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HUMAN FIBROBLASTS RELEASE REACTIVE OXYGEN SPECIES IN RESPONSE TO TREATMENT WITH SYNOVIAL FLUIDS FROM PATIENTS SUFFERING FROM ARTHRITIS

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Human fibroblasts in primary culture released reactive oxygen species upon exposure to synovial fluid obtained by joint aspiration from twelve patients suffering from rheumatoid arthritis. The primary radical produced was O_2^- as determined by ESR spin trapping and cytochrome c reduction. In contrast to the oxidative burst in granulocytes and monocytes, radical formation proceeded continuously for at least four hours.

Low-level chemiluminescence was increased upon exposure to inflammatory human synovial fluids. Spectral characteristics and effects of azide and 1,4-diazabicyclo-(2,2,2)-octane led to the conclusion that the photoemissive species were excited carbonyls. Radical production and light emission were not altered either by xanthine or allopurinol, nor by azide, cyanide or rotenone. The O_2^- production increased in the presence of NADH or NADPH, making an NAD(P)H oxidase a likely source.

The liberation of reactive oxygen species correlated with the number of leukocytes present in the inflammatory joint fluids, but not with the concentrations of immunoglobulins and complement factor C3.

KEY WORDS: Human fibroblasts, superoxide production, hydrogen peroxide production, low-level-chemiluminescence, arthritis, inflammation.

INTRODUCTION

The aetiology of rheumatoid arthritis and the molecular events triggering this pathophysiological process remain unknown. Various biogenic amines, kinins, prostaglandins, leukotrienes, activated complement factors, immunoglobulins, lytic enzymes and reactive oxygen species are released in inflamed tissues and interact in a complex manner. However, there is one feature common to all types of inflammation, infiltration of the tissue by freely moving cells. Evidence has accumulated to support the concept that leukocytes, especially granulocytes and lymphocytes, are involved in the pathogenesis of rheumatoid arthritis, because they are present in large numbers at the sites of inflammation within the synovial fluid. Augmented numbers of HLA-

DR positive, which means activated T-lymphocytes were demonstrated in the synovial fluid and synovial tissue of patients with rheumatoid arthritis and juvenile rheumatoid arthritis.[1-3] Connective tissue cells, fibroblasts, synovial cells and possibly chondrocytes increase the destructive processes by loosening the tissue and permit rapid proliferation, leading to pannus formation.

The basic stimulus of rheumatoid arthritis that alters cellular functions is unknown, but reactive oxygen species might be involved in both initiation and progression of inflammation. They may interfere by degrading the extracellular connective tissue matrix,[4-7] by inactivating natural protease inhibitors,[8-11] moderating chemotactic activity, and by directly affecting the immune system.[12-15]

In the present study, we investigate whether connective tissue fibroblasts themselves are capable of releasing reactive oxygen species upon stimulation with joint fluids from patients suffering from rheumatoid arthritis, and whether the capacity to release these oxygen species is correlated with clinical parameters.

MATERIALS AND METHODS

Patients

Synovial fluids from twelve patients suffering from an idiopathic arthritis (mostly rheumatoid origin), with at least one aspirable joint have been included in this study (Table I). Grouping of the patients followed the capability of their synovial fluids to stimulate in vitro primary cultures of human fibroblasts for the release of reactive oxygen species (see later). All patients were under the care of the Rheumatology Department of Medizinische Hochschule Hannover. Informed consent was obtained from all patients.

Patients with a post-traumatic, haemorrhagic joint swelling, degenerative joint diseases, septic or crystal-induced synovitis were excluded. After puncture of the joint with a 18 to 20 gauge needle synovial fluid was aspired aseptically into syringes. The joint fluids were either tested within one hour after aspiration or frozen immediately after removal without further treatment and stored at $-20°C$ until used. In addition a routine analysis was performed as described (Table I).

