Peripheral blood lymphocytes from patients with rheumatoid arthritis are differentially sensitive to apoptosis induced by anti-tumour necrosis factor-alpha therapy

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Abstract

Objective

The efficacy of anti-tumour necrosis factor-alpha (TNF-alpha) therapies in rheumatoid arthritis (RA) has been mainly attributed to TNF-alpha neutralisation. Other mechanism as immune cell apoptosis, which is impaired in RA, may also be induced by anti-TNF-alpha therapies. The aim of our study was to investigate whether TNF-alpha inhibitors could induce apoptosis in vitro of the peripheral blood lymphocytes of RA patients.

Methods

Peripheral blood mononuclear cells (PBMC) isolated from 24 patients with RA and 18 healthy donors were incubated with anti-TNF-alpha agents, infliximab or etanercept, in comparison with no agent and including an isotypic control, for 48 hours. Apoptosis was detected and quantified by annexin V labelling of phosphatidylserine externalization using cytofluorometric analysis and compared with PBMC production TNF-alpha in vitro.

Results

In healthy donors, induced apoptosis was observed in 0.3% to 3.8% of lymphocytes with both therapies. In RA patients the treatment induced lymphocyte apoptosis in 17 of 24 patients with a percentage of annexin V-positive lymphocytes ranging from 0.1% to 25%. Among these 17 RA patients, a significant in vitro lymphocyte apoptosis (> 4%) was observed in 11 patients (46%) compared with healthy donors ($p < 0.01$). The variability of the response to anti-TNF-alpha within the RA population was not dependent on TNF-alpha synthesis or disease activity.

Conclusions

In vitro induction of lymphocyte apoptosis by anti-TNF-alpha was observed in a subgroup of RA patients. Based on these data, it would be of interest to further study the interindividual variations of sensitivity to apoptosis induced by TNF alpha inhibitors in relation to treatment efficacy or resistance observed in RA patients.

Key words

Anti-TNF-alpha therapies, rheumatoid arthritis, lymphocytes, apoptosis.

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Abbreviations:

CD:	Crohn's disease
DAS 28:	Disease Activity Score in 28 joints
ELISA:	enzyme-linked immunosorbent assay
EDTA:	ethylenediamine tetraacetic acid
FITC:	fluorescein isothiocyanate
PBMC:	peripheral blood mononuclear cells
PBS:	phosphate buffered saline
RA:	rheumatoid arthritis
SLE:	systemic lupus erythematosus
TNF:	tumour necrosis factor

Competing interests: none declared.

Introduction

There have been major advances in the management of rheumatoid arthritis (RA), leading to the development of tumour necrosis factor (TNF) inhibitors. These agents, infliximab, an anti-TNF-alpha antibody, and etanercept, a soluble TNF-alpha receptor, make it possible to arrest joint damage and even to prevent it by treating early in the disease course (1-3). The efficacy of anti-TNF-alpha therapies highlights the key role of TNF-alpha in the pathogenesis of RA. However, their mechanisms of action are not yet fully understood. An emerging hypothesis is that TNF-alpha inhibitors could induce apoptosis of immune cells, which is impaired in RA. Several lines of evidence support this hypothesis.

In addition to the recruitment of inflammatory cells, impaired apoptosis of macrophages and T cells has been demonstrated in RA synovium with a reduced rate of apoptosis of these cells. The paucity of synovial apoptotic cells may play an important role in the development of the synovial hyperplasia observed in RA (4-7). The resistance of these immune cells to cell death has been associated with decreased expression of some genes encoding for pro-apoptotic proteins such as TRAP-1, 2, CASP6, 8, TP53, OSIVA and TRIP, and with increased expression of the anti-apoptotic *Bcl-2* protein (4, 8). This phenomenon has been also well studied in Crohn's disease (CD). T lymphocytes from the lamina propria have been shown to be resistant to the induction of apoptosis, and restoration of apoptosis was demonstrated *in vivo* after an infusion of infliximab (9). In parallel, *in vitro* infliximab treatment of CD peripheral blood monocytes and lamina propria T lymphocytes induced apoptosis of these cells (9-12). The induction of apoptosis seems to depend on the molecular structure of the anti-TNF-alpha agent, as no such effect was observed using etanercept. These data were correlated with the *in vivo* therapeutic effect, as etanercept was not effective in CD (13, 14).

