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Role of reactive oxygen species in rheumatoid arthritis synovial T lymphocytes

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CHAPTER 2:

Intracellular free radical production in synovial T lymphocytes of patients with rheumatoid arthritis.

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Abstract

Objective: To investigate the cellular and molecular sources of oxidative stress in patients with rheumatoid arthritis (RA) through analysis of reactive oxygen species (ROS) production in synovium.

Materials: Cytochemical procedures based on the 3,3'-diaminobenzidine (DAB)-Mn^{2+} deposition technique were applied on unfixed cryostat sections of synovium from RA patients and disease controls. For immunophenotyping, sections were fixed after incubation, followed by immunofluorescence staining with FITC-labeled antibodies. FACS analysis of the ROS-reactive dye DCF was used to measure intracellular ROS in T lymphocytes from peripheral blood and synovial fluid. To determine which enzymes produced ROS, different inhibitors were tested.

Results: Large quantities of DAB precipitated in the majority of RA synovial T lymphocytes, indicative of intracellular ROS production. These ROS producing T lymphocytes were observed throughout the synovium. To a lesser extent polymerization of DAB was also observed in other forms of chronic arthritis but was absent in osteoarthritis. DAB staining on cytospins of purified synovial fluid T cells from RA patients confirmed the presence of ROS-producing cells. One of the ROS involved appeared to be hydrogen peroxide, as catalase suppressed intracellular ROS production. Superoxide dismutase, which uses superoxide as a substrate to form H_2O_2, DPI (an inhibitor of NADP(H) oxidase), L-NMMA (an inhibitor of NO synthesis), NDGA (an inhihitor of lipoxygenase) and rotenone (which inhibits mitochondrial ROS production), failed to suppress ROS production.

Conclusion: The present study shows that chronic oxidative stress observed in synovial T lymphocytes is not secondary to exposure to environmental free radicals, but originates from intracellularly-produced ROS. Additionally, our data suggest that one of the intracellularly-generated ROS is H_2O_2, although the oxidase(s) involved in its generation remains to be determined.

Introduction

Reactive oxygen species (ROS) play an important role in a variety of pathological conditions, such as ischemia-reperfusion, carcinogenesis, AIDS and aging. In rheumatoid arthritis (RA), oxidative stress has been described as an important mechanism underlying destructive proliferative synovitis (1). In addition, oxidative stress was found to influence functional characteristics of synovial T lymphocytes with critical implications on proximal and distal T cell receptor (TCR) signaling events (2-5). Chronic oxidative stress in synovial fluid (SF) T lymphocytes inhibits TCR-dependent phosphorylation of pivotal signaling molecules required for efficient T cell proliferation, thus contributing to severe hyporesponsiveness of these cells to antigenic stimulation. Oxidative stress in RA SF lymphocytes also plays a role in NF-κB-dependent gene transcription, e.g. resulting in upregulation of TNF-α and IL-1 (6).

Several sources of ROS in the synovial joint that could lead to the disturbed redox homeostasis in SF T lymphocytes have been proposed. These include exposure to free radicals liberated by activated phagocytic cells at the site of inflammation (7), ischemia/reperfusion compromised oxygen radical tension in the inflamed joint (8), and generation of hydroxyl radicals by Fe^{2+} released from dying cells (9). Recent evidence however, suggests that T lymphocyte oxidative stress originates from intracellular enzyme activity controlled by the small GTPases Ras and Rap1 (10).

The specific detection of free radicals is hampered by several methodological problems due to high reactivity and short life of free radicals. Ongoing oxidative stress is therefore generally

analyzed by measurement of secondary products such as oxidized proteins, peroxidized lipids and their breakdown products, or oxidized DNA. These methods give only limited information however on the cellular source(s) of ROS production *in situ*. Therefore, we attempted to identify the source(s) of synovial oxidative stress using a cytochemical technique based on the principles described for the histochemical localization of ROS production in polymorphonuclear leukocytes (developed by Karnovsky 1994), using 3,3' diaminobenzidine (DAB) and manganese ions (11). Free radicals directly react with DAB, forming an insoluble DAB polymer in a reaction catalyzed by the presence of Mn^{2+}. This technique has been successfully used to identify ROS producing sites (12,13,14).

Methods

Reagents

KCN, NaN_3 and $CoCl_2$ were obtained from Merck (Darmstadt, Germany); polyvinylalcohol (weight average Mr 70 000-100 000), menadione, catalase, diphenyleneiodonium (DPI), rotenone and $MnCl_2 \cdot 4\ H_2O$ were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was obtained from PGM Chemicals (New Germany, RSA); 3,3'-diaminobenzidine tetrahydrochloride (DAB) from Fluka Chemika (Buchs, Switzerland), Cu/Zn superoxide dismutse (SOD), nordihydroguaiaretic acid (NDGA) from Alexis Biochemicals Benelux (Breda, the Netherlands) and N^G-methyl-L-arginine, acetate salt (L-NMMA) from Molecular Probes Europe (Leiden, the Netherlands).

