Neutrophil Migration and Production of Reactive Oxygen Species During Treatment with a Fully Human Anti-Tumor Necrosis Factor-α Monoclonal Antibody in Patients with Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** To evaluate the effects of therapy with a fully human anti-tumor necrosis factor (TNF)-α monoclonal antibody on the production of superoxide and other reactive oxygen species (ROS) and on the migration capacity of neutrophils in patients with rheumatoid arthritis (RA).

Methods. A total of 29 patients with active RA and 25 healthy controls participated. Assessments were performed at baseline and 2 weeks after the first administration of anti-TNF-α. The production of ROS was studied in unstimulated conditions and after stimulation of receptor dependent (serum treated zymosan, STZ) and receptor independent (phorbol mystrate acetate, PMA) pathways by luminol enhanced chemiluminescence. As well, the PMA induced burst production of superoxide was measured using the cytochrome-c reduction assay. Potential changes in neutrophil migration to joints were assessed by scintigraphy with autologous leukocytes.

Results. Baseline production of ROS (both spontaneously and after STZ stimulation) and superoxide and the *ex vivo* chemotaxis were similar in RA patients (n = 25) and controls (n = 25) and remained unchanged after administration of anti-TNF-α. The production of ROS after PMA stimulation was slightly higher in patients than in controls ($p = 0.04$) and this difference disappeared 2 weeks after the first dose of anti-TNF-α ($p < 0.05$). The scintigraphic study showed that a single dose of anti-TNF-α, but not placebo, markedly decreased the influx of leukocytes to inflamed joints.

Conclusion. In patients with RA, anti-TNF-α therapy rapidly decreases the influx of leukocytes into inflamed joints but does not impair neutrophil chemotaxis and production of ROS. (J Rheumatol 2003;30:232–7)

Key Indexing Terms:
ANTI-TUMOR NECROSIS FACTOR-α RHEUMATOID ARTHRITIS ADALIMUMAB
NEUTROPHIL MIGRATION PMN REACTIVE OXYGEN SPECIES

The efficacy of tumor necrosis factor-α (TNF-α) neutralizing approaches in rheumatoid arthritis (RA) is well recognized[1,2]. Despite the excellent efficacy/toxicity ratio of these agents, there are still concerns about the risk for infections including, but not limited to, tuberculosis[3-8], carcinogenesis[9-11], and autoimmune disorders[12,13] during TNF neutralization. Some of these potential adverse effects could be at least partly related to an impaired function of neutrophils and other phagocytic cells during TNF-α neutralization.

On the other hand, modulation of the function of neutrophils and phagocytic cells could be beneficial in RA and other chronic inflammatory disorders. TNF-α is involved in the priming, chemotaxis, and production of reactive oxygen species (ROS) by neutrophils[14-16]. These cells are abundant in RA synovial fluid, and their production of ROS and other inflammatory mediators may induce cartilage damage[17,18]. Reduction in neutrophil priming, ROS production, and chemotaxis have been implicated in the mechanism of action of other rapidly-acting antirheumatic drugs such as methotrexate (MTX), leflunomide[19-22], and steroids[23]. Whether this also holds true for TNF-α blocking agents is unknown. In patients with RA, administration of infliximab, a chimeric anti-TNF-α monoclonal antibody, has been reported to reduce the influx of neutrophils into joints[24]. It has been hypothesized that this could be due to a decreased expression of adhesion molecules[25,26]. Whether blocking TNF-α alters the physiological migration of neutrophils to inflammatory foci has not been assessed yet. Such effect could be relevant for the risk of infections during TNF-α neutralization, espe-

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cially since this strategy is often combined with agents that alter neutrophil chemotaxis such as MTX and steroids[23,27].