In RA patients, infliximab decreased synovial cellularity and inflammation as soon as 48 hours after the start of treatment (15). Two studies have suggested that this effect could be due to induction of macrophage apoptosis (16) or to early inhibition of cell migration (17). Although there are indications that infliximab exerts its effects on monocytes from synovial fluid or from peripheral blood in RA (16), there are, as yet, no studies demonstrating the same mechanism of action on the peripheral blood lymphocyte population.

In the present study, we analysed the apoptotic effect of infliximab and etanercept *in vitro* on the lymphocyte population of RA patients, compared with healthy donors.

We then sought a relationship between the efficacy of anti-TNF-alpha in inducing apoptosis and firstly the capacity of PBMC to produce TNF-alpha *in vitro,* and secondly the clinical features of RA.

Patients and methods

Patients

Eighteen healthy donors and 24 patients with RA were included in the study. All RA patients fulfilled the American Rheumatism Association 1987 revised criteria. There were 19 women and 5 men with a mean age of 59.3±13.5 years (range 32-80 years). None of the patients had been treated with anti-TNF-alpha. Disease activity was assessed with the Disease Activity Score in 28 joints (DAS 28). Clinical and demographic characteristics of the RA patients are reported in Table I. Healthy donors were 15 women and 3 men with a mean age of 46.1±14.1 years (range 24-73 years). Informed consent was obtained from each patient. This study was approved by the Research Committee for the Hospices Civils de Lyon.

Isolation and culture of peripheral lymphocytes

PBMC were isolated by density gradient centrifugation on a layer of Ficoll-Paque Plus® (Amersham, Sweden) from blood samples from healthy donors or RA patients. These suspensions contained 53 to 57% T lymphocytes, 10 to 13% B lymphocytes, 8 to 14% NK cells and 9 to 17% monocytes as defined by CD3, CD19, CD16/CD56 and CD14, respectively. 95% cell viability was

Table I. Clinical and demographic characteristics of RA patients.

Patient	Sex (M/F)	Age (years)	Disease duration (years)	Medication			DAS 28 score
				MTX (mg/week)	Other (mg/week)	Prednisone (mg/day)	
RA1	F	79	15	7.5	none	5	4.2
RA2	M	57	13	10	none	none	2.2
RA3	F	62	10	none	leflunomide	none	4.8
RA4	F	57	ND	15	leflunomide	none	5
RA5	F	71	42	12.5	chloroquine	10	3.5
RA6	F	59	7	10	none	none	2.4
RA7	F	37	2	10	none	none	2.3
RA8	F	66	10	none	none	5	ND
RA9	F	49	0.66	15	none	1 pulse	ND
RA10	F	49	29	15	none	7.5	2
RA11	F	60	8	7.5	none	10	3.2
RA12	F	78	11	15	none	5	5.6
RA13	F	76	ND	10	none	none	ND
RA14	F	32	41	none	none	none	4.5
RA15	M	73	5	10	none	none	2.9
RA16	F	56	1	10	none	none	5.6
RA17	F	51	24	10	none	none	ND
RA18	M	44	1	12.5	none	5	2.1
RA19	M	67	14	10	none	none	0.8
RA20	M	69	1	15	none	none	ND
RA21	F	58	36	10	leflunomide	none	1.8
RA22	F	55	22	10	none	none	4.2
RA23	F	80	16	10	none	none	2.8
RA24	F	39	6	15	none	none	1.5

*ND: not done; MTX: methotrexate; M: male; F: female.

confirmed by trypan blue exclusion. PBMC were resuspended in RPMI 1640 (Gibco, GB) supplemented with 10% fetal calf serum (PAN Biotech GmbH, Australia), 10 mM Hepes (Gibco), 2 mM L-glutamine (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma, Taufkirchen, Germany).