Patients and tissue processing.

Synovial tissue samples were obtained from patients during knee arthroscopy performed under local anaesthesia. These included 30 patients with established RA (mean disease duration 14 (SD ± 11 years), 3 RA patients with early, DMARD-naive disease (joint symptoms 3 months or less), and 25 control patients (8 psoriatic arthritis, 7 reactive arthritis, and 10 osteoarthritis (OA)). Tissue fragments were snap frozen by immersion in methylbutane (-80°C) and stored in liquid nitrogen until further use.

Peripheral blood (PB) and synovial fluid (SF) T cells from 5 RA patients (mean disease duration of 6 (SD ± 16) years) were purified from mononuclear cells using a negative isolation procedure (T Cell Negative Isolation Kit, Dynal Biotech Norway), which resulted in a > 90% $CD3^+$ cell population. Purified T cells were subsequently spun onto slides using a cytospin centrifuge (Shandon) for histochemical ROS staining or used for ROS detection on FACScan.

Histochemical procedures

Cryostat sections (8µm thick) were freshly cut on a cryostat at a cabinet temperature of -25°C. The sections were placed on Star Frost® adhesive slides (Optic Labor, Friedrichsdorf, Germany) and immediately used for staining. Before use, sections were air dried for 3 min at room temperature. The incubation medium contained 10% w/v polyvinyl alcohol (PVA), dissolved in 100 mM Tris-maleate buffer (pH 8.0). Sodium azide (5mM) was added to inhibit endogenous myeloperoxidase activity. The following components were added shortly before incubation of the cryostat sections: 0-12.5 mM DAB, 0- 6.5 mM $MnCl_2$ and 0-100mM CoCl2 (1). All the compounds were added in strict order and thoroughly mixed from stock solutions to the PVA-containing medium. Incubation lasted for 60 min at 37 °C. After incubation, sections were washed in distilled water to stop the reaction, and mounted in glycerol for light microscopical inspection.

To further characterize DAB^+ cells, an immunohistochemical staining was performed using a double-staining immunofluorescence procedure following incubation in DAB-Mn^{2+} solution.

The synovial cryostat sections were subsequently washed, air-dried and fixed with 4% paraformaldehyde. They were incubated for 30 min at 4°C with unconjugated mouse anti-human monoclonal antibodies against CD3, CD15, CD19 and CD68 (CLB, Amsterdam, the Netherlands), or the appropriate isotype control antibodies. FITC-labeled sheep anti-mouse secondary antibodies (BD 1:1000) were used for visualization. Tissue sections were scored independently by 2 observers for the presence of DAB precipitate in 50-200 FITC-positive cells. Data were expressed as mean value ± SD.

ROS detection on FACScan

Purified T cells were resuspended at 5×10^6 cells/ml in phenol red-free DMEM medium and loaded with 28 μM 6-carboxy-2', 7'-dichlorodihydrofluorescein diacetate - di(acetoxymethyl ester) (6-carboxy-DCF) (Molecular Probes) for 20 min at 37°C. Cells were analyzed on a FACScan (Becton Dickinson) for the mean fluorescence intensity (MFI) of oxidated 6-carboxy-DCF in the FL1 channel at 3 different time points (0, 10 and 20 minutes). The inhibitors of the different enzymes SOD (1000U/ml), catalase (1000U/ml), DPI (5 μM), NMMA (5 μM) and NDGA (10 μM) were added 30 min before DCF. Rotenone (5 μM) was added 60 min before ROS measurement.

Results

Production of free radicals in rheumatoid synovium.

Diaminobenzidine (DAB) precipitates in the presence of free radicals to form an insoluble final reaction product, visible as a brown DAB polymer. Manganese ions (Mn^{2+}) can be added to catalyze DAB polymerization, as Mn^{2+} is oxidized to Mn^{3+} by $O_2^{\bullet-}$ and enabling Mn^{3+} to oxidize DAB. In synovial tissue from 30/30 RA patients, a large number of DAB^+ cells were detectable perivascularly, in leukocyte aggregrates, and in the intimal and subintimal synovial lining (Figure 1). The presence of DAB in the incubation medium already led to strong formation of the insoluble final reaction product (Figure 1A). Addition of Mn^{2+} (0.5 – 5 mM) only marginally enhanced the amount of DAB precipitate, without affecting the localization pattern of the precipitate (Figure 1B). The highest amounts of final reaction product were found when the incubation medium contained 12.5mM DAB and 2.5mM $MnCl_2$. This composition was then used for all further experiments. Addition of cobalt ions (Co^{2+}) to DAB or DAB/ Mn^{2+}-containing media led to the formation of a blue final reaction product, but also did not affect the amount nor the localization pattern of the precipitate (Figure 1C).

