Biochem. J. (1989) 263, 539–545 (Printed in Great Britain)

Human fibroblasts release reactive oxygen species in response to interleukin-1 or tumour necrosis factor-α

Beate MEIER,*|| Heinfried H. RADEKE,† Susanne SELLE,* Maged YOUNES,‡ Helmut SIES,§ Klaus RESCH,† and Gerhard G. HABERMEHL*

*Chemisches Institut, Tierärztliche Hochschule Hannover, Bischofsholer Damm 15, D-3000 Hannover 1, †Institut für Molekularpharmakologie, Medizinische Hochschule Hannover, Konstanty-Gutschow-Straße 8, D-3000 Hannover 61, ‡Institut für Toxikologie, Medizinische Universität Lübeck, Ratzeburger Allee 160, D-2400 Lübeck, and §Institut für Physiologische Chemie I, Universität Düsseldorf, Moorenstraße 5, D-4000 Düsseldorf, Federal Republic of Germany

Human fibroblasts in primary culture released reactive oxygen species upon stimulation with cytokines such as interleukin-1α (IL-1) or tumour necrosis factor-α (TNF). The primary radical produced was $O_2^{\cdot-}$ as determined by e.s.r. spin trapping and cytochrome *c* reduction. In contrast to the oxidative burst in granulocytes and monocytes, radical formation took place continuously for at least 4 h. Low-level chemiluminescence was increased by stimulation with IL-1 and TNF. Spectral characteristics and tests with azide led to the conclusion that the photoemissive species were excited carbonyls and not singlet oxygen. Further, there was a liberation of ethane from the cells. Radical production and light emission were not altered by either xanthine or allopurinol, nor by azide, cyanide or rotenone. $O_2^{\cdot-}$ production increased in the presence of NADH or NADPH, making an NAD(P)H oxidase a likely source.

INTRODUCTION

The cytokines interleukin-1α (IL-1) and tumour necrosis factor-α (TNF) are released by activated monocytes and macrophages. Both are proteins with an almost identical molecular mass of 17.5 kDa. TNF additionally occurs in a trimeric native form of 45 kDa. IL-1 exhibits a variety of stimulatory activities on maturation, differentiation and growth of many cell types involved in development and inflammation, such as fibroblasts, synovial cells, endothelial and epithelial cells, bone marrow cells, and T- and B-lymphocytes [1–4]. IL-1 possesses fibroblast growth factor activity, induces the acute-phase protein synthesis in hepatocytes and the proliferation of immunologically important cells like thymocytes and B-lymphocytes, as well as stimulating their maturation. As a local pro-inflammatory agent, IL-1 stimulates the release of prostaglandins, collagen and collagenase from synovial cells and fibroblasts. Systemically its effects were described as endogenous pyrogen activity and as catabolin. Serum concentrations of IL-1 in patients with inflammatory joint diseases were shown to be elevated [5]. Moreover, the synovial fluids in arthritic joints contained high amounts of IL-1 and TNF. For a variety of cells including fibroblasts and T- and B-lymphocytes, the activity is mediated by an apparently identical receptor (IL-1 receptor) molecule [6].

TNF was first described by its anti-tumour activity [7]. Recently, it was observed that TNF also shows important effects on several non-tumour cells, increasing the interferon-γ-mediated expression of class II major histocompatibility antigens which are necessary to induce an immune response [8,9]. It also enhances the numbers of epidermal growth factor receptors on fibroblasts [10]. A recent report supports the idea of the participation of reactive oxygen products in TNF-mediated tumour cell killing [11].

Both cytokines may have profound effects on inflammatory reactions [5,12–16]. TNF-specific antibodies inhibited pro-inflammatory activities of TNF [17]. IL-1 and TNF both induce oxygen radical formation in neutrophils and macrophages [18–21]. This has not been described so far in other cells which carry IL-1 and TNF receptors. Here we report that these cytokines induce the formation of reactive oxygen species in human fibroblasts.