$1x10^6$ cells/mL plated on 24-well plates (Falcon, Becton Dickinson, New Jersey) were incubated for 48 hours in the absence (culture medium with no molecule *i.e.*, control medium) or presence of infliximab (Remicade®; Centocor Inc, Malvern, PA, USA) (100 or 200 µg/mL), etanercept (Enbrel®; Immunex Corp., Seattle, WA) (12.5 or 25 µg/mL) or a human IgG1κ (PHP010 clone, Serotec Ltd, GB) as an isotypic control.

After 48 hours of culture, the cellular suspension contained 60 to 75% T lymphocytes, 12 to 15 % B lymphocytes, 4 to 10% NK cells and less than 2% monocytes. Within the lymphocytes, 7.0±4.0% of cells were labelled by propidium iodide dye (Sigma, Steinheim, Germany) used as a marker for cell death.

Flow cytometric analysis of apoptosis

Cells were resuspended in buffer containing FITC-conjugated annexin V for 15 minutes following the instructions of the manufacturer (Bender MedSystems, Vienna, Austria). To differentiate between apoptotic and necrotic cells, propidium iodide was added and cell suspension was immediately analysed by flow cytometry using an Epics XL Coulter cytometer. Cells were gated for lymphocyte characteristics using both forward and sideward scatter plot. Annexin V-positive cells were considered as apoptotic. Propidium iodide -positive cells were considered as necrotic. Results were expressed as a percentage of specific apoptosis according to the following formula: percentage of apoptosis induced by anti-TNF-alpha minus percentage of spontaneous apoptosis observed with the control medium. A positive control of apoptosis was performed using mitoxantrone in each experiment.

Quantitative measurement of TNF-alpha synthesis by PBMC in vitro

TNF-alpha concentration was measured in PBMC culture supernatant using an ELISA kit (TNF-alpha EASIA kit, BioSource Europe S.A, Nivelles, Belgium) according to the manufacturer's instructions.

Statistical analysis

Differences between the number of controls and RA patients showing apoptosis were evaluated by the χ^2 test. A p value of < 0.01 was considered statistically significant. The difference between percentages of peripheral blood lymphocyte apoptosis in the two groups was evaluated by Student's t-test with a level of significance $p<0.05$.

Results

Anti-TNF-alpha induced lymphocyte apoptosis

We first investigated whether anti-TNF-alpha induced apoptosis of PBMC from healthy donors or RA patients. PBMC were exposed for 48 hours to the control medium, isotypic control, etanercept or infliximab, and then subjected to annexin V labelling. A representative annexin labelling experiment is shown in Figure 1.

Spontaneous lymphocyte apoptosis was measured in the cells after 48 hours of culture in the control medium and reached 16.1±10.8%. The percentage of annexin V-positive lymphocytes from RA cultured with the isotypic control (IgG1κ) (11.7±5.8%) was not significantly different from that with the control medium ($p>0.05$). There was no significant difference between spontaneous apoptosis of lymphocytes from RA (16.1±10.8%) and healthy controls (15.5±7.5%).

Infliximab induced specific apoptosis in 17 RA patients with 4.2±4.0% and 5.4±4.0% of annexin V-positive lymphocytes, treated with 100 or 200 µg/ml respectively, and in 11 out of 18 healthy donors with 1.8±1.2% and 1.4±1.2% respectively (Fig. 2A). Etanercept induced specific apoptosis in 18 RA patients with 5.6±6.3% and 3.7±3.3% of annexin V-positive lymphocytes, treated with the dose of 12.5 or 25 µg/ml respectively, and in 8 out 15 healthy donors with 2.5±1.3% and 2.1±0.5% respectively (Fig. 2B).

