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Novel aspects of the activation of NADPH oxidase in neutrophils of rheumatic patients on biological therapy

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ABSTRACT

The relationship between inflammation and formation of reactive oxygen species (ROS) is still not completely understood and excessive inflammatory reaction is attributed to increased yet also to reduced ROS formation. To compare ROS formation in severe and low inflammation, neutrophil oxidative burst was analyzed in rheumatic patients before and during therapy with TNFα- or interleukin-6 receptor-neutralizing antibodies.

Intracellular and extracellular ROS productions were evaluated on the basis of luminol- and isoluminol-enhanced chemiluminescence in isolated peripheral neutrophils. Disease activity score DAS28 and platelet to lymphocyte ratio were used as markers of arthritis activity and the intensity of systemic inflammation.

Biological therapy effectively reduced the intensity of inflammation. Of the twenty-six patients studied eighteen achieved remission or low disease activity. Highly active arthritis persisted only in one patient, though prior to the therapy it was evident in all subjects tested. In patients receiving biological therapy, intracellular chemiluminescence was significantly higher than in patients before this therapy; ROS produced by neutrophils extracellularly were not affected.

The increased ROS formation associated with reduced inflammation supports the need to revise the view of the role of ROS in inflammation - from toxic agents promoting inflammation towards a more complex view of ROS as regulators of immune pathways with inflammation-limiting capacity. From this perspective, the interference with neutrophil-derived oxidants may represent a new mechanism involved in the anti-inflammatory activity of biological therapy.

1. Introduction

Activation of neutrophils (polymorphonuclear leukocytes) by environmental factors is considered to be one of the initiation processes involved in the pathogenesis of rheumatoid arthritis [1,2]. These cells can release, expose and generate neoepitopes that have the potential to break immune tolerance and result in the generation of autoantibodies [3]. Neutrophils represent > 90% of cells infiltrating the synovial fluid during the active phases of arthritis [4], yet they are relatively underestimated in this disease. This seems rather surprising in view of the fact that of all immune cells, neutrophils possess the strongest cytotoxic potential and hence the greatest capacity to inflict tissue damage [4,5].

Neutrophils of rheumatic patients have an activated phenotype – exhibiting delayed apoptosis, active expression of genes and membrane receptors, and increased capacity to produce reactive oxygen species (ROS) [6,7]. ROS are formed by NADPH oxidase after association of cytosolic and membrane-bound components of the enzyme [8,9]. The oxidase assembly at the plasma membrane leads to the release of ROS extracellularly or into phagosome, whereas the assembly on granule membranes results in ROS that are retained within intracellular organelles [10]. Extra- and intracellular ROS are differently involved in neutrophil functions and their formation is separately activated and regulated [10,11].

For their highly reactive nature, ROS are considered mediators of tissue destruction and enhancing factors for autoimmune and inflammatory diseases [6,7,12,13]. However, recent findings indicate that ROS formed in phagocytes are involved in limiting the inflammatory response and they may be beneficial in both inflammatory and autoimmune conditions. The protective role of ROS and their capability to diminish inflammation was indicated by the hyper-

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inflammatory responses found in patients or in experimental animals whose phagocytes displayed severely depressed ROS production [14–19]. Yet the relationship between inflammation and formation of ROS is still not completely identified and excessive inflammatory reaction is attributed to increased yet also to reduced ROS production. To compare ROS formation in severe and low inflammation, neutrophil oxidative burst was analyzed in rheumatic patients before and during biological therapy.

Biological therapy represents a modern targeted treatment of rheumatic diseases that blocks the actions of specific cytokines or immune regulators [1]. However, administration of biologics is often accompanied by infectious complications that are attributed to the reduced activity of blood phagocytes [20]. The measurement of ROS production should disclose the effect of the therapy on this important antimicrobial mechanism.

2. Subjects and methods

2.1. Patients

Twenty-nine patients were recruited from the Centre for Biological Treatment in Rheumatology in Bratislava (Table 1). The patients tested before biological therapy showed signs of highly active arthritis not responding to conventional therapy with methotrexate, methylprednisolone, sulfasalazine or with the combination of these drugs. The group consisted of 15 subjects; twelve of them were evaluated for the second time after administration of biologics. The group of patients tested during biological therapy involved 26 subjects treated with TNFα-neutralizing antibodies (adalimumab, golimumab, etanercept, certolizumab pegol) or with an antibody against the interleukin-6 receptor (tocilizumab). Fifteen healthy donors, who had not received any medication for at least seven days, served as controls. Smokers were excluded from the study; the tested patients had no other inflammatory rheumatic or cardiovascular diseases and did not suffer from infection at the time of blood collection. All subjects gave informed consent and the study was approved by the Ethics Committee of the University Hospital Bratislava.