MATERIALS AND METHODS

Materials

RPMI 1640 medium (R10SP) and trypsin (0.125%, w/v)/EDTA (0.01%, w/v) were obtained from Gibco (Eggenstein, Germany), fetal calf serum (4K03) from Biochrom (Berlin, Germany), sterile plastic material for the cell cultures from Nunc (Wiesbaden, Germany) and plastic cover slips (22 mm × 60 mm) and 4-well multiplates from Lux (Newbury Park, CA, U.S.A.). Human recombinant TNF, with a specific activity of 10^8 units/mg, was kindly supplied by Bissendorf Peptides (Wedemark, Germany). Human recombinant IL-1, with a specific activity of 2×10^7 units/mg, was from Dainippon (Osaka, Japan). Peroxidase type II and cytochrome *c* grade III were obtained from Sigma (Deisenhofen, Germany), Nitroblue Tetrazolium (NBT) and scopoletin were from Serva (Heidelberg, Germany), and 5,5′-dimethylpyrroline-*N*-oxide (DMPO) from Aldrich (Steinheim, Germany). Calibration gas was obtained from Messer Griesheim (Düsseldorf, Germany).

Abbreviations used: IL-1, interleukin-1α; TNF, tumour necrosis factor-α; NBT, Nitroblue tetrazolium; DMPO, 5,5′-dimethylpyrroline-*N*-oxide; PBS, phosphate-buffered saline; SOD, superoxide dismutase; PMA, phorbol 12-myristate 13-acetate.

|| To whom correspondence should be addressed.

Superoxide dismutase (SOD) was a gift from Grünenthal (Aachen, Germany). All the other chemicals were from Merck (Darmstadt, Germany).

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 (informed consent having been obtained from all patients), were collected and immediately transported to the laboratory. The skin tissues were cut into small pieces of less than 1 mm^3, placed in sterile Petri dishes and incubated for 30–45 min at 37 °C in an atmosphere of 5 % CO_2. Afterwards, 1 ml of medium was added and non-atttached tissues were removed. After further incubation for 4 h, 4–5 ml of medium was added. The medium was changed for the first time after 1 week, and subsequently every 3 days until the cells reached confluency. These cells were subcultivated four times and then frozen as stock culture in liquid nitrogen. Fibroblasts from three different individuals (age 6 months, 12 years and 36 years) were used for the experiments. The cells were taken from different regions of the body, but each cell line from only one region. The results were additionally checked with an established human fibroblast cell line, CLR-1147 ATCC, (American Type Culture Association, Rockville, MD, U.S.A.). No significant differences were observable between these cell lines. Contamination with phagocytes, which would interfere with the experiments, is excluded because they cannot survive in the culture conditions used. To exclude even low contamination, only cells between ten and twenty passages were used. The cells were checked for contamination (virus, mycoplasma and other cell types) by electron microscopy. The fibroblasts exhibited normal radiation sensitivity, indicating that no transformation had occurred during the passages. More detailed information is described in ref. [22]. The phenotype and the stimulated radical production remained stable between passages.

Culture conditions

Cells were cultured in RPMI 1640 medium, which additionally contained 10 % heat-inactivated fetal calf serum and 2 mmol of glutamine/l. For subcultivation of confluent fibroblasts, the medium was removed, cells were washed with phosphate-buffered saline (PBS; NaCl, 140 mmol/l; KCl, 2.7 mmol/l; Na_2HPO_4, 56 mmol/l; KH_2PO_4, 1.5 mmol/l, pH 7.2) and treated with a thin film of trypsin/EDTA solution for 5 min. Cells were washed twice with culture medium and divided into new flasks in a ratio of 1:4.

To measure $O_2^{\cdot-}$ and H_2O_2 production, fibroblasts were cultured as a monolayer on all four sides of glass cuvettes. The fibroblasts were grown to confluence in plastic flasks (125 cm^2), washed twice with PBS, detached with trypsin/EDTA, washed and resuspended in culture medium. This cell suspension was added to autoclaved glass cuvettes with Teflon stoppers (Kontron, Hannover, Germany), which were then 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 in 5 % CO_2 in air. The density and homogeneity of the cells were checked before the experiments by counting the cells on an equivalent part of the cuvette using a phase-contrast microscope. The cells remained adherent during the experiments. At the end of the experiments the cells were detached with trypsin/EDTA and counted in a Neubauer chamber to obtain the number of cells/ml. The adherence of the fibroblasts, i.e. mimicking the 'natural' condition, is a prerequisite in determining the formation of reactive oxygen species.