Strong staining intensity did not allow recognition of the shape of the nuclei when counterstained with haematoxylin or Giemsa (data not shown) for differentiation between polymorphonuclear cells and mononuclear cells. To further characterize the cellular sources of ROS, cryostat sections of synovium were incubated with Mab against CD3, CD15, CD19 and CD68. Within the synovial $CD15^+$ population (neutrophils), 36% (mean, SD ± 19) of the cells contained intracellular DAB final reaction product. Additionally, 67% (mean, SD ± 22) of $CD3^+$ cells (T lymphocytes) also contained high intracellular DAB precipitate (Figure 2). No DAB precipitate was found in B-cells (CD19) or macrophages (CD68) (data not shown).

Figure 1

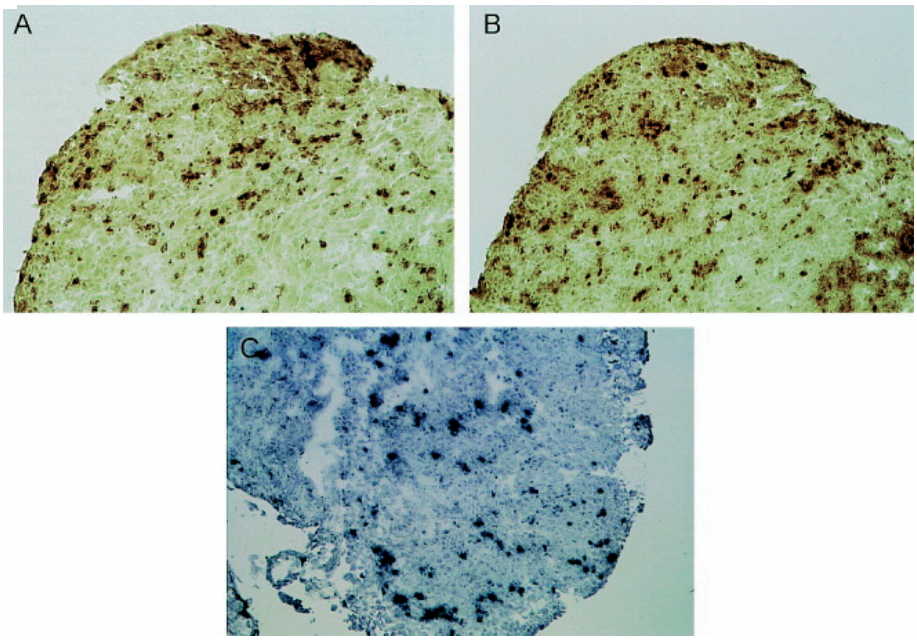

FIGURE 1: DAB staining of RA synovial tissue. DAB precipitates in the presence of free radicals to form an insoluble final reaction product, visible as a brown DAB polymer. A large number of DAB-positive cells can be identified (1A). Addition of Mn^{2+} only marginally enhances the amount of DAB precipitate, without affecting the localization pattern of the precipitate (1B). Addition of cobalt ions (Co^{2+}) to DAB or DAB/ Mn^{2+}-containing media leads to the formation of a blue final reaction product (1C).