Materials

RPMI-medium (R 10 SP) and trypsin/EDTA (0.125%/0.01%; w/v) were obtained by Gibco BRL (Eggenstein, FRG), fetal calf serum (4K03; FCS) by Biochrom (Berlin, FRG), sterile plastic material for cell cultures was from Nunc (Wiesbaden, FRG) and the plastic cover slips 22 × 60 mm and 4-well multiplates from lux (Newbury Park, CA, USA). Peroxidase type III, cytochrome c grade III, and 1,4-diazabicyclo-(2,2,2)-octane (DABCO) were obtained from Sigma (Deisenhofen, FRG), nitroblue tetrazolium (NBT) and scopoletin were from Serva (Heidelberg, FRG), and 5,5′-dimethylpyrroline-N-oxide (DMPO) from Aldrich, (Steinheim, FRG). Superoxide dismutase was a gift of Grünenthal, (Aachen, FRG). Test kits for fluorescence immunoassays, acid phosphatase, May-Grünwald-Giemsa staining solutions and other chemicals were from Merck, (Darmstadt, FRG).

METHODS

Determination of leukocytes in the inflammatory synovial fluids

The synovial cell count of leukocytes in the inflammatory human joint punctates was determined microscopically by counting in a Neubauer-chamber. Differentiation of the leukocytes was performed after staining according to Pappenheim (May-Grünwald-Giemsa).

Determination of immunglobulins and complement

The rheumatoid factors (immunglobulin IgA, IgG, IgM and complement factor C3) in the inflammatory human joint fluids were determined by fluorescence immunoassays with a Laser Nephelometer 37062 (Behring, Berlin, FRG).

Determination of protein concentration and acid phosphatase

The protein concentration in the aspired joint fluids was assayed with the Biuret reagent,[16] and acid phosphatase according to.[17]

Human fibroblast cell culture

Primary cultures of human fibroblasts were prepared by the method of explantate culture. Small pieces of healthy human skin, which had been removed during surgery, were collected and immediately transported to the laboratory. Informed consent was obtained from all patients. The skin tissues were cut into small pieces (less than 1 mm^3), placed in sterile petri dishes and incubated for 30–45 min at 37°C with 5% (v/v) CO_2 in air. Afterwards 1 ml of medium was added and non attached tissue was removed. After a further incubation for 4 h, 4–5 ml medium were added. One week later the medium was changed for the first time, and subsequently every 3 days until cells reached confluency. These cells were subcultivated 4 times and then frozen as stock culture in liquid nitrogen. The fibroblasts were free of other cell types.[18] For the experiments cells of the 10th to 20th passage were used.

Culture conditions

Cells were cultured in RPMI 1640 medium, which additionally contained 5% heat-inactivated fetal calf serum and L-glutamine (2 mmol/l). For subcultivation of confluent fibroblasts the medium was removed, cells were washed with phosphate-buffered saline (PBS) and treated with a thin film of trypsin/EDTA solution for 5 min. Cells were washed twice with culture medium and split 1:4 in new flasks.

To determine the O_2^- and H_2O_2 production, fibroblasts were cultured as a monolayer on all four sides of glass cuvettes. For this purpose the fibroblasts were grown to confluency in plastic flasks (25 cm^2), washed twice with PBS-buffer, detached with trypsin/EDTA, washed and resuspended in 4 ml culture medium. This cell suspension was transferred into autoclaved glass-cuvettes with teflon stoppers (Kontron, Hannover, FRG), closed and incubated at 37°C on one side for 60 min. The medium was removed and the same procedure was carried out for the three other sides. Afterwards, the medium was changed to remove non-adherent cells. The cuvettes

were opened and incubated in an upright position overnight at 37°C with 5% (v/v) CO_2 in air. The adherence of the fibroblasts, that means a "natural" condition, is a prerequisite to determine the formation of reactive oxygen species.[19]

For the photographic documentation of O_2^- production, the cells were cultured on glass slides in 4-well multiplates. To determine ultraweak spontaneous light emission, the fibroblasts were cultured to confluency (5×10^4 cells/cm^2) on plastic strips in a 4-well-multiplate.