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

Determination of superoxide formation

Fibroblasts were cultured in the cuvettes as described. The activity tests were performed in 2 ml of potassium phosphate buffer (50 mmol/l, pH 7.2) which additionally contained 0.15 mol of NaCl/l, 1 mmol of $MgCl_2$/l, 0.6 mmol of $CaCl_2$/l and 10 mmol of glucose/l (test buffer) at 37 °C. $O_2^{\cdot-}$ radical formation was determined by reduction of cytochrome *c* (5×10^{-5} mol/l) at 550 nm [23], or of NBT (0.1 mmol/l) to Blue Formazan [24] for photographic documentation. Addition of SOD (0.1 μmol/l) confirmed that the reduction of cytochrome *c* and NBT was caused exclusively by $O_2^{\cdot-}$. Photographs were taken using an Olympus OM-2 camera on an Olympus ITM-2 inverted microscope.

The reduction of cytochrome *c* was measured using a double-beam spectrophotometer with automatic cuvette changing for six cuvettes and six reference cuvettes (Kontron). The cells were incubated with cytochrome *c* for 1 h before stimulation to avoid a spontaneous formation of $O_2^{\cdot-}$, and were then stimulated with IL-1 or TNF. The integration time for each cuvette was 40 s and the cycle time was 5 min. The sensitivity was high, because any turbidity causing non-specific light scattering was excluded by working with adherent cells. As the cells remained adherent during the experiments, no shift of the baseline was observable. At the end of each experiment the test solution was removed and measured in a cuvette without cells against an unstimulated reference to avoid disturbance caused by the fibroblasts. The absorbance of each cuvette was printed every 5 min and the mean values and standard deviations for four different experiments were calculated by a graphic program including statistical functions (PlotIT, S. P. Eisensmith, 1985; ICS, G.m.b.H., Frankfurt, Germany). The cuvettes were either preincubated for 15 min with TNF or IL-1 in the culture medium and afterwards washed twice with test buffer (37 °C), or the stimulants were added directly to the test solution.

Radical formation was measured by e.s.r. spin-trapping with DMPO. DMPO was purified by filtration through charcoal [25]. The experiments were carried out in a flat 200 μl cell at room temperature with an X-band cavity (Bruker-Analytic B-ER 420, Karlsruhe, Germany). The cells were cultured in 16-well multiplates, washed twice with the test buffer, and a final volume of 200 μl of the buffer containing 50 mmol of DMPO/l and the stimulants was added. The cells were incubated at room temperature for between 30 min and 2 h with the test solution, put on ice for 3 min, scraped off with a soft rubber policeman, 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 an n.m.r. oscillator.

Determination of H_2O_2 formation

H_2O_2 was determined fluorimetrically using scopoletin [26] with adherent fibroblasts in cuvettes cultured as described above in a Kontron Fluorimeter. The following compounds were added to 2 ml of test buffer: scopoletin, 40 nmol/l and peroxidase, 1 μmol/l. The excitation wavelength was 381 nm and the emission wavelength 436 nm. The decrease in fluorescence was recorded directly and the graphs (means $\pm$ S.D. for four different experiments) were calculated with PlotIT. Calibration was done with authentic H_2O_2.