2.2. Disease activity

Disease activity was assessed on the basis of the disease activity score DAS28 - a parameter involving the evaluation of 28 joints with regard to tenderness upon touch and swelling, erythrocyte sedimentation rate and patient subjective assessment of disease severity. According to the DAS28 values, the activity of rheumatoid arthritis was evaluated as follows: high (DAS28 above 5.1), middle (from 3.2 to 5.1), low (from 2.6 to 3.2) and remission (below 2.6). Platelet to lymphocyte ratio was used as a parameter reflecting the intensity of systemic inflammation [21,22]. The number of blood cells was determined by the hematological Analyzer ABX Pentra 60 (Horiba Medical, Irvine, CA, USA).

Table 1
Characteristics of patients and control subjects involved in the study.

Parameter	Rheumatic patients	Healthy controls
Number of persons	29	15
Mean age (yrs)	56	40
Range	24–73	24–61
Women	23	6
Men	6	9
Mean disease duration (yrs)	12.4	
Mean DAS28 score		
Before therapy	5.72	
During therapy	2.55	
Anti-TNFα treated patients	19	
Anti-IL-6R treated patients	10	

3. Material and methods

3.1. Chemicals and solutions

Luminol, isoluminol, PMA (4β-phorbol-12β-myristate-13α-acetate), superoxide dismutase and dextran (from *Leuconostoc mesenteroides*, catalog number D1037, average mol wt 425,000–575,000) were from Sigma-Aldrich Chemie (Deisenhofen, Germany). Horseradish peroxidase (HRP) and catalase were obtained from Merck (Darmstadt, Germany) and lymphoprep (density 1.0077 g/ml) was purchased from Nycomed Pharma AS (Oslo, Norway).

Phosphate buffered saline (PBS) consisted of 136.9 mmol/l NaCl, 2.7 mmol/l KCl, 8.1 mmol/l Na_2HPO_4 and 1.5 mmol/l KH_2PO_4, pH 7.4. Calcium- and magnesium-containing PBS (PBS+) involved 1.8 mmol/l $CaCl_2$ and 0.5 mmol/l $MgCl_2$.

3.2. Blood collection and isolation of neutrophils

Neutrophils were isolated as described previously [23]. Fresh blood was obtained by venepuncture and anticoagulated with 3.8% trisodium citrate (blood to citrate ratio 9:1). Erythrocytes were allowed to sediment in 1% dextran solution (35 min, 22 °C). Suspension of leukocytes and platelets was centrifuged (10 min, 170 ×*g*, 22 °C), the pellet was resuspended in PBS, layered on Lymphoprep and neutrophils were separated by centrifugation (30 min, 170 ×*g*, 22 °C). After hypotonic lysis of contaminating erythrocytes, neutrophils were washed, resuspended in PBS and their count was adjusted to 10^4/μl. The purity of isolated neutrophils was 91.9 ± 0.3% (determined by ABX Pentra 60) and the final suspension contained > 96% of viable neutrophils, as evaluated by trypan blue exclusion.

3.3. Extra- and intracellular formation of reactive oxygen species

Spontaneous and PMA stimulated ROS formation were evaluated on the basis of enhanced chemiluminescence in a microtiter plate luminometer Immunotech LM-01T (Immunotech, Prague, Czech Republic). The method is based on the use of two luminophores – luminol and isoluminol, characterized by different abilities to cross biological membranes [24,25]. The soluble stimulus PMA was chosen to avoid phagocytosis of luminophores and its interference with the measurement of intracellularly produced oxidants.

Extracellular chemiluminescence (CL) was measured in samples containing neutrophils (5×10^5), isoluminol (5 μmol/l), horseradish peroxidase (8 U/ml) and PBS+ (spontaneous CL) or 0.01 μmol/l PMA (stimulated CL). Peroxidase was added to ensure complete ROS detection. The enzyme is essential for the oxidation of luminophores [26] and the formation of chemiluminescence signal may be limited by insufficient secretion of peroxidase from neutrophil granules. In the measurement of intracellular chemiluminescence, luminol (5 μmol/l) was used as a luminophore and superoxide dismutase (100 U/ml) and catalase (2000 U/ml) were added instead of peroxidase [27,28].