Determination of superoxide formation

Fibroblasts were cultured in the cuvettes as described. To avoid an unspecific perturbation of the test systems by the inflammatory joint fluids the fibroblasts were pre-incubated with the fluids, diluted to a tenth with potassium phosphate buffer (50 mmol/l, pH 7.2) which additionally contained 0.15 mol/l NaCl, 1 mmol/l $MgCl_2$, 0.6 mmol/l $CaCl_2$, and 10 mmol/l glucose (test-buffer) at 37°C for 5 min. A 1:10 dilution of the fluids was necessary, as fibroblasts which came into contact with the undilated inflammatory joint fluids immediately detached. Afterwards the cells were washed twice with the test buffer at 37°C. Activity tests were performed photometrically (UVIKON 820, Kontron, Hannover) in 2 ml test buffer at 37°C. O_2^- formation was determined by the reduction of cytochrome c, (50 μmol/l) at 550 nm[20] or nitroblue tetrazolium (100 μmol/l) to the blue formazan[21] for photographic documentation. The additon of superoxide dismutase (100 nmol/l) confirmed that the reduction of cytochrome c and nitroblue tetrazolium was completely caused by O_2^-. Photos were taken with an Olympus OM-2 camera at an Olympus ITM-2 inverted microscope.

Radical formation was measured by electron spin resonance (ESR) – spin-trapping with 5,5′-dimethyl-l-pyrroline-N-oxide (DMPO). DMPO was purified by filtration through charcoal.[22] The experiments were carried out in a flat cell of 200 μl at room temperature with an X-band cavity (Bruker-Analytic B-ER 420, Karlsruhe FRG). The cells were cultured in 16-well multiplates, pre-incubated with the diluted fluids (1:10 v:v with test buffer) at 37°C, washed twice with the test buffer, and 200 μl joint final volume of the buffer including 50 mmol/l DMPO were added. The cells were incubated at room temperature for 15 min with the test solution, placed on ice for 3 min, scraped off with a soft rubber, transferred to the flat cell and measured under following conditions: amplitude, 100 kHz; field modulation, 0.5 mT; microwave power, 150 mW; receiver gain, 4×10^6; recording time, 2000 s with a response time of 0.5 s; field centre, 0.342 T; sweep width, 50 mT. Only 10 mT around the centre were recorded. The magnetic field was measured with a nuclear magnetic resonance oscillator.

Determination of hydrogen peroxide formation

Hydrogen peroxide was determined fluorometrically using scopoletin[23] with adherent fibroblasts in cuvettes cultured as described above in a fluorimeter (SFM 23, Kontron, Hannover, FRG). The following compounds were added to 2 ml test-buffer: scopoletin, 40 nmol/l; and peroxidase 1 μmol/l. The excitation wavelength was 381 nm and the emission wavelength 436 nm. Calibration of the fluorescence was performed with hydrogen peroxide.

Low-level chemiluminescence

Chemiluminescence of fibroblast monolayer cultures (5×10^4 cells/cm^2) on plastic slides was measured according to[24] with a red sensitive photomultiplier cooled to -25°C by a thermoelectric cooler (EMI Gencom, Plainview, NY) in order to decrease the dark current. The plastic strips were pre-incubated with the synovial fluids (1:10 v/v diluted with the test buffer) at 37°C, washed twice with the test buffer at 37°C and positioned into a cuvette of $3 \times 4.5 \times 0.7$ cm with the cell-covered side towards the photomultiplier. The assays were carried out in test buffer, at 37°C. For a spectral analysis of the emitted light, a cutoff filter (Jenaer Glaswerke Schott, FRG.) with transmission > 620 nm was placed into the light path.

Statistical methods

Regression and correlation analysis were performed using a linear regression analysis (directly and after logarithmic transformation) and a Spearman rank-order test.