Determination of ethane production

The fibroblasts, cultured in plastic flasks (20 cm^2), were washed twice with test buffer (37 °C). Then the stimulants, dissolved in 1 ml of test buffer, were added to the cultures. The flasks were closed gas-tight with a Teflon seal in the stopper and incubated for 30 min at 37 °C. Samples of 7 ml were removed from the gas space by piercing through the Teflon seal and injected via a 5 ml sampling loop into an aluminum oxide column (80–100 mesh). Ethane production was determined by gas chromatography (Carlo Erba model 2151, Hannover, Germany) with a flame ionization detector. Nitrogen of the purest grade available was used as a carrier gas with a flow rate of 40 ml/min. The analysis was run isothermally at 60 °C. A mixture of methane, ethane, propane, butane, 2-methylpropane, pentane and hexane, each 100 p.p.m by vol. in nitrogen, was used for calibration.

Low-level chemiluminescence

Chemiluminescence of fibroblast monolayer cultures (5×10^4 cells/cm^2) on plastic slides was measured according to [27] with a red-sensitive photomultiplier cooled to −25 °C by a thermoelectric cooler (EMI Gencom, Plainview, NY, U.S.A.) in order to decrease the dark current. The plastic strips were positioned into a cuvette of 3 cm × 4.5 cm × 0.7 cm, with the cell-covered side towards the photomultiplier. The assays were carried out in test buffer at 37 °C. For spectral analysis of the emitted light, a cut-off filter (Jenaer Glaswerke, Schott, Mainz, Germany) with transmission > 620 nm was placed into the light path.

RESULTS

Effects of cytokines on superoxide production

Human fibroblasts released $O_2^{\cdot -}$ upon stimulation with 280 nmol of IL-1/l or 290 nmol of TNF/l. This was documented photographically by the reduction of NBT to the blue, insoluble formazan (Figs. 1*a* and 1*b*). Without stimulation, NBT was not reduced by the fibroblasts; the stimulated reduction of NBT was completely abolished in the presence of 0.1 μmol of SOD/l (Fig. 1*c*). The cells remained viable over a period of 4 h, as tested by Trypan Blue exclusion. The distribution of the Blue Formazan particles was rather homogeneous over the plasma membrane and could not be correlated to any specific cellular compartment by light microscopy.