Figure 2

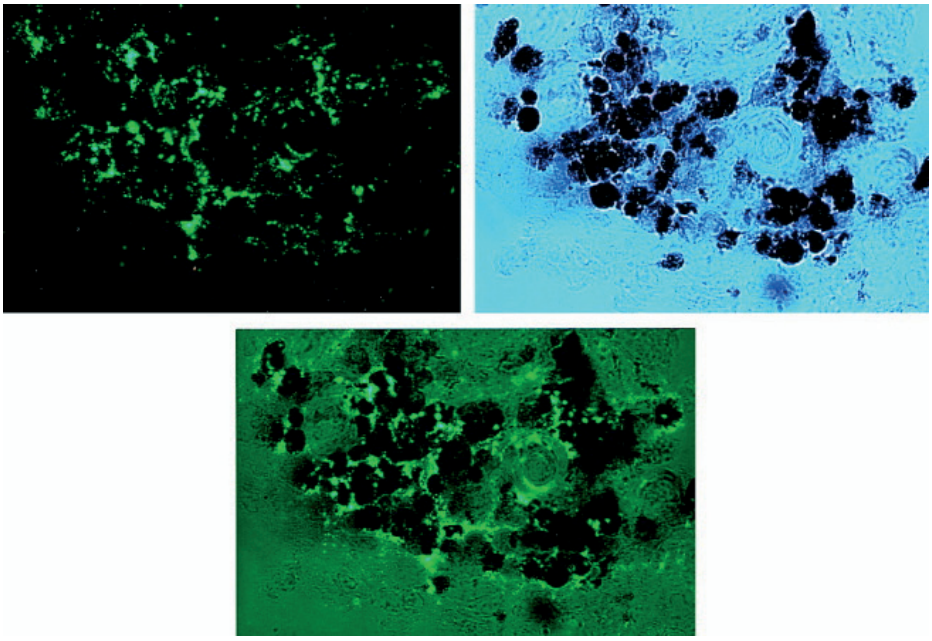

FIGURE 2: Combined DAB and CD3 immunofluorescence of RA synovial tissue. CD3-positive cells contained high intracellular DAB precipitate. Fluorescence microscopy (left panel) and corresponding light microscopy image (middle panel). Combined fluorescence and bright field imaging is shown in lower panel (X400).

To eliminate possible effects of disease modifying anti-rheumatic drugs (DMARD), and to investigate whether intracellular oxidation of DAB in T cells also occurs in early arthritis, we performed the DAB staining on sections of 3 patients with recent onset, DMARD-naive RA. ROS staining in cryostat sections from these patients was indistinguishable from the other RA patients (Figure 3, right panel). To examine the disease specificity of ROS production by synovial T lymphocytes, cryostat sections of RA and non-RA synovial tissue were compared for DAB precipitation products. No polymerization of DAB was observed in any of the T cells in OA tissue samples (n=10) (Figure 4, upper panels). A minimum of 50 $CD3^+$ DAB^- cells were detected in each of the OA tissue samples. Rare DAB^+ cells were identified as $CD15^+$ neutrophils (Figure 4, middle panels). In 1/8 patients with reactive arthritis and 3/7 patients with psoriatic arthritis DAB precipitation was observed in 73% (mean, SD ± 3) and 48% (mean, SD ± 12) of the $CD3^+$ cells respectively (Figure 4, lower panels). There was no correlation between disease activity, disease duration, the presence of RF, disease pattern or erosiveness on the one hand and DAB precipitation on the other hand.

