturnal awakenings, n, median (range)	0 (0–5)	1 (0–5)
Spontaneously painful articulations†, median (range)	2 (0–42)	2 (0–42)
Painful articulations after palpation†, median (range)	5 (0–28)	4 (0–28)
Synovitis, no./patient†, median (range)	2 (0–24)	2 (0–24)
Erythrocyte sedimentation rate, mm, median (range)	24 (5–80)	18 (2–78)
C-reactive protein, mg/l, median (range)	12 (0–112)	11 (0–112)
Associated MTX treatment (no. of patients)	16	ND
Associated leflunomide treatment (no. of patients)	6	ND
No DMARD associated treatment (no. of patients)	4	ND

Significant difference in patients with FANA < 1/1280: * $p = 0.048$ and ** $p = 0.014$. † In 44 articulations studied. ND: non determined, VAS: visual analog scale of 0 (no pain) to 10 (intense pain). MTX: methotrexate, DMARD: disease modifying antirheumatic drug.

Table 3. Characteristics of patients with RA treated with infliximab, showing fluorescent antinuclear antibody (FANA) and anti-ssDNA IgG serum levels as a function of infliximab discontinuation.

	Discontinuation of Infliximab (1 lupus-like syndrome excluded), n = 14						Uninterrupted Infliximab Treatment, n = 43	
	Inefficacy, n = 3		Infection, n = 4		Anaphylactoid Skin Reaction, n = 7			
Women/men	2/1		1/3		6/1		35/8	
Mean age, yrs, ± SD	63 ± 10		59 ± 8		57 ± 11		54 ± 12	
Duration of infliximab treatment, mo	11.6 (8–14)		14.3 (11–18)		10.8 (6–18)		14.2 (1–18)	
FANA (last positive dilution)	Baseline	Stop*	Baseline	Stop	Baseline	Stop	Baseline	12–18 mo
< 1/80	1	1	0	0	0	0	9	0
1/80	2	1	3	2	6	2	24	11
1/320	0	0	1	1	0	2	10	16
> 1/1280	0	1	0	1	1	3	0	16
Anti-ssDNA, antibody units								
< 200	3	2	3	4	2	1	37	20
200–499	0	0	1	0	2	0	6	9
> 500	0	1	0	0	3	6	0	14

Results are numbers of patients. * At the time infliximab was stopped.

possible influence on clinical evolution were investigated retrospectively in 58 patients with RA undergoing longterm infliximab treatment and compared with control RA patients treated with CS and MTX. In patients with an uninterrupted infliximab regimen, lower age and positive personal assessment of therapy were found to be associated with high FANA levels. In patients in which infliximab was discontinued due to localized rash/edema or systemic anaphylactoid reactions, high anti-ssDNA IgG antibody levels were present both at baseline and at the time of stopping infliximab.

Our data expand previous reports[1,6-10] by providing sequential quantitative FANA evaluation. A noncommercial ELISA was used to detect anti-dsDNA antibodies of the IgG class, which appear to be more specific in predicting renal failure in lupus, while IgM antibodies may represent natural antibodies induced or amplified during inflammatory, infectious, or lymphoproliferative disorders[24]. Although less specific than anti-dsDNA IgG antibodies in lupus, anti-ssDNA IgG were measured, since they are commonly observed at very high levels in active phases and persist during periods of low disease activity[24]. In addition, their determination provides a control for the absence of DNA denaturation in our anti-dsDNA antibody ELISA.

Groups 1, 2, and 3 were closely matched for age, sex ratio, and disease duration. At baseline, the ratios of patients without FANA in Group 1 and control Groups 2 and 3 (17.2%, 16.2%, and 35.7%, respectively) were lower than in a previous study[25], in which 58.8% of RA patients were FANA-negative, 38.5% had low or moderate levels, and only 2.7% had high levels (i.e., > 1/320), possibly due to recent improvement of HEp-2 test sensitivity. Increase in FANA levels in the course of anti-TNF-α therapies was commonly reported, with some variations according to series[6,7,11,26,27]. In 2000, a retrospective, open-label study on RA treated with infliximab showed a 29% to 53% increase in FANA-positive patients compared to 30% to 32% in the control group[7]. A FANA increase over 1/40 was also observed in patients with AS, from 17.1% to 82.3% after 30 weeks' infliximab, and in Crohn's disease, from 7.2% to 56.8% after 2 years[11,19]. Lower increases were also observed under etanercept and adalimumab therapies[3,28]. In our study, an increase of FANA in infliximab treated RA patients was very significant from Month 6 to Month 30, with homogenous and finely speckled FANA patterns and strong chromatin staining, similar to patients with lupus[22].