3.4. Data analysis

Production of ROS by neutrophils was evaluated on the basis of integral values of chemiluminescence (area under chemiluminescence curve over 1800 s). All values are given as means ± SEM. The statistical significance of differences between means was established by Student's *t*-test.

4. Results

A high activity of rheumatoid arthritis (DAS28 above 5.1) was found in all patients before biological therapy. In these subjects, systemic inflammation was confirmed by the double increased ratio of platelets to lymphocytes (Fig. 1). During biological therapy, remission was

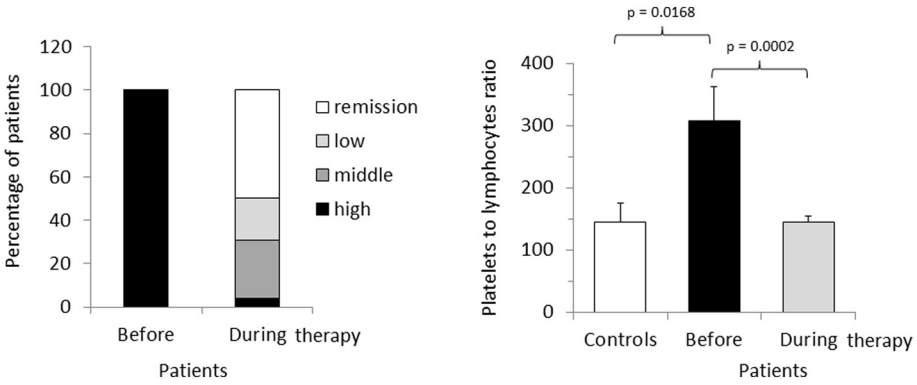

Fig. 1. Activity of rheumatoid arthritis (left) and platelets to lymphocytes ratio as a parameter reflecting the intensity of systemic inflammation (right) before and during biological therapy. Disease activity was assessed on the basis of the DAS28 values as follows: remission (DAS28 < 2.6), low (from 2.6 to 3.2), middle (from 3.2 to 5.1) and high (above 5.1). The number of platelets and lymphocytes was determined by the hematological Analyzer ABX Pentra 60 (HoribaMedical, Irvine, CA, USA). Mean ± SEM, n = 15–26.

Table 2
Number of white blood cells, neutrophils, lymphocytes and platelets in arthritic patients before and during biological therapy and in control subjects.

Cells (10^3/μl)	Controls	Patients	
		Before therapy	During therapy
White blood cells	5.81 ± 0.39	7.43 ± 0.55*	6.79 ± 0.50
Neutrophils	3.19 ± 0.35	4.36 ± 0.34*	3.34 ± 0.33
Lymphocytes	1.79 ± 0.10	2.18 ± 0.28	2.39 ± 0.18*
Platelets	230.79 ± 40.60	522.79 ± 74.66**	318.20 ± 22.81*,++

Mean ± SEM, n = 15–26.
* $p < 0.05$.
** $p < 0.01$ (vs controls).
++ $p < 0.01$ (before vs during therapy).

achieved in 50% of the patients tested, low and middle disease activity was observed in respective 19% and 27% of the patients. The beneficial effect of biological therapy was reflected by the reduction of platelets to lymphocytes ratio to control values.

Numbers of white blood cells, neutrophils, lymphocytes and platelets are given in Table 2. Blood of rheumatic patients showed markedly elevated platelet count (by 127%) and this increase was significantly reduced during the therapy. The number of other elements exceeded the control values only slightly.

Biological therapy significantly increased the formation of ROS inside neutrophils (Fig. 2). Compared with patients before the therapy, the intracellular chemiluminescence rose by 120% (spontaneous) and by 65% (stimulated). The increase was observed after administration of both anti-TNFα as well as anti-IL-6R antibodies. Concentration of ROS produced by neutrophils extracellularly was higher in rheumatic patients than in control subjects. Biological therapy did not cause any significant differences (Fig. 3).

5. Discussion

5.1. Decreased inflammation in rheumatic patients receiving biological therapy

Biological therapy represents a highly effective treatment of rheumatic diseases that blocks the actions of pro-inflammatory cytokines, modulates lymphocyte function or targets intracellular signalling of inflammatory cells [1]. The application of biologics has revolutionized the therapy of rheumatoid arthritis, so that not only clinical remission but also structural and functional remission has become the target of treatment [29]. The presented results showed high efficiency of the biological therapy based on TNFα or IL-6 receptor blockade: of the twenty-six patients tested, eighteen patients (69%) achieved remission or low disease activity. The highly active arthritis persisted only in one patient, whereas prior to the therapy, it was evident in all the subjects tested.