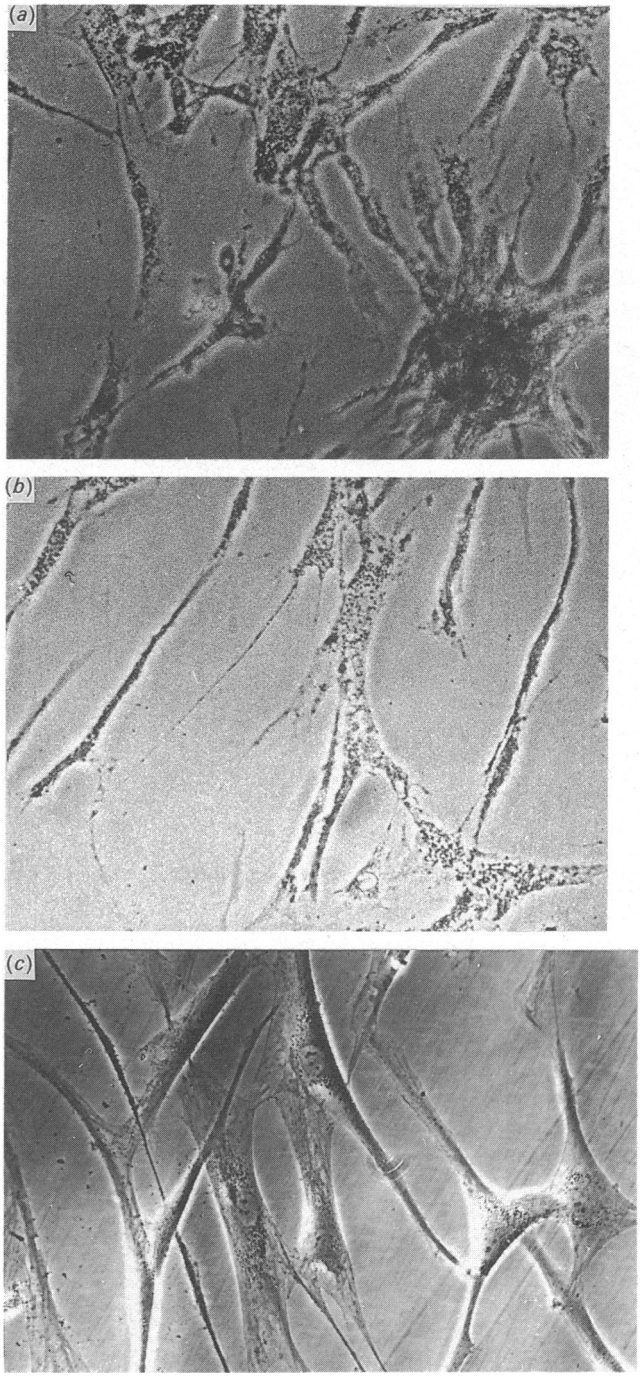

Fig. 1. Oxygen radical-dependent reduction of NBT by stimulated human fibroblasts

The fibroblasts were stimulated with (*a*) IL-1 (280 nmol/l) or (*b*) TNF (290 nmol/l), or were unstimulated (*c*). The cells were incubated for 2.5 h (37 °C, 5% CO_2) with the stimulants in the test buffer.

The time- and dose-dependent formation of $O_2^{\cdot -}$ was determined by cytochrome *c* reduction [23] after stimulation with IL-1 (Fig. 2) or TNF (Fig. 3). The reduction of cytochrome *c* was observable directly after addition of the stimulants and increased linearly in the case of IL-1 and with a slightly sigmoidal shape for TNF for at least

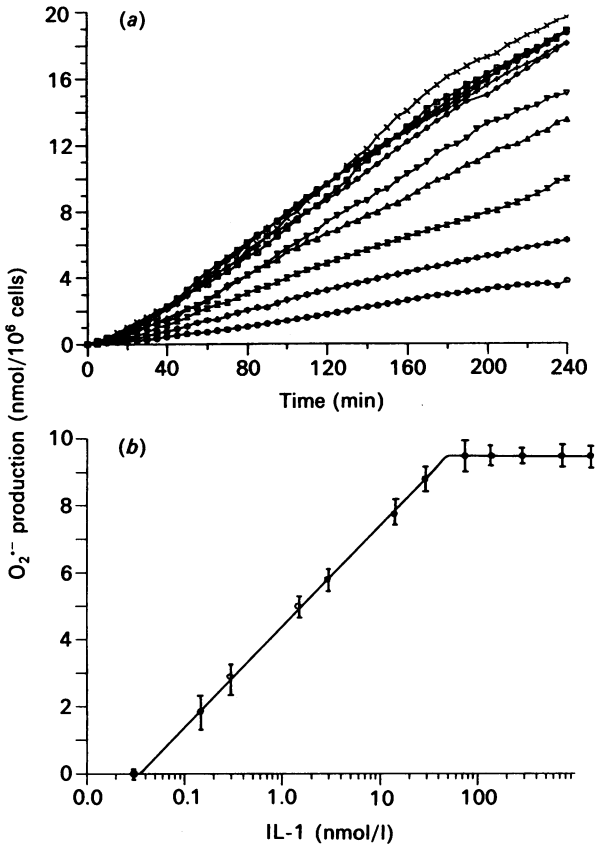

Fig. 2. Time- and dose-dependent formation of $O_2^{\cdot-}$ after stimulation with IL/l

(*a*) Time course of $O_2^{\cdot-}$ release after stimulation with various concentrations of IL-1 (nmol/l) (from top to bottom): ●, 1450; ×, 725; □, 290; +, 145; ◇, 72.5; ◆, 29; ▽, 14.5; △, 2.9; ⊻, 1.45; ✡, 0.29; ○, 0.145. Unstimulated controls were always below the detection limit. Each point shows the mean value of four experiments. (*b*) Dose–response curve of $O_2^{\cdot-}$ formation after 2 h of incubation with IL-1. Each point is the mean value of four different experiments, ±S.D.