RESULTS

Pre-incubation time and dilution of the aspirates

When adherent human fibroblasts were brought into contact with the inflammatory joint fluids they immediately detached. Therefore, the joint fluids were previously diluted 10-fold before being added to the fibroblasts. The fibroblasts remained adherent and visibly intact after incubation in the presence of the diluted joint fluid up to one hour.

To avoid interference of compounds present in the inflammatory joint fluids of the patients with the test systems, the fibroblasts were carefully washed with the test buffer after a pre-incubation period with the synovial fluids of 5 min up to one hour. Afterwards the production of reactive oxygen compounds was determined as described. A pre-incubation time of 5 min was sufficient to induce a complete stimulation of the fibroblasts to release reactive oxygen compounds.

In parallel experiments the inflammatory synovial fluids of three patients were either tested directly without further treatment within one hour after aspiration, or frozen for 5 min, causing a destruction of the leukocytes present in the synovial fluid, and tested afterwards for their capacity to induce the formation of reactive oxygen species in fibroblasts. No differences were observable between frozen and non-frozen samples.

Additionally, synovial fluids (10-fold diluted) were incubated for 5 min in a cuvette which was washed carefully twice after the incubation with the test buffer. No cells had attached to the glass walls by this time and no superoxide or hydrogen peroxide formation was observable.

The experiments described below were performed with frozen inflammatory synovial fluids, batched in small aliquots and stored frozen for up to one year. Within this time the capacity to stimulate the formation of reactive oxygen species by fibroblasts was not changed, as checked by repeated assays.

Effects of aspirates on superoxide production

Human fibroblasts released O_2^- upon exposure to diluted synovial fluids of patients suffering from arthritis. This was documented photographically by the reduction of

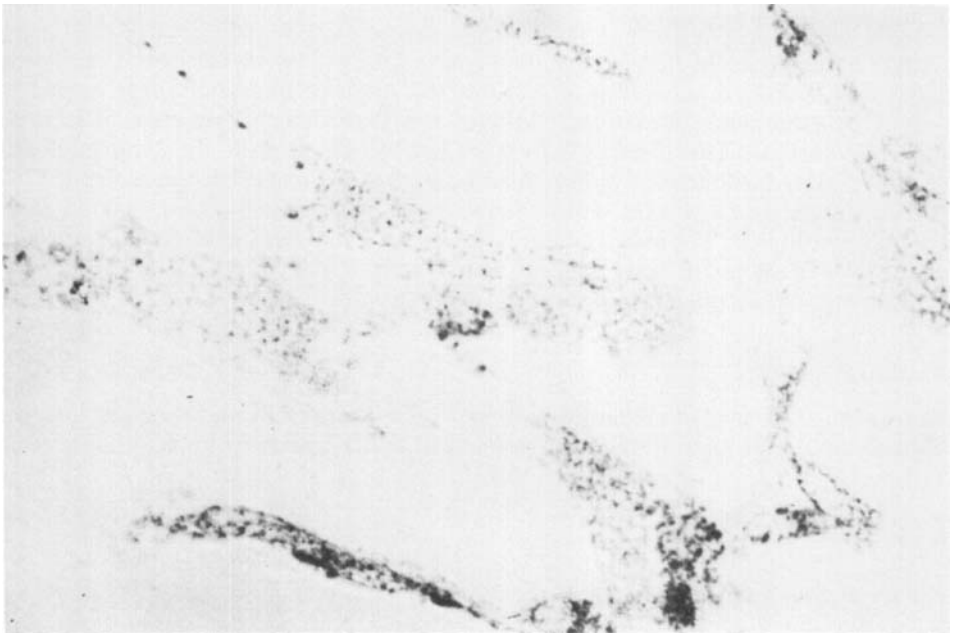

FIGURE 1 Oxygen radical-dependent reduction of nitroblue tetrazolium by human fibroblasts exposed to synovial fluid from patients suffering from rheumatoid arthritis. The cells were exposed for 5 min to synovial fluid number 1, diluted 1:10 (v:v) the in test-buffer, washed twice with the test buffer and incubated for 1 h with NBT in the test-buffer at 37°C and 5% CO_2 in air (see methods).