The first occurrence of anti-dsDNA antibody during anti-TNF-α therapy was described in 1993 in 2/20 patients[1]. Anti-dsDNA antibodies were subsequently found at low levels in 16% (*Crithidia lucilia* fluorescence test, CLIFT) and at high levels in 4% (commercial Farr assay) of infliximab treated RA patients[29]. Antibody isotype characterization revealed anti-dsDNA IgM in 22/156 (14%) patients and anti-dsDNA IgG, IgA, and IgM in one woman who developed a lupus syndrome[7]. Recently, anti-dsDNA IgM were detected in 11% of RA patients (by CLIFT), 17% of AS patients (commercial ELISA), and 13% (commercial ELISA) or 11% (CLIFT and commercial Farr assays) of Crohn's patients treated for 2 years[11,19]. In RA patients treated with etanercept and adalimumab, anti-dsDNA antibodies were observed in 15% and 3.9% of patients, respectively (commercial Farr assay)[2,3]. In our study, only 3/58 patients (5%) developed anti-dsDNA IgG antibodies, which preced-

ed the appearance of symptoms possibly associated with lupus in 2 patients. In all cases, the occurrence of anti-dsDNA IgG was preceded by high level anti-ssDNA IgG.

In one study, anti-ssDNA antibodies (by commercial ELISA) were surveyed in infliximab treated Crohn's patients, showing a clear increase in 17/71 (39.5%) FANA-positive patients[11]. Neither anti-ssDNA isotypes nor clinical associations were given[11]. In our study, a significant increase in anti-ssDNA IgG was observed in almost 50% of Group 1 patients. Seventy-four percent of patients with high FANA levels exhibited high anti-ssDNA IgG levels. In other patients, FANA targets could be histones and/or nucleosomal structures, and no correlation was found between FANA and anti-ssDNA antibody levels ($p > 0.05$)[30]. Associated with infliximab, MTX did not influence the increase in autoantibodies ($p > 0.05$), as previously reported in patients with RA[7].

In Group 1, only the patient's and not the rheumatologist's positive assessment of efficacy of infliximab was associated with higher FANA levels. As in one other report, neither the initial presence of FANA nor their increase at values ≥ 1/1280 was associated with interruption of treatment[31]. In contrast, high anti-ssDNA IgG levels (≥ 500 AU) were more frequent in Group 1 patients who stopped infliximab for rash or systemic anaphylactoid reaction than in other infliximab treated patients, both at the onset of therapy and at the time of stopping infliximab. This identifies sequential anti-ssDNA IgG monitoring as a possible predictor of rash or adverse systemic reactions in infliximab treated patients with RA.

Only one Group 1 patient developed a lupus-like syndrome. While he presented only 3 ACR criteria, lupus was supported by the appearance of migratory arthralgias associated with anti-dsDNA IgG, both worsening with increased dose of infliximab and improving with CS therapy. The role of TNF-α inhibition in the occurrence of lupus is supported by previous observations of lupus induction in patients with RA and Crohn's disease[8,9,11,12]. The frequency of lupus may be underestimated in cases with mild presentation, since the predominant clinical symptoms include asthenia, rashes, photosensitivity, arthralgia, edema, myalgia, pleuritis, and pericarditis, without renal disorders, which are not restricted to lupus[7-17]. Before the use of TNF-α therapy, reports of an association of RA with lupus symptoms ("rhupus syndrome") were uncommon. A retrospective study over 11 years detected only 6/7000 (0.09%) patients simultaneously presenting sufficient criteria for both RA and lupus, suggesting a mere coincidence[32]. However, another study of 11 lupus cases in patients with RA suggested that the concurrence of RA and systemic lupus erythematosus (SLE) was not as rare as previously considered[33].

A direct role for TNF-α modulation in the pathogenesis of lupus is supported by experimental and clinical evidence. Low TNF-α production was observed in NZB×NZW F1 mice, a lupus experimental model, in which development of nephritis was reduced by TNF-α supply[34]. Lupus patients with lower *in vitro* TNF-α production by blood mononuclear cells and decreased TNF-α/TNF-α receptor ratios exhibited more renal damage[35-37]. Apoptosis appears to be a central mechanism triggering lupus, since infliximab binding on cellular membranes could lead to activation of apoptosis and release of nuclear antigen[38]. CRP, which plays a role in apoptotic fragment-clearing, strongly decreases in patients treated with anti-TNF-α, and could result in an important increase of nuclear antigens[8]. However, etanercept, a chimeric TNF-α receptor with a lower apoptosis-inducing capacity than infliximab, has also been considered a lupus inducer, and thalidomide, which exhibits anti-TNF-α activity, has been successfully used in treatment of SLE[39]. This suggests the alternative involvement of mechanisms such as indirect release of the Th2 cytokines interleukin 4 (IL-4) and IL-10, resulting in B cell activation and production of autoantibodies[40].

Our data from patients with RA undergoing longterm infliximab treatment suggest that sequential assessment of serum FANA, anti-dsDNA, and anti-ssDNA IgG antibodies provides predictors of a patient's personal assessment of the efficacy of infliximab, of possibly overlooked lupus-like symptoms, and/or of skin/systemic anaphylactoid reactions.

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