The reduced inflammation was confirmed by a markedly decreased number of platelets. Blood platelets, besides their role in hemostasis, are also potent immune and inflammatory cells and actively participate in the pathogenesis of rheumatic diseases. These cells contribute to

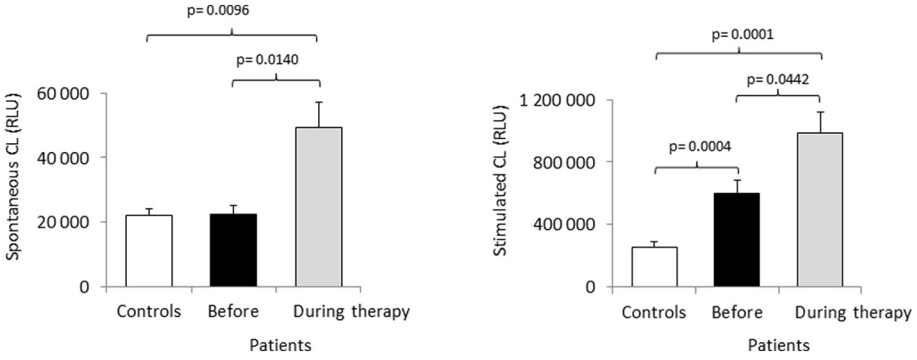

Fig. 2. Intracellular formation of reactive oxygen species. Spontaneous (left) and stimulated (right) intracellular chemiluminescence (CL) examined in arthritic patients before or during biological therapy and compared with controls. Chemiluminescence was measured in samples containing isolated neutrophils (5×10^5), luminol (5 μmol/l), superoxide dismutase (100 U/ml), catalase (2000 U/ml) and PBS + (spontaneous CL) or 0.01 μmol/l PMA (stimulated CL). Mean ± SEM, n = 15–26, RLU – relative light units.

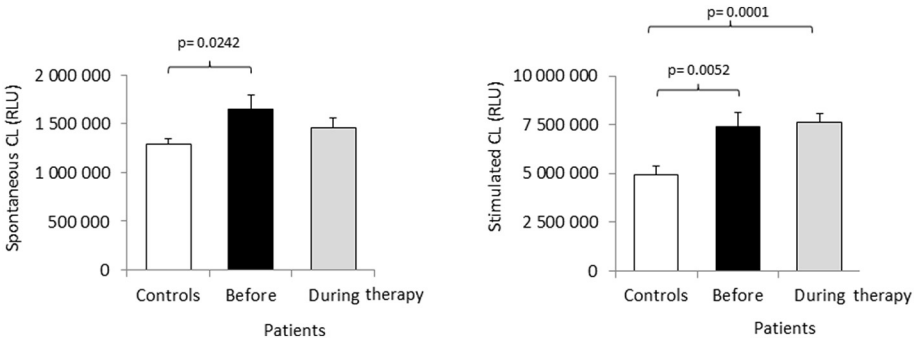

Fig. 3. Extracellular formation of reactive oxygen species. Spontaneous (left) and stimulated (right) extracellular chemiluminescence (CL) examined in arthritic patients before or during biological therapy and compared with controls. Chemiluminescence was measured in samples containing isolated neutrophils (5×10^5), isoluminol (5 µmol/l), horse radish peroxidase (8 U/ml) and PBS + (spontaneous CL) or 0.01 µmol/l PMA (stimulated CL). Mean ± SEM, n = 15–26, RLU – relative light units.

synovial inflammation as a source of prostaglandins and serotonin. Platelet-derived microparticles, abundant in arthritic joint fluid, can elicit production of inflammatory mediators from resident synovial fibroblasts [30,31]. Increased platelet-to-lymphocyte ratio has recently been introduced as a biomarker of systemic inflammation associated with arthritis severity, erythrocyte sedimentation rate and concentration of C-reactive protein [21,22,32]. In patients receiving biological therapy, this parameter was decreased to control values, indicating a substantially reduced inflammatory reaction.

5.2. Decreased inflammation was associated with elevated formation of ROS inside neutrophils

Neutrophil-derived reactive oxygen species are formed by NADPH oxidase after association of cytosolic and membrane-bound components of the enzyme [8,9]. A long-standing paradigm has been that ROS production occurs exclusively in the plasma membrane. A growing body of evidence points to the possibility that phagocytes are capable to generate oxidants within intracellular organelles, in a process that most likely involves fusion of at least two types of distinct granules [10,15].