Comparing induced apoptosis in RA

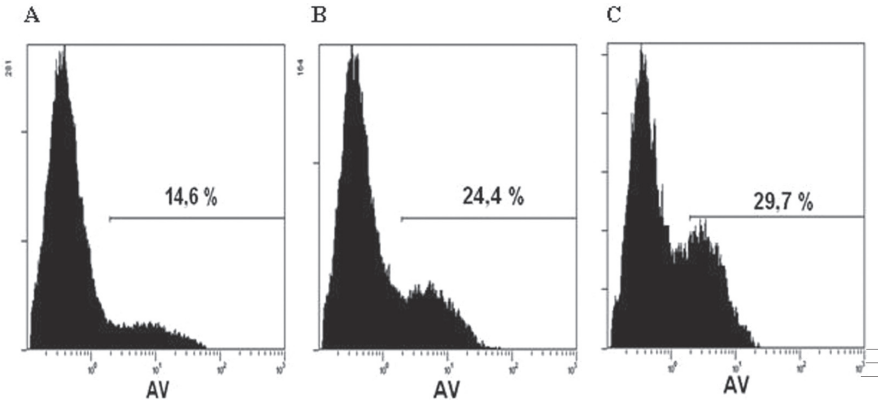

Fig. 1. Apoptosis induction in peripheral blood lymphocytes from an RA patient by infliximab and etanercept, shown by annexin V labelling. Freshly purified PBMC from an RA patient were incubated with culture medium **(A)**, infliximab 100 μg/mL **(B)** or etanercept 12.5 μg/mL **(C)**. Apoptosis was measured by surface binding of annexin V-FITC among lymphocytes. Results are expressed as the percentage of cells labelled with annexin V-FITC.

patients and healthy donors, apoptosis was slightly increased for RA with both infliximab and etanercept ($p<0.05$) (Fig. 2). However, we observed considerable heterogeneity in the apoptotic effect of anti-TNF-alpha, which ranged from 0.1% to 14% in PBMC of RA patients treated with infliximab and from 0.5% to 25% in PBMC of RA patients treated with etanercept. In contrast, in healthy donors induced apoptosis was observed in 0.3% to 3.8% of lymphocytes with both infliximab and etanercept.

We then established a threshold value of 4% beyond which the specific apoptosis of RA patients was considered as different from controls. This value was obtained by the 95% confidence interval of specific apoptosis measured in PBMC of healthy donors with infliximab. We further distinguished two groups of RA patients: those with insensitive PBMC or induced apoptosis in less than 4% of cells, similar to healthy donors, and those with sensitive PBMC, or induced apoptosis in more than 4% of cells (Fig. 2). Overall, the lymphocytes from 11 of 24 RA patients showed apoptosis. Within this population, 10 and 9 RA patients were sensitive to infliximab (mean specific apoptosis: 8.3±3.3%) and to etanercept (mean specific apoptosis: 8.7±6.8%), respectively. Comparing induced apoptosis in sensitive RA patients and healthy donors, apoptosis was significantly increased for RA with both infliximab and etanercept ($p<0.01$) (Fig. 2). There was no statistically significant difference between the different doses of a same molecule or between the two molecules.

Anti-TNF-alpha induced apoptosis is not related to TNF-alpha synthesis

We next compared the capacity of PBMC from RA patients and healthy donors to synthesize TNF-alpha in the culture supernatant. Mean TNF-alpha synthesis in non-treated PBMC was higher in RA (34.1±20.8 pg/mL) than in healthy donors (15.2±17.4 pg/mL). But because of the heterogeneity of TNF-alpha levels the difference did not reach statistical significance ($p>0.05$).

Comparing the ability of PBMC from insensitive RA and sensitive RA to produce TNF-alpha, we did not observe any difference between the two groups of RA patients ($p>0.05$). There was no correlation between the highest TNF-alpha concentration and the levels of spontaneous or specific apoptosis in RA patients.

Anti-TNF-alpha induced apoptosis is not related to clinical RA features

Finally, clinical features of insensitive and sensitive RA were compared using the DAS 28 as an indicator of disease activity. The DAS 28 was not significantly different between the two groups of RA patients (3.4±1.3 and 3.1±1.6, $p=0.45$). No correlation between the DAS 28 and the percentage of spontaneous apoptosis was found ($r = 0.077$, $r^2= 0.006$).