Adalimumab (D2E7, Knoll-BASF, Ludwigshafen, Germany) is a fully human, IgG1 monoclonal ant-TNF-α antibody developed using phage display techniques[28] that is now undergoing phase III studies in RA. Its efficacy has been demonstrated in thousands of patients[29-32] and can be measured within days from therapy initiation. We evaluated the effect of a single dose of adalimumab on neutrophil function in patients with active RA. To this aim, the production of ROS and the chemotactic capacity of neutrophils were assessed using *ex vivo* assays. The influx of leukocytes into the synovial joints was analyzed *in vivo*.

MATERIALS AND METHODS

Patients. We studied consenting patients with RA enrolled in double blind, placebo controlled studies with adalimumab monotherapy at our center. All patients fulfilled American Rheumatism Association criteria[33], had active disease, defined as a disease activity score (DAS)[34] > 3.2, and had undergone a 4 week washout period for disease modifying antirheumatic drugs (DMARD). Nonsteroidal antirheumatic drugs (NSAID) and steroids, up to 10 mg daily, were kept stable 4 weeks prior to and during the study. Patients were randomized to initiate treatment with adalimumab administered subcutaneously (20 to 80 mg dose) or placebo. *Ex vivo* and *in vivo* neutrophil function test and white blood cell (WBC) counts were assessed at baseline and at Week 2. At this point, clinical response according to the EULAR criteria[35] was also assessed. All *ex vivo* neutrophil function tests were also performed in age and sex matched healthy controls. The study was approved by the ethical committee of the University Medical Center Nijmegen.

Ex vivo neutrophil function tests. All reagents used were from Sigma Chemicals (St. Louis, MO, USA), unless stated otherwise. Neutrophils were isolated from heparinized blood (Vacutainer, Becton Dickinson, Rutherford, NJ, USA) as described[36]. Briefly, whole blood, diluted 1:1 in 0.4% trisodium citrate in phosphate buffered saline (PBS), was subjected to Percoll gradient centrifugation (δ = 1.076 g/ml; Pharmacia, Uppsala, Sweden; 700 g, 18 min, 25°C). The neutrophil-containing cell pellet was resuspended in 50 ml ice-cold isotonic lysis solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA), centrifuged (5 min, 400 g, 4°C), and remaining erythrocytes were lysed (lysis solution, 5 min). Cytospin and trypan blue staining showed > 97% neutrophils and > 99% viable cells in all procedures. Neutrophils (final concentration 2×10^6 cells/ml) were kept at room temperature until assay.

The "longterm" (120 min) spontaneous and stimulated respiratory burst were measured as the production of ROS that excite luminol (luminol enhanced chemiluminescence, LECL). To this aim, 200 µl neutrophil suspensions (2×10^6 cells/ml) and 20 µl luminol (5×10^{-4} M) per well were incubated in 96 well microplates at 37°C for 120 min in the absence or presence of a neutrophil stimulus. The latter consisted of serum treated zymosan (STZ), which induces ROS production through complement C3b receptor activation[37], or phorbol myristate acetate (PMA) which yields receptor independent activation of protein kinase C. STZ and PMA were used at final concentrations of 1 mg/ml and 100 ng/ml, respectively. Chemiluminescence was monitored every 30 s for 120 min using an automated LB96V Microlumat Plus plater luminometer (EG&G Berthold, Bad Wildberg, Germany). The integral area under the curve (AUC) over this period is expressed in relative light units (RLU). The peak luminescence, expressed in RLU/s, was calculated using Winglow software (EG&G Berthold).

The "short term" burst production of superoxide (O_2) was measured after stimulation with PMA for 5 min using the cytochrome-c reduction assay. The latter measures the superoxide dismutase inhibitable reduction of ferricytochrome c[38], which is expressed as the maximum rate of cytochrome c reduction (in nmol/min/10^6 neutrophils at 550 nm) using 21.1 $mM^{-1} \cdot cm^{-1}$ as extinction coefficient[39]. The assay was performed at 37°C on a thermostatted spectrometer (Perkin-Elmer Lambda 12, Perkin-Elmer, Norwalk, CT, USA).