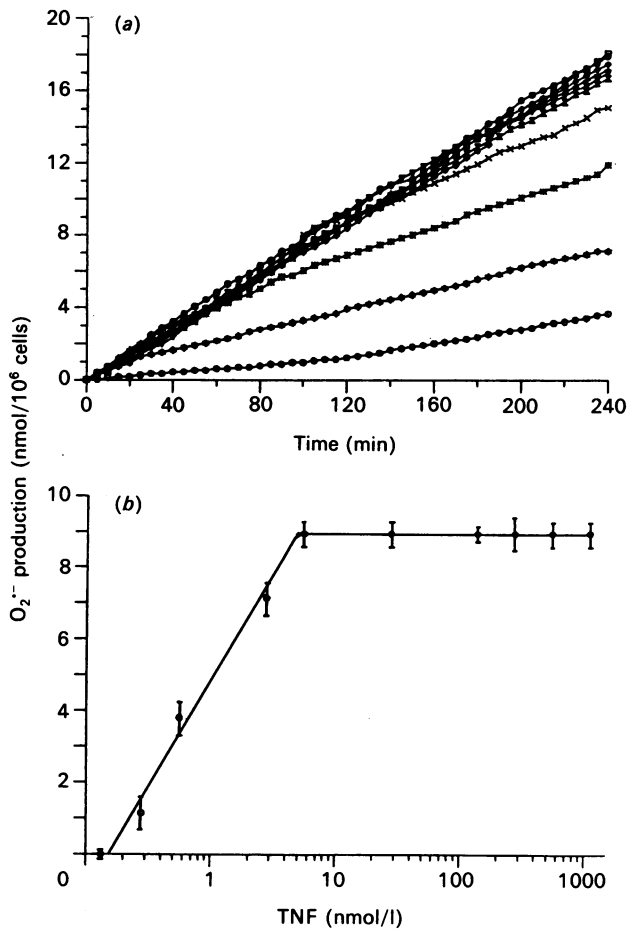

Fig. 3. Time- and dose-dependent formation of $O_2^{\cdot-}$ after stimulation with TNF

(*a*) Time course of $O_2^{\cdot-}$ release after stimulation with various concentrations of TNF (nmol/l) (from top to bottom): ●, 2800; ▽, 1400; □, 280; △, 140; ⊻, 56; ×, 5.6; ✱, 2.8; ✡, 0.56; ○, 0.28. Unstimulated controls were always below the detection limit. Each point shows the mean value of four different experiments. (*b*) Dose–response curve of $O_2^{\cdot-}$ formation after 2 h of incubation with TNF. Each point is the mean value of four different experiments, ±S.D.

4 h. The basal production was below the detection limit; $O_2^{\cdot-}$ production was even observable when the stimulants were added at a concentration of about 0.1 nmol/l, which is in the range of the K_d of the IL-1 and TNF receptors. Half-maximal stimulation was reached at about 0.3 nmol/l for IL-1, and 0.9 nmol/l for TNF. Maximal $O_2^{\cdot-}$ production was observable on addition of 50 nmol of IL-1/l or 5 nmol of TNF/l. The reduction of cytochrome *c* was completely inhibited by the addition of 0.1 μmol of SOD/l.

Effects of cytokines on H_2O_2 production

Human fibroblasts were preincubated with IL-1 or TNF at different concentrations in the culture medium for 15 min. Then the stimulants were removed by washing twice with test buffer at 37 °C. Alternatively, IL-1 or TNF were added directly to the cuvettes in the test buffer. H_2O_2 production was determined fluorimetrically by the peroxidase-mediated oxidation of scopoletin [26]. The treatment of fibroblasts with these stimulants led to production of H_2O_2 as shown in Fig. 4. As H_2O_2 is the product of the dismutation of $O_2^{\cdot-}$, the effects of time and dose on the response were similar to those described above. No differences were observed between pre-incubated and non-preincubated cells. Untreated fibroblasts did not alter the fluorescence of scopoletin; in addition, no alteration of fluorescence was observed in the presence of 1 μmol of catalase/l.