The relationship between inflammation and ROS production is still not completely recognized and the excessive inflammatory reaction has been associated with both increased [3,33] and reduced [14,19] ROS formation. The analysis of neutrophil oxidative burst before and during biological therapy enabled to compare ROS formation in conditions of severe and low inflammation. In arthritic patients receiving biological therapy, intracellular chemiluminescence was higher than in patients before this therapy, i.e. less intensive inflammation was associated with increased ROS production inside neutrophils. These results are in contrast with the traditional view of ROS as agents that enhance inflammation.

The pro-inflammatory activity was assumed from high reactivity of ROS and from their ability to initiate cellular damage and tissue destruction and to activate transcription factors and cytokine synthesis or to induce the formation of autoantigens by oxidative modification of host proteins and other targets [3,6,33,34]. Nevertheless, the available data provides no consistent support for a beneficial effect of substances decreasing ROS concentration on inflammatory conditions and in some studies antioxidants have been shown to act as pro-inflammatory factors [14,35]. Moreover, absent or compromised phagocytic ROS production is often connected with hyper-inflammation instead of a milder inflammatory response, as would be expected based on the traditional view of ROS as mediators of tissue destruction and inflammation.

The protective role of ROS and their capability to diminish inflammation was indicated by hyper-inflammatory responses found in patients whose phagocytes displayed severely depressed ROS production, e.g. in patients suffering from chronic granulomatous disease, granulomatous colitis or Crohn's disease [14–17]. ROS deficiency associated with increased susceptibility to autoimmune diseases and chronic inflammation was confirmed in animal models for rheumatoid arthritis, multiple sclerosis, psoriasis, gout, and lupus [18,19]. In these studies, the stimulation of ROS formation decreased the autoimmune response and ameliorated both the acute and chronic phases of arthritis [36,37].

Several mechanisms have been suggested as mediating the hyper-inflammatory state associated with the lack of ROS: (i) impaired redox regulation of autoreactive T cells, (ii) activation of IFN signalling, (iii) hyper-reactivity of ROS deficient macrophages followed by increased IL-17 production in lymphocytes and by neutrophil stimulation, (iv) impaired ROS dependent attenuation of Ca^{2+} signalling, (v) lack of ROS induced apoptosis, (vi) lack of NETs formation associated with less absorbance of inflammatory cytokines, (vii) impaired ROS dependent inhibition of dendritic cell functions [14,18,19,38].

5.3. Biological therapy significantly increased formation of ROS inside neutrophils

The administration of biological therapy significantly elevated intracellular ROS production in neutrophils. The increase was observed after administration of both anti-TNFα and anti-IL-6R antibodies, i.e. independently of the cytokine affected. As several clinical findings indicate that ROS formed inside neutrophils are essential to keep inflammatory reactions under control [10,15,18], the observed effect of biologics may be considered beneficial.

An increased ROS formation was observed by Capsoni et al. [39] in isolated neutrophils of rheumatic patients receiving adalimumab therapy. In other studies, the biological therapy did not exert any effect [20,40,41], yet none of these investigations included a separate detection of extra- and intracellular ROS.

The antibodies against TNFα or IL-6 receptor increased the intracellular but not extracellular chemiluminescence. This suggests an interference with some factors selectively associated with formation of ROS inside neutrophils, e.g. $p40^{phox}$, phosphatidylinositol 3-phosphate (PI3P), phosphoinositide 3-kinase (class III PI3K), isoform δ of protein kinase C (PKCδ), cytoskeleton, phospholipase A_2 or interferon-β pathway. All these components may control the assembly of NADPH oxidase in intracellular membranes [10,11,37]. Increased ROS formation may result from several mechanisms and from the simultaneous modulation of several types of immune cells, as the activities of multitasking cytokines TNFα and IL-6 were blocked by the therapy tested.

5.4. Biological therapy had no effect on extracellular ROS formation

Biological therapy is based on the blockade of actions of specific cytokines or regulators on immune cells [1]. These effects may reduce the antimicrobial activity of blood phagocytes and increase the risk of infection. Infectious complications are the most severe and common adverse effects of biological therapy [20]. Our measurements showed that the concentration of ROS produced by neutrophils extracellularly was not affected by biological therapy. This indicated that the antimicrobial capacity of blood neutrophils was not reduced, at least in terms of ROS production.

6. Conclusion

The increased ROS formation observed in patients with reduced inflammation emphasizes the need to revisit the view of the role of ROS in inflammation - from toxic agents promoting inflammation towards a more complex view of ROS as regulators of immune pathways with inflammation-limiting capacity.

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Conflict interests

The authors declare that they have no conflict interests.

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