nitroblue tetrazolium to formazan (Figure 1). Without the exposure to synovial fluid there was no reduction. The reduction of nitroblue tetrazolium was completely abolished in the presence of superoxide dismutase (100 nmol/l) (not shown). The cells remained viable over a period of 2 h; assessed by exclusion of trypan blue. The distribution of the blue formazan particles was homogeneous over the plasma membrane and could not be correlated with any specific cellular compartment by light microscopy.

The time-dependent formation of O_2^- was determined by cytochrome c reduction[20] after stimulation with the synovial fluids (Figure 2). The reduction of cytochrome c took place continuously for at least four hours. The basal production of O_2^- was below the detection limit. The reduction of cytochrome c was completely inhibited by the addition of superoxide dismutase (100 nmol/l).

Effects of aspirates on hydrogen peroxide production

The H_2O_2 production of human fibroblasts pre-incubated with the joint punctates was determined fluorimetrically by the peroxidase-mediated oxidation of scopoletin.[23] The treatment of fibroblasts with these stimulants led to a production of H_2O_2 as shown in Figure 3. As H_2O_2 is the product of the disproportionation of O_2^-, time courses were similar to that described above. Untreated fibroblasts did not alter the fluorescence of scopoletin.

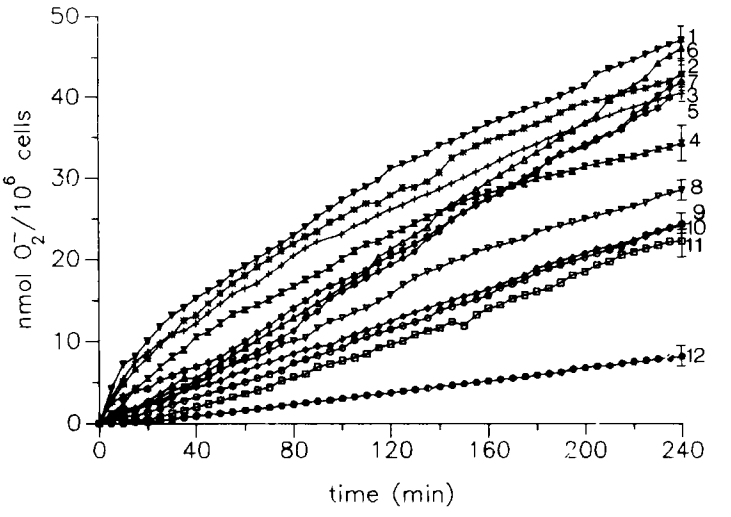

FIGURE 2 Formation of O_2^- by human fibroblasts after exposure to synovial fluids from 12 patients suffering from rheumatoid arthritis. Controls without stimulation were below the detection limit. Each point shows the mean value and standard deviation of four different experiments.

Determination of radicals by ESR spectroscopy

The nature of the radicals generated was analyzed by ESR spin-trapping with DMPO.[22] After a pre-incubation of 5 min with the joint fluid and a further incubation period of 15 min with DMPO (50 mmol/l) in the test-buffer, a spectrum of overlapping signals of the DMPO–OOH and DMPO–OH adduct was observable (Figure 4). The