Neutrophil chemotaxis was evaluated using a modification of the Boyden chamber assay[37]. Briefly, neutrophils (1×10^6/ml, 400 µl per well) and N-formyl-methionin-leucin-phenylalanin (fMLP, 10^{-8} M, 600 µl per well) were loaded in the upper and bottom chamber of MilliCell-PC culture plates separated by isopore polycarbonate membranes (12 mm diameter, 10 µm thickness, 3 µm pore size) and incubated at 37°C for 1 h. The number of neutrophils that reached the bottom chamber was counted using a hemocytometer (Coulter Counter, Coulter Electronics, Mijdrecht, Netherlands).

All *ex vivo* neutrophil tests were simultaneously assessed in patients with RA and age and sex matched healthy controls to correct for interassay variation.

Neutrophil function in vivo. Scintigraphy with technetium-99 labeled autologous WBC. Aliquots of 50 ml venous blood were drawn and mixed with 10 ml methylcellulose containing 0.33% acid citrate dextrose. Red blood cells were allowed to sediment for 1 h. The WBC-containing supernatant was centrifuged (10 min, 150 g) and the resulting cell pellet was washed, centrifuged (10 min 150 g), and resuspended in 1.5 ml PBS with 1% human serum albumin. No isolation of the neutrophil subset was performed and WBC were labeled with 1 GBq Tc-99m-hexamethylpropyleneamine oxide (HMPAO) at room temperature for 30 min. After centrifugation (10 min, 150 g), the WBC pellet was resuspended in 5% glucose and microscopically checked for cell integrity. Labeling efficiency (cell associated activity/total activity) was always > 75%.

A dose of 200 MBq Tc-99m-WBC was administered intravenously. Whole body scintigraphy was obtained 1 and 4 h after injection, using a single head gamma camera equipped with a parallel hole low energy collimator (Siemens Orbiter, Siemens Inc., Hoffman Estate, IL, USA). Joint uptake was scored semiquantitatively using a 0–2 scale (0 = no, 1 = equivocal, 2 = clearly increased uptake) by a blinded observer[40]. The following joints were scored: sternoclavicular, acromioclavicular and mandibular joints, shoulder, elbow, wrist, metacarpophalangeal joints, proximal interphalangeal joints, hip, knee, ankle joint (scored combined with subtalar joint), forefoot and metatarsophalangeal joints. For each patient, a total joint score was calculated by adding the values of all joint regions at baseline and this was set as 100%. Changes in scintigraphic scores at Week 2 are expressed as percentage change from baseline.

Statistical analysis. Data are expressed as mean or median according to their distribution. Statistical analysis for paired observations was performed using Student t tests and Wilcoxon signed rank tests as appropriate. Between-group comparisons were tested using the Mann-Whitney U test. Correlation was tested using the Spearman rank correlation test. Analyses were performed using the Astute Base Module, version 1.50.

RESULTS

Patients. A total of 29 patients gave informed consent for different studies on the *ex vivo* and *in vivo* neutrophil function tests. After unblinding, 21 patients had been randomized to receive active treatment and 8 placebo. Baseline patient characteristics in these 2 groups were similar (Table 1). At 2 weeks after the first administration of anti-TNF Mab, 58% of the treated patients, but none receiving placebo, fulfilled the EULAR criteria for clinical response.

Ex vivo neutrophil tests. *Ex vivo* neutrophil function tests were performed in 25 patients with RA (18 receiving anti-TNF-α and 7 placebo) and 25 age and sex matched controls. Total leukocyte counts and WBC subset counts in peripheral blood showed no significant increase in neutrophil counts 2 weeks after the first administration of anti-TNF Mab (median neutrophil counts 5.86×10^9/l and 6.13×10^9/l at baseline and 2 weeks, respectively; p = 0.64).

Table 1. Baseline patient characteristics.