Figure 3

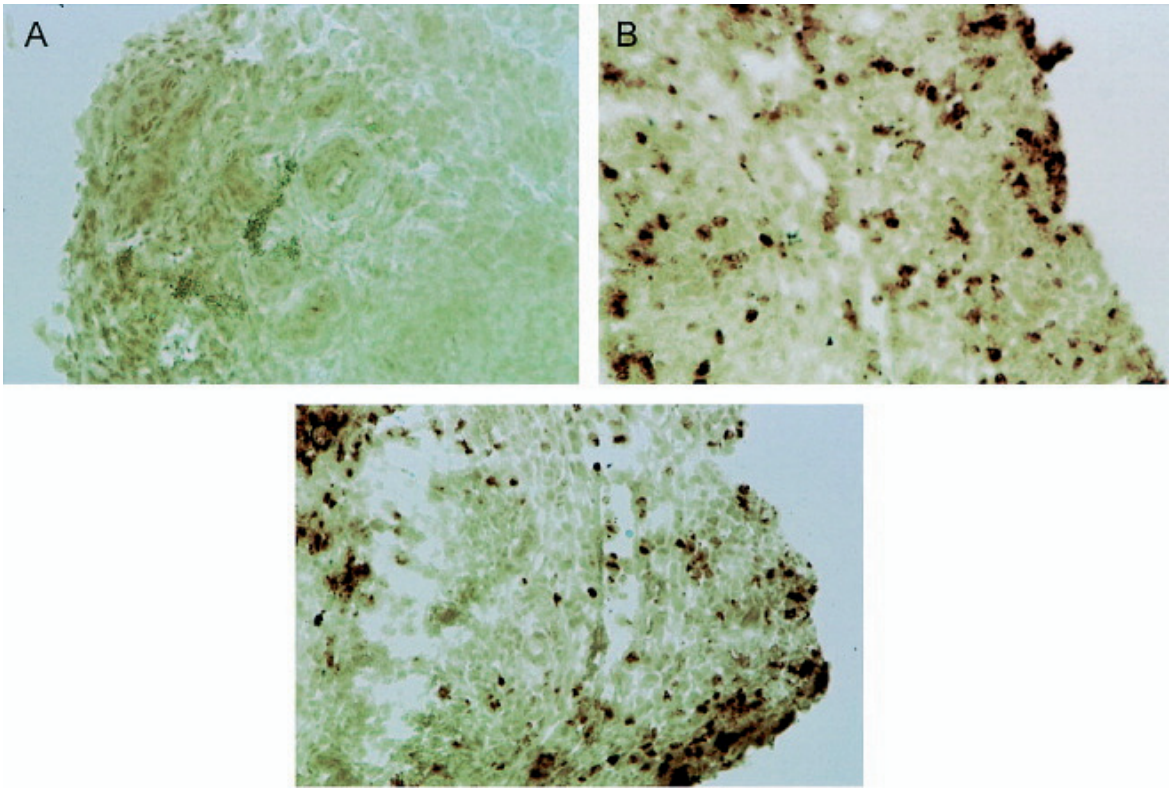

FIGURE 3: DAB staining of sections of synovium from patients with early RA and patients with long standing RA. Tissue section from patients with early RA (Fig 3A) and long standing RA (Fig 3B) both stained equally positive for DAB (X200).

Figure 4

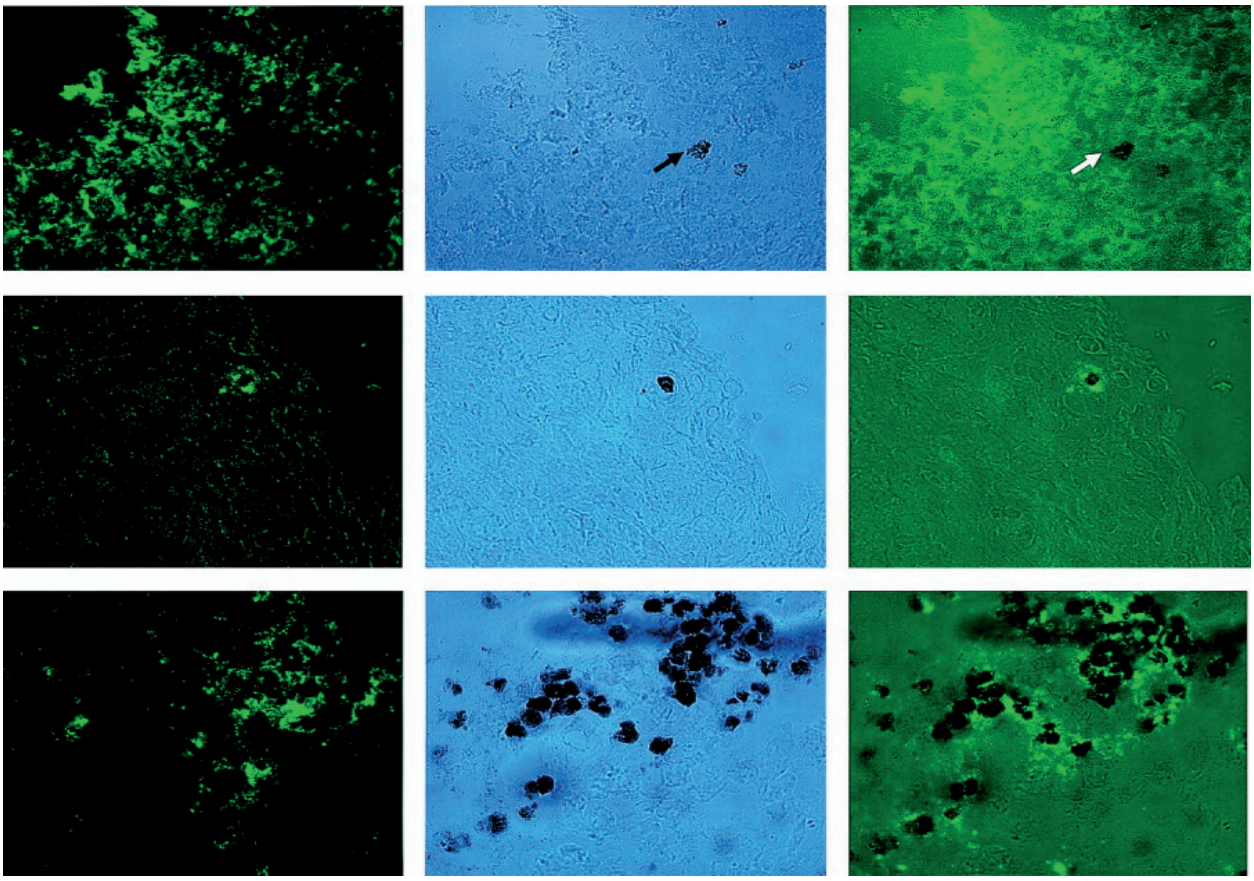

FIGURE 4 : In OA tissue sections DAB precipitate was observed rarely and only in $CD15^+$ neutrophils. Although a minimum of 50 $CD3^+$ cells per patient were counted, no DAB^+ T lymphocytes were found in OA patients. The upper panels show OA tissue with a significant number of $CD3^+$ lymphocytes and corresponding light microscopy image (middle panel). Combined fluorescence and bright field imaging is shown in right panels (X400). All DAB positive cells corresponded with $CD15^+$ neutrophils (middle lanes). $CD3^+DAB^+$ lymphocytes in a psoriatic arthritis patient are shown in lower panels.