Discussion

Restoration of apoptosis of the immune cells impaired in RA may be one of the mechanisms of action of anti-TNF-alpha therapies. In our experiment, *in vitro* treatment of peripheral blood lymphocytes with infliximab and with etanercept significantly increased apoptosis in RA patients in comparison with healthy controls. In a previous study, peripheral blood lymphocytes have been shown to be less susceptible to infliximab than monocytes/macrophages in RA (16). However, in that study, the duration of the culture was 24 hours, which may be too short to observe an effect in the lymphocyte population. Forty-eight hours of incubation was chosen in our study as previous analyses showed that apoptosis can be revealed after this duration of treatment and that changes occurred in synovial tissue of RA patients treated with infliximab within 48 hours of initiation of therapy (15).

Comparing the two molecules, there was no statistically significant difference between infliximab and etanercept either in the number of patients with apoptotic lymphocytes or in the percentage of these cells. This result is in disagreement with a report showing that adalimumab and infliximab induced apoptosis of monocytes *in vitro* to the same extent but that monocytes treated with etanercept survived (18). We suggest that monocytes and lymphocytes may respond differently to different molecules. Furthermore the same effect with both molecules can be explain as soluble TNF receptors can bind membrane TNF and transmit an intracellular signal (19).

With regard to anti-TNF-alpha concentrations, the effect does not seem to be dose-dependent, as we did not observe any difference between the level of specific apoptosis and the dose for either etanercept or infliximab. A previous study demonstrated that the effect of anti-TNF-alpha is dose-dependent, but this concerned lamina propria T lymphocytes (12). It has also been demonstrated that infliximab induced monocyte apoptosis in CD patients in a dose-dependent manner; however, this effect was observed between 1 to