Percentage, Mean ± SD or Median (p25–p75) as Appropriate	Adalimumab, n = 21	Placebo, n = 8
Age, yrs	55 ± 12	57 ± 10
Sex, % female	62	75
Rheumatoid factor, % > 10 IE	100	100
Disease duration, yrs	13 ± 8	9 ± 6
Number of previous DMARD	6 (4–8)	5 (3–6)
NSAID use, %	95	100
Prednisone use, % of patients	71	75
Disease Activity Score	5.4 ± 0.8	5.3 ± 0.9
Swollen joints 44/66*	18.8/21.6	25.5/20.3
RAI/tender joints 68*	25.3/37	25.5/29.3
CRP, mg/l	53 ± 42	71 ± 32
ESR, mm/h	33 ± 25	32 ± 20
EULAR responses after 2 weeks, %	58	0

* In Study 1 Ritchie Articular Index and swollen 44 were measured; in Study 2 swollen 66 and tender joints 68 were used. No statistically significant differences were present at baseline.

Data on the capacity for ROS production and chemotaxis of neutrophils are shown in Table 2. As shown, the production of ROS, including superoxide (O_2), and the *ex vivo* chemotactic capacity of neutrophils were largely similar in RA patients and controls (Table 2). At baseline, only the peak production of ROS after PMA stimulation was slightly higher in RA patients than in controls (8.6 ± 3.2 vs 6.8 ± 2.5 RLU/s, $p < 0.05$; patient/control ratio 1.46 ± 0.28). No such difference was observed 2 weeks after anti-TNF-α administration. The chemotactic activity and the burst production of O_2 after PMA stimulation did not change during the study (Table 2).

In vivo neutrophil function. Scintigraphic evaluation with radiolabeled leukocytes was performed in 4 patients who had been randomized to anti-TNF-α (n = 2) and placebo (n = 2). At baseline, a polyarticular pattern of uptake mirroring the clinically inflamed joints was observed in all patients (Figure 1). Scintigraphic joint scores obtained at baseline (absolute values ranging from 6 to 18) correlated with swollen joint counts (within-patient values ranging from R = 0.3 to 0.64; p = 0.008 to < 0.0001).

Scintigraphic images obtained 2 weeks after the first dose of anti-TNF-α showed a markedly decreased uptake of leukocytes into inflamed joints (Figure 1). In the treated patients 30% of the joints showing clearly increased leukocyte uptake (score 2) at baseline had become normal by Week 2 (score 0), and 64% decreased to score 1. In contrast, no change from baseline values was observed in patients receiving placebo. Scintigraphic scores normalized for the baseline values were 53% and 33% in treated patients and 100% and 106% in the placebo group, respectively.

DISCUSSION

Our results show that treatment with adalimumab drastically reduces neutrophil migration into synovial joints shortly after initiation of therapy without inducing an increase in neutrophils in peripheral blood. The decreased homing of neutrophils to the target organ is not the result of downmodulation in leukocyte function, as adalimumab does not impair the production of ROS or the chemotactic capacity of neutrophils *ex vivo* or *in vivo*.

In our study, the reduction of neutrophil homing to the joints was observed using scintigraphy with radiolabeled WBC. This is a sensitive method for assessing joint inflammation and correlates well with clinical signs[41,42], in our case especially with joint swelling scores. The scintigraphy was performed using radiolabeled leukocytes without isolation of the neutrophil subset. However, we obtained results similar to a study by Taylor, *et al* with another anti-TNF-α Mab[24] using isolated radiolabeled neutrophils. Although only a limited number of patients were studied, the scintigraphic results were consistent (observed only in treated patients and not in the

Table 2. Neutrophil chemotaxis and ROS production, patients versus controls (n = 25), treated (n = 18) versus placebo (n = 7).