ROS production in RA synovial fluid T cells

When DAB staining was performed on cytospins of purified paired PB and SF T cells, DAB precipitate was not found in any of the PB T lymphocytes (Figure 5, upper panels), but was easily detectable in T cells from SF (Figure 5, middle and lower panels). The formation of ROS-dependent DAB polymerization in synovial T lymphocytes is likely due to intracellular enzymatic activity. First, DAB polymerization was not affected by pre-incubation of cytospins or cryostat sections in aqueous medium at 37 °C, but was completely inhibited by preincubation for 10 min at 100 °C. Second, prefixation with paraformaldehyde 4% also abolished formation of final reaction product, as did overnight airdrying at room temperature. Third, the amount of DAB polymerization increased with increasing incubation times from 15 to 60 minutes. On cytospins, the initial DAB precipitate (15 min) in T lymphocytes was detected in the cytoplasm, but not the nucleus of the SF T cells. With increasing incubation periods, DAB precipitate could be detected throughout the cell.

Catalase-sensitive generation of hydrogen peroxide in synovial T lymphocytes

Because it is difficult to quantitate DAB precipitation, we measured intracellular ROS production in purified SF and PB T cells, using FACS analysis of the ROS-reactive dye DCF. SF T cells displayed a higher basal rate of ROS production than PB T cells (Figure 6A). In order to determine which oxidants and enzymes are responsible for the free radical production, inhibitors of the different enzyme systems proposed to regulate ROS production in T lymphocytes were tested (Figure 6B).

The addition of exogenous catalase, which uses H_2O_2 as substrate clearly diminished the DAB polymerization in synovial T cells, reducing basal ROS production in SF T cells to levels observed in PB T cells. A majority of intracellular H_2O_2 originates from the dismutation of $O_2^{\bullet-}$ by superoxide dismutases (SOD). However, when SOD was added to the incubation medium, no significant inhibition of ROS production was observed. Also, addition of DPI (an NADPH oxidase inhibitor), L-NMMA (an NO synthetase inhibitor) or NDGA (a lipoxygenase inhibitor) did not result in significant inhibition of intracellular ROS production. Addition of rotenone (the mitochondrial complex I inhibitor) also did not inhibit increased ROS production in SF T cells.