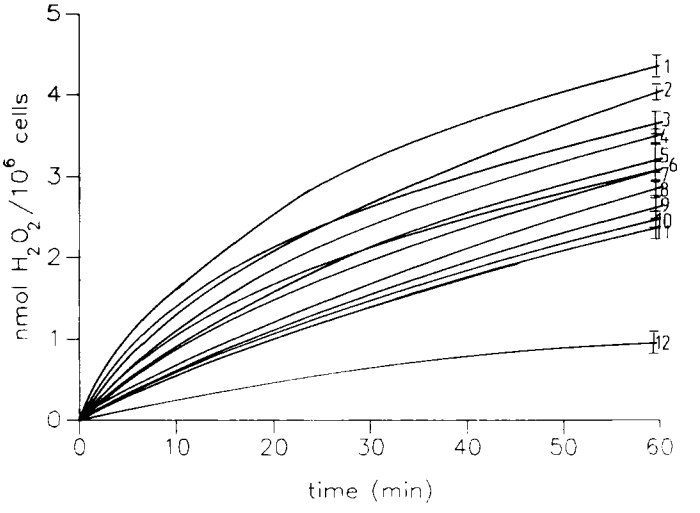

FIGURE 3 Formation of H_2O_2 by human fibroblasts after exposure to synovial fluids from 12 patients suffering from rheumatoid arthritis. Controls without stimulation were below the detection limit. Each graph shows the mean value and standard deviation of four different experiments.

FIGURE 4 Radical production by human fibroblasts after exposure to synovial fluids from patients suffering from rheumatoid arthritis, determined by ESR-spin-trapping with DMPO. *Upper line.* The fibroblasts were exposed to synovial fluid for 5 min and then incubated with 200 μl test-buffer supplemented with 50 mmol/l DMPO for 15 min (see methods). *Lower line.* The fibroblasts were treated as described above, but superoxide dismutase (1 μmol/l) was added to the test solution.

amplitudes of the ESR spectra correlated with the O_2^- production of the different joint fluids, as determined by the cytochrome c assay, whereas the hyperfine splitting was not altered. These signals were not observed when superoxide dismutase (100 nmol/l) was added to the test system, whereas heat inactivated SOD was without effects. It is concluded that the primary radical induced was O_2^- and the DMPO–OH adduct was due to decompositon of the DMPO–OOH adduct.[22]

Low-level-chemiluminescence

Fibroblasts stimulated with synovial fluids of patients suffering from arthritis showed spontaneous chemiluminescence (Figure 5). The addition of azide and DABCO up to 10 mmol/l had no effect on light emission. Because azide is a quencher of singlet oxygen and DABCO is described to enhance the $^1O_2$-mediated low-level chemiluminescence, the photoemissive species may be excited carbonyl compounds rather than

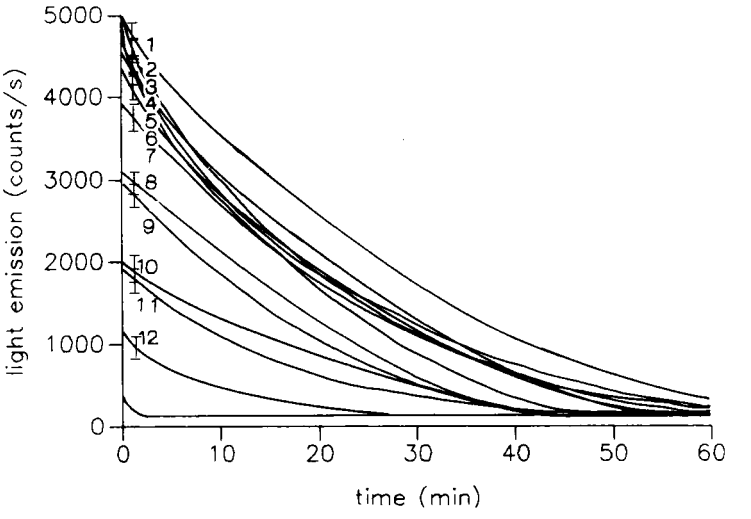

FIGURE 5 Low-level-chemiluminescence of human fibroblasts after exposure to synovial fluids from 12 patients suffering from rheumatoid arthritis. Each graph shows the mean value and standard deviation of three different experiments.

singlet oxygen. This was supported by the spectral characteristics; the 620 nm cutoff filter completely absorbed the light emitted by the fibroblasts, so that the photoemission is in the visible spectral region attributed to excited carbonyls.