Mean ± SD or Median (p25–p75)	Before				After		
		Patients		Controls	Patients		Controls
	All Patients	Anti-TNF-α	Placebo		Anti-TNF-α	Placebo	
Superoxide production	8.6 ± 2.3	8.5 ± 2.5	9.0 ± 1.9	10.0 ± 3.2	8.5 ± 1.8	7.1 ± 2.6	9.7 ± 2.5
Chemiluminescence							
Spont. AUC	5.7 (3.7–11.5)	6.4 (3.1–11.6)	5.0 (3.8–7.2)	7.4 (4.5–10.8)	6.9 (3.6–15.7)	6.5 (4.3–7.8)	9.4 (5.6–15.9)
Spont. peak	2.0 (1.0–2.7)	2.1 (0.9–3.5)	1.4 (1.1–2.3)	1.8 (1.2–3.1)	1.6 (1.0–3.6)	1.6 (1.0–2.3)	2.5 (1.6–4.5)
STZ AUC	25.8 ± 10.1	23.5 ± 8.6	31.6 ± 12.0	26.3 ± 10.9	22.4 ± 5.7	27.3 ± 6.1	24.7 ± 7.5
STZ peak	12.5 ± 3.9	12.2 ± 3.5	13.4 ± 5.0	12.4 ± 4.3	10.8 ± 1.8	12.6 ± 4.4	11.2 ± 1.9
PMA AUC	11.6 ± 3.7	11.2 ± 3.5	12.8 ± 4.8	10.8 ± 4.0	9.8 ± 3.5**	11.3 ± 4.9	10.4 ± 4.2
PMA peak	8.6 ± 3.2	8.5 ± 3.4	9.7 ± 4.2	6.8 ± 2.5*	7.1 ± 2.6**	8.9 ± 4.4	6.8 ± 2.5
Chemotaxis	53.6 ± 18.5	49.1 ± 18.8	61.4 ± 18.8	49.9 ± 17.6	39.6 ± 25.3	46.2 ± 12.2	42.5 ± 20.0

Superoxide production is expressed as nmol/min/10^6 neutrophils; chemiluminescence is expressed as RLU/s for peak value and AUC of the RLU/s for AUC; chemotaxis is expressed as percentage of cells that passed the membrane. STZ: serum treated zymosan, RLU; relative light units.
* $p < 0.05$, patients compared to controls at baseline. ** $p < 0.05$, comparison patient/control ratio after treatment versus before treatment and treated patients versus placebo group.