Figure 5

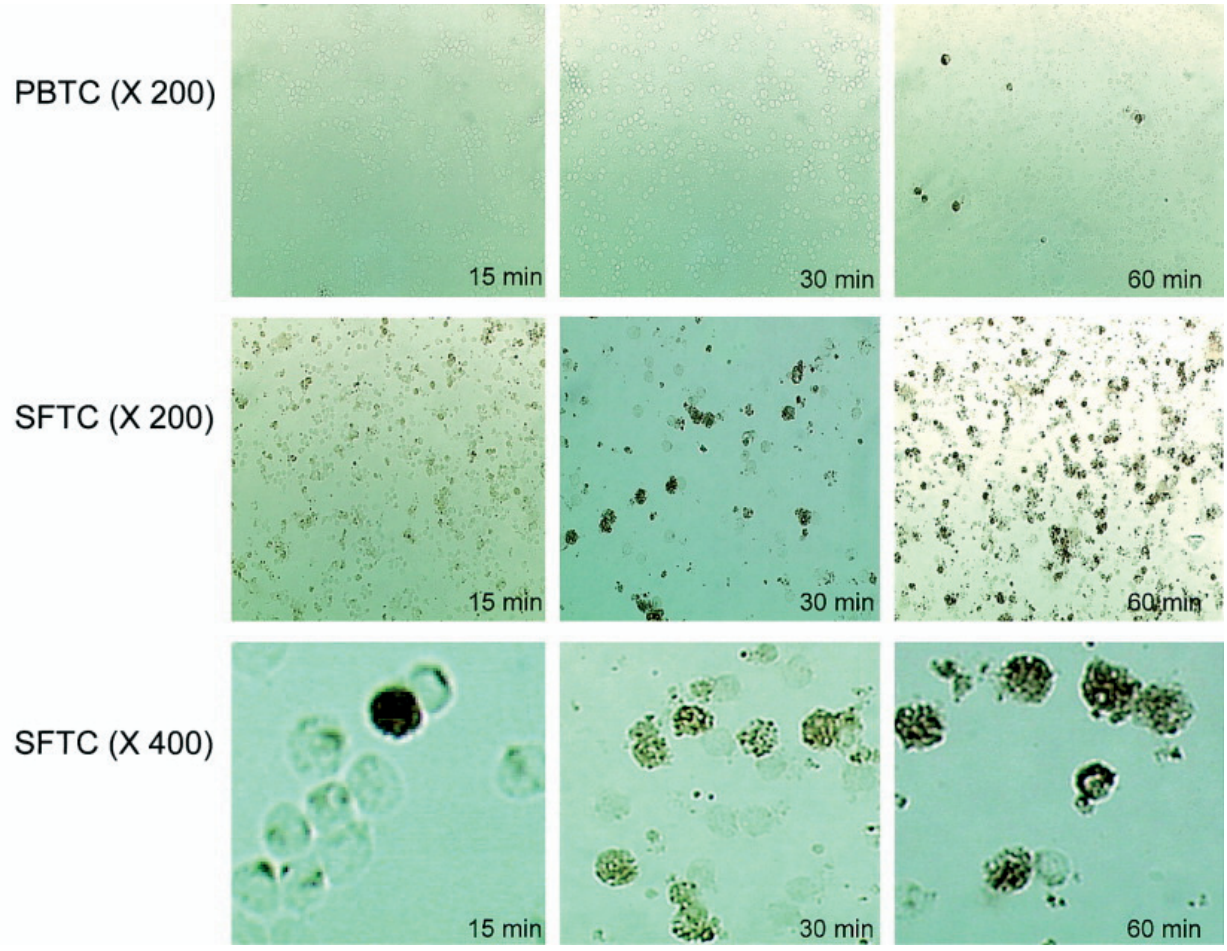

FIGURE 5: DAB staining on cytospins of purified PB TC (Upper panels) and SF TC (middle panels x 200; lower panels: x 400) after different incubation periods. DAB precipitation in SF T cells originates in the cytoplasm of SF T cells (15 min, left panels). Longer incubation period shows a time-dependent increase in intracellular DAB final reaction product (30-60 min, middle and right panels).