Inhibition and stimulation of oxygen radical production and light emission

Since the nature of the oxygen radical generating system in fibroblasts is unknown, we added a series of inhibitors and possible substrates to the stimulated fibroblasts. Xanthine (100 μmol/l) or allopurinol (100 μmol/l) did not alter the oxygen radical production, making xanthine oxidase unlikely as a source.

Likewise, azide (1 μmol/l to 1 mmol/l), cyanide (1 μmol/l to mmol/l) or rotenone (10 μmol/l) were added as inhibitors of the mitochondrial electron transport chain and were without any effect on light emission and cytochrome c reduction.

In the presence of NADH (1 mmol/l) or NADPH (1 mmol/l), O_2^- production increased sixfold. As both substrates are thought to be unable to penetrate an intact cell membrane, the radical generating system might be localised in the outer plasma membrane, as is known for the NADPH oxidase system.[25] Moreover, radical initiated lipid peroxidation may change the permeability of the cell membranes.

Correlation of stimulatory effects with clinical data

The synovial fluids from patients were classifed in numerical order according to their potency to stimulate O_2^- production in human fibroblast cell cultures within 2 hours after exposure to the fluids (Table I). Statistical analysis using a linear regression and Spearman rank order test was performed to obtain information about the relation of oxygen radical production to clinical data (Table II). High correlation was obtained between the number of leukocytes (granulocytes and lymphocytes) present in the inflammatory joint fluids, whereas no significant correlations were observable between immunoglobulins and the complement factor C3. The correlation to acid phosphatase as a "marker enzyme" released during inflammation was not significant.

DISCUSSION

Rheumatoid arthritis leads to irreversible destruction of joints, cartilage, bones, capsules and tendons. The aetiology is unknown, as are the self-perpetuating and self-accelerating mechanisms of destruction. However, the release of reactive oxygen species by activated granulocytes and macrophages represents one important destructive element.

The results presented in this study show that not only phagocytes but also fibroblasts release reactive oxygen species into the environment upon exposure to diluted synovial fluids from patients suffering from various forms of arthritis. The absolute amount of superoxide radicals released by the fibroblasts is comparable to superoxide release by monocytes and granulocytes, making it likely that fibroblasts may contribute to a radical-induced tissue destruction. In contrast to phagocytes, the formation of singlet oxygen was not detectable.[26,27]

It became clear that the total number of cellular components of synovia, especially the leukocytes (granulocytes and lymphocytes), correlates well with the capacity of the synovial fluid to induce oxygen radical formation in fibroblasts. In contrast, no

TABLE I
Clinical data of the patients

diagnosis	leukocytes cells/μl	granulocytes cells/μl		lymphocytes cells/μl		monocytes cells/μl	
1. seroneg.C.P.	13000	10920	(84%)	1040	(8%)	1040	(8%)
2. C.P.	8200	3690	(45%)	4510	(55%)	410	(5%)
3. C.P.	7000	5320	(76%)	1470	(21%)	210	(3%)
4. seroneg.C.P.	3750	2550	(68%)	1050	(28%)	150	(4%)
5. C.P.	2300	1390	(60%)	736	(32%)	184	(8%)
6. C.P.	2950	531	(18%)	1505	(51%)	915	(31%)
7. C.P.	1300	65	(5%)	936	(72%)	299	(23%)
8. undiff.Mono.A	1050	546	(52%)	335	(32%)	168	(16%)
9. undiff.Sp.A.	2000	480	(24%)	960	(48%)	460	(28%)
10. oligoarthr.	1000	200	(20%)	640	(64%)	160	(16%)
11. M. Reiter	2900	2320	(80%)	348	(12%)	232	(8%)
12. M. Bechterew	320	77	(24%)	192	(16%)	51	(16%)

C.P., chronic Polyarthritis; seroneg. seronegative; undif., undifferentiated; Sp.A., Spondarthritis; Mono.A., Monoarthritis; M. Reiter, Morbus Reiter; M. Bechterew, Morbus Bechterew