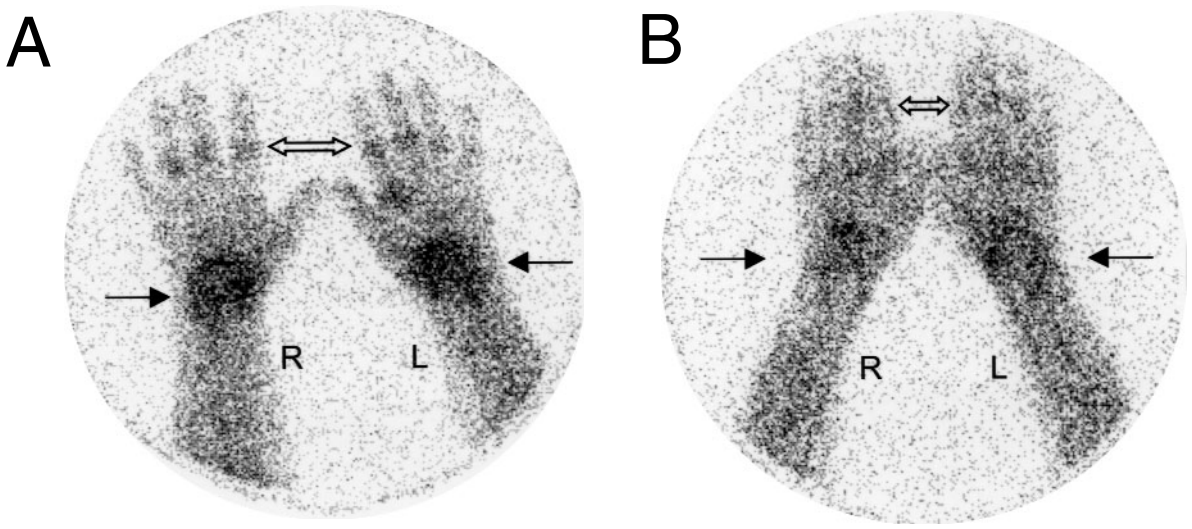

Figure 1. Scintigraphic image of a hand taken 4 h after administration of Tc-99m labeled autologous leukocytes in a patient with RA. Before treatment (A) uptake is seen in the wrists (black arrows), the 2nd MCP joint of the left hand, and several PIP joints (white arrows). Two weeks after the first injection of anti-TNF-α (B), the activity in the wrists is almost normal and the increased leukocyte uptake in the finger joints has disappeared.

placebo group) and concomitant with the clinical response and the decrease in acute phase reaction (data not shown).

In view of our results, a direct systemic effect on the intrinsic chemotactic capacity of neutrophils seems very unlikely. Instead, the decreased influx of neutrophils after anti-TNF-α may be mediated through local deactivation of the synovial endothelium, as suggested by the decrease of local and systemic levels of adhesion molecules[25,26] and by a decreased local production of chemoattractants such as interleukin 8[43,44]. The study by Taylor, *et al* also corroborates this hypothesis, as the observed decrease in granulocyte homing to inflamed joints was not accompanied by a similar change in granulocyte influx in noninflamed organs like the lungs and the spleen.

The findings described here have several potential consequences. First, it does not seem probable that suppression of neutrophil function or motility is implicated either in the mechanisms of action or in some of the potential adverse effects of anti-TNF-α therapy. This is especially true for the susceptibility to bacterial infections, since the killing of these microorganisms by neutrophils and other phagocytes is largely dependent on the production of reactive oxygen metabolites. However, care should be taken in extrapolating these findings to the active site of inflammation in RA, as different conditions may precipitate different behavior of the inflammatory cells.

Second, the knowledge that TNF blockade does not downregulate neutrophil functions can be used to design combination therapies with synergistic effects. In this context, several studies do suggest that some antirheumatic drugs such as steroids, leflunomide, and MTX and certain NSAID can modulate neutrophil ROS production and migration[19-22]. The mode of action of anti-TNF-α does not seem to include modulation of neutrophil function, which provides a rationale for the combination of TNF blocking strategies with agents (e.g., MTX) that do influence neutrophil activity.

Some other observations in our study deserve further mention. Comparison of the neutrophil ROS production and migration between patients with RA and healthy controls did not show important differences. Only the peak production of ROS after stimulation by receptor-independent pathways (PMA) was slightly higher in RA than in controls. This difference was not apparent 2 weeks after the first administration of anti-TNF-α and was also not detected using time-integrated measures (AUC) or by the cytochrome-c reduction assay. Our findings suggest the neutrophil function in patients with RA is not impaired and corroborate findings in several previous studies[45]. Potential bias due to concomitant therapies cannot be definitively ruled out, but these remained unchanged during the whole study period.

One potential limitation of our study might be the short followup period. Therefore, these observations cannot be directly extrapolated to the situation during longterm TNF neutralization. Nevertheless, the effect of this anti-TNF-α Mab and other TNF blocking agents is extremely rapid and the drug half-life of adalimumab is roughly 12 days[46]. Moreover the lifespan of neutrophils is only a few hours for nonactivated neutrophils, somewhat longer under inflammatory conditions[47]. This explains the time-schedule used in our studies and the hypothesis that short and longterm anti-TNF-α treatments will not differ much concerning their effects on neutrophils.

We found that treatment with the human anti-TNF-α Mab adalimumab effectively suppressed neutrophil migration into inflamed joints in RA. This therapy did not interfere with the physiologic functions of neutrophils such as oxidative burst and chemotaxis.

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