Figure 6

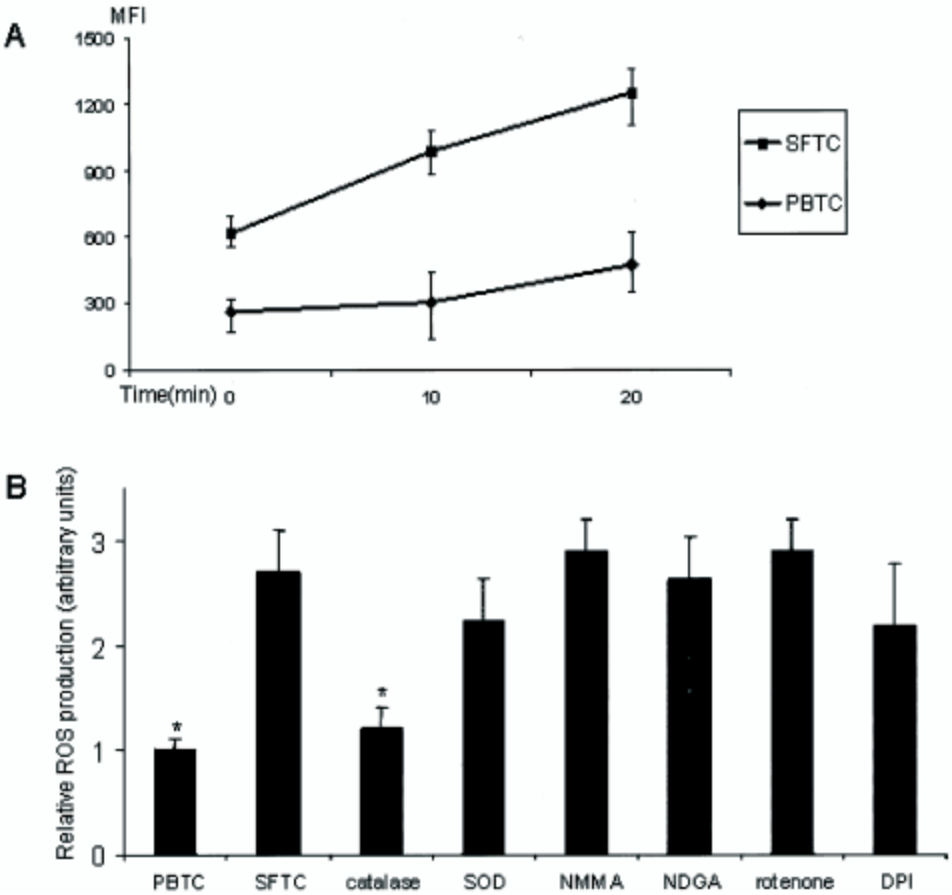

FIGURE 6: A: ROS production is constitutively upregulated in SF T cells, when compared to PB T cells. Mean fluorescence (MFI) of DCF in PB and SF T cells. After 20 min pre-incubation at 37 °C with DCF, MFI was measured at three different timepoints. The data shown are the mean (±SD) values of three independent experiments.
B: Catalase reduced basal ROS production in SF T cells to levels observed in PB T cells. No significant inhibition of intracellular ROS production was seen after pre-incubation with DPI, NMMA, NDGA, and rotenone.
The ROS generation is expressed as the increased ROS production in the different conditions compared to a basal level of ROS production in PBTC =1. The data shown are the mean values of three independent experiments.

Discussion

T lymphocytes are regarded to play a key role in the pathogenesis and the perpetuation of rheumatoid arthritis. Synovial T lymphocytes display features of severe oxidative stress, which results in a number of proliferative and signaling abnormalities (2-5). While there is general consensus about the presence of oxidative stress in synovial T cells, the origin of this oxidative stress is unknown.

Our present data demonstrate that chronic oxidative stress observed in synovial T cells mainly originates from free radicals generated by intracellular sources. This conclusion is at odds with suggestions that exposure of synovial T cells to environmental free radicals, or proximity to ROS-producing neutrophils and/or macrophages is responsible for oxidative stress in synovial T cells. Moreover, perivascular T cells, which have recently entered the synovial milieu, as well as T cells distributed in the intima, subintima and in lymphocyte clusters all produced ROS. This suggests that acquisition of intracellular ROS production is a very early event following extravascularization of T lymphocytes into synovial tissue, and again indicates that in synovial T cells free radicals originate from intracellular sources, rather than environmental free radicals. Although DAB^+ cells were clearly identified as T lymphocytes, not all synovial T lymphocytes contained DAB precipitate, suggesting only a specific subpopulation of synovial T cells are under oxidative stress. The presence of DAB precipitate in over 90% of SF T cells on cytospin versus 68% on tissue sections could be due to a difference in CD3 subset composition between synovial fluid and synovial tissue, or technical issues. Further analysis of specific T cell subpopulations within synovial fluid and tissue should provide insight into these possibilities.
The presence of ROS producing T cells was not entirely specific for rheumatoid arthritis as synovial tissue specimens from a proportion (5 out of 15) of patients with other forms of chronic arthritis also contained DAB^+ synovial T cells. No DAB precipitate was found in synovial tissue from OA patients.
During the past decade, reduction-oxidation (redox) reactions that generate ROS have been identified as important chemical mediators in the regulation of signal transduction processes (14). In particular ROS appears to have a central role in the balance between cell growth, survival and apoptosis. The specific cellular response is dependent on the species of oxidant(s) produced, their subcellular source and localization, the kinetics of production, and the quantities produced. Therefore, the identification of intracellular free radicals in synovial T cells may not only provide an explanation for the altered behavior of synovial T cells, but also prove a pivotal hallmark in understanding underlying pathophysiological mechanisms in rheumatoid arthritis. Jackson at all recently identified 3 different ROS producing events following T cell receptor activation (15): first a rapid H_2O_2 production independent of Fas or NADPH oxidase; second a sustained H_2O_2 production dependent on both Fas and NADPH oxidase; and third a delayed superoxide production that was dependent on Fas ligand and Fas, yet independent of NADPH oxidase. Our results favor the first oxidase as the primary source in synovial T lymphocytes since the intracellular ROS production was catalase-dependent and DPI-independent. Under physiological conditions however, intracellular ROS production is a transient phenomenon, occurring only up to 15 minutes after T cell stimulation. Therefore, the sustained intracellular ROS production in SF T cells might be a pathognomonic hallmark for chronic arthritis, which is not found in PB T cells isolated from RA patients or healthy controls. Identifying the oxidase responsible for the intracellular ROS in SF T cells, and the proteins that regulate the oxidase, could provide new therapeutic targets in RA.

Conclusion

There is a growing body of evidence demonstrating the critical role of ROS in the regulation of mitogenesis, differentiation and apoptosis in physiological and pathophysiological conditions. Our present results indicate that the chronic oxidative stress observed in synovial T lymphocytes from RA patients originates from intracellular generated free radicals, rather then environmental influences.

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