Chemical parameters of the synovial fluid

	protein g/l	acid phosphatase nkat/l	IgG mg/l	IgA mg/l	IgM mg/l	complement C3 mg/l	O_2-production/ 10^6 cells nmol/l × 2h
1.	4.70	16.83	7.4	1.2	0.8	0.35	30.6
2.	4.41	12.12	10.8	0.7	0.6	0.40	27.0
3.	4.00	21.00	7.5	2.1	1.2	0.36	26.0
4.	5.49	19.33	7.7	1.9	0.9	0.36	23.0
5.	5.00	16.67	7.7	1.1	< 0.2	0.38	20.5
6.	4.20	28.83	7.4	2.9	0.6	0.46	20.0
7.	3.15	11.83	n.d.	n.d.	n.d.	n.d.	19.5
8.	5.40	7.50	n.d.	n.d.	n.d.	n.d.	16.0
9.	3.50	14.33	7.1	7.1	1.2	0.45	13.0
10.	5.20	6.67	n.d.	n.d.	n.d.	n.d.	17.0
11.	4.50	11.83	18.9	3.3	1.9	< 0.20	10.0
12.	4.20	5.00	n.d.	n.d.	n.d.	n.d.	3.3

n.d. not determined.

correlation was obtained between radical production and the concentration of the immunoglobulins, IgA, IgM (acute phase immunoglobulin) and IgG (chronic phase immunoglobulin) or complement factor C3. The involvement of these compounds in the stimulation of granulocytes to release reactive oxygen species is well documented.[29,30] In contrast to granulocytes and monocytes, fibroblasts possess no Fc- or complement receptors, making a receptor-mediated stimulation unlikely. No correlation, however, was observable between the concentration of immunoglobulins or complement factor C3 and the severity of the disease.

On the other hand, as lymphokines (TNF-α and IL-1α) are potent stimulants of radical formation in fibroblasts,[19] the significant correlation between the number of leukocytes present in synovia and radical formation might agree with the concept that secretory products of these cells are the source of radical-stimulating activity in the inflamed synovia. In fact, high concentrations of cytokines have been demonstrated in synovial fluids of arthritic joints.[31–34]

The synovial fluids of arthritic joints contain numerous potent stimulants of oxygen

TABLE II
Correlation of O_2^- release by fibroblasts and clinical parameters

parameter	r	p	r_1	p	r_2	p
leukocytes	0.666	0.018	0.875	0.000	0.860	0.000
granulocytes	0.662	0.019	0.655	0.021	0.755	0.006
lymphocytes	0.573	0.052	0.785	0.003	0.783	0.004
monocytes	0.397	0.040	0.397	0.040	0.295	0.342
acid phosphatase	0.579	0.048	0.679	0.015	0.708	0.012
protein	0.076	0.814	0.108	0.739	0.100	0.749
IgG	−0.538	0.169	−0.482	0.226	−0.013	0.948
IgA	−0.716	0.049	−0.879	0.004	−0.762	0.031
IgM	−0.558	0.151	−0.083	0.846	−0.149	0.706
complement C3	−0.268	0.521	−0.301	0.469	−0.339	0.392

r = correlation coefficient; r_1 = logarithmic correlation coefficient; r_2 = Spearman correlation coefficient for rank correlation; p = probability.

radical formation in phagocytic cells including decomposed hyaluronic acid, collagen and elastin. The amount of superoxide production observed by the fibroblasts upon stimulation with joint fluids was doubled in comparison to IL-1 or TNF in the optimal concentration,[19] making a participation of other compounds likely.

Whatever the precise mode of activation may be, our study clearly demonstrates that compounds present in the synovia of patients suffering from various forms of arthritis are potent stimulants triggering the release of activated oxygen species from fibroblasts, which may contribute to tissue destruction in inflammatory diseases.

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