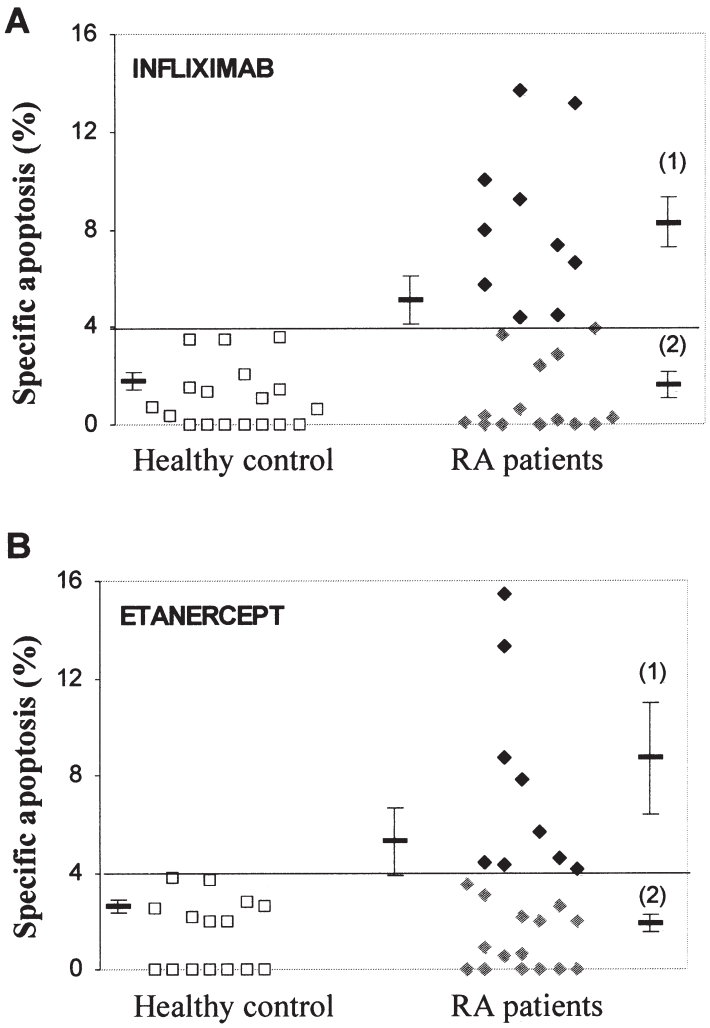

Fig. 2. Anti-TNF-α sensitivity of peripheral blood lymphocytes from healthy donors and RA patients.
Freshly purified PBMC from healthy donors or RA patients were incubated with infliximab **(A)** (healthy donors n = 18; RA patients n = 24) or etanercept **(B)** (healthy donors n = 15; RA patients n = 24). Apoptosis was measured by surface binding of annexin V-FITC on lymphocytes after 48 h of culture. Results were expressed as percentage of specific apoptosis (% of the apoptosis induced by anti-TNF-α minus % of the spontaneous apoptosis with the control medium). A threshold value of 4% was determined from the 95% interval confidence of healthy donors-specific apoptosis and allowed to distinguish 2 populations among RA patients. The "sensitive RA" (1) showed a specific apoptosis in more than 4% of lymphocytes cells after 48 hours of incubation with infliximab or etanercept. The "unsensitive" RA" (2) showed an apoptosis in less than 4% of lymphocytes cells in these conditions. Bars and vertical lines indicate the means ± SEM of the values for each group of patients. Statistical significance of the difference between percentages of peripheral blood lymphocytes apoptosis in the different group compared to healthy donors was determined by the student test with a level of significance $p < 0.05$ (*).

10 mg whereas no clear difference was apparent between 10 mg and 100 mg, suggesting that there is a maximal dose beyond which a cumulative effect no longer occurs (9-12).

We did not observe induced apoptosis of more than 4% of cells in healthy controls. Our findings do not agree with those of Vigna-Perez *et al.* demonstrating that adalimumab can induce apoptosis in some healthy individuals (2 out of 5) and with those of Balog *et al.* demonstrating in 4 patients that infliximab induced a specific apoptosis of 19% of the PBMC (20, 21). This discrepant result may be due to the different molecule used for the first study and to the different ethnic origin of the patients for the second one.

In contrast, 46% of RA patients displayed anti-TNF-alpha-induced apoptosis in more than 4% and up to 25% of the lymphocyte population. With regard to spontaneous apoptosis, there was no difference between this group and the group of RA patients showing an apoptosis of less than 4% or no apoptosis at all, suggesting that induced apoptosis does not depend on the spontaneous apoptosis observed at baseline. As there is a clear relationship between apoptosis and systemic lupus erythematosus (SLE) (22, 23), we searched for SLE-like symptoms or biological markers in the group of RA patients with apoptosis-sensitive PBMC to anti-TNF-alpha. These patients did not show any clinical symptoms characteristic of SLE. Three out of eleven patients (27%) displayed antinuclear antibodies but with no anti-double strain DNA autoantibodies (data not shown).

The modestly enhanced TNF-alpha secretion in RA patients did not further explain the difference in induced apoptosis between the two RA groups and between RA and healthy donors. In addition, there was no correlation between the highest TNF-alpha concentration and the level of spontaneous apoptosis. Nor was there any significant difference in DAS 28 scores between the two groups of RA patients. Lymphocytes from severe RA did not seem more sensitive to the two agents than less severe disease. However, it will be interesting to follow up the two groups of patients to observe their further response to anti-TNF-alpha therapies. Differences in lymphocyte susceptibility to anti-TNF-alpha induced apoptosis may explain the resistance observed in some patients (24, 25).

In conclusion, infliximab and etanercept induce an *in vitro* apoptosis within the peripheral lymphocyte population in a subgroup of RA patients. Further studies are warranted to clarify the relationship between this effect and the therapeutic activity of the anti-TNF alpha treatment and to determine whether this *in vitro* apoptotic response could help to predict which RA patients will be responders or non-responders to therapies.

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