Determination of radicals by e.s.r. spectroscopy

The nature of the radicals generated was determined by e.s.r. spin-trapping with DMPO [25]. After an incubation period of 30 min with 280 nmol of IL-1/l or 290 nmol of TNF/l and 50 mmol of DMPO/l in the test buffer, a spectrum of overlapping signals of the DMPO-OOH and DMPO-OH adducts was observable (Fig. 5). The signal of the DMPO-OH adduct increased subsequently with longer incubation time in relation to the DMPO-OOH adduct. These signals were not observed when SOD (1 μmol/l) was added to the test system,

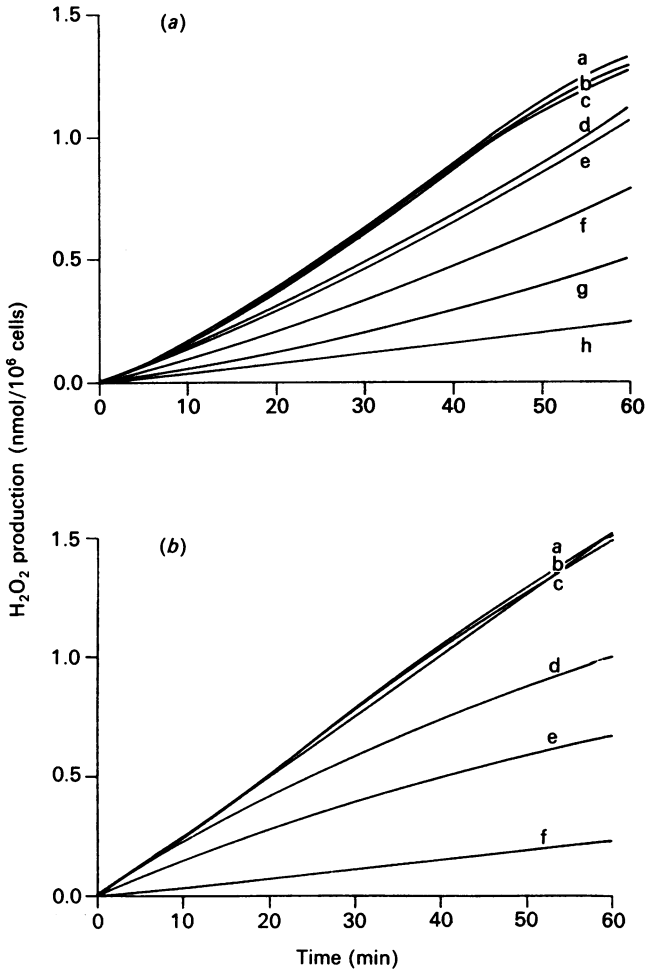

Fig. 4. H_2O_2 production by stimulated fibroblasts

(*a*) H_2O_2 production on stimulation by various concentrations of IL-1 (nmol/l): a, 1450; b, 290; c, 145; d, 14.5; e, 2.9; f, 1.45; g, 0.29; h, 0.145. (*b*) H_2O_2 production on stimulation by various concentrations of TNF (nmol/l): a, 280; b, 56; 5.6; d, 2.8; e, 0.56; f, 0.28. Incubations were carried out for 60 min. Each graph shows the mean values of four different experiments, ±S.D.

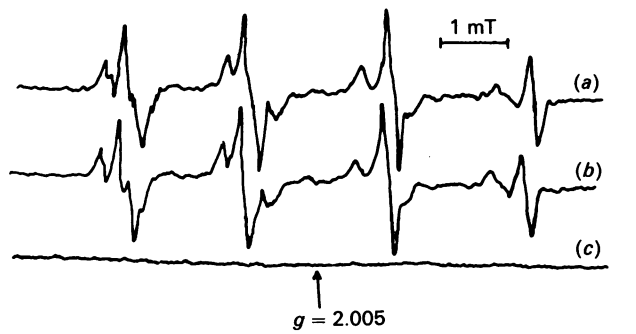

Fig. 5. Radical production by stimulated fibroblasts determined by e.s.r. spin trapping with DMPO

(*a*) Fibroblasts stimulated with 280 nmol of IL-1/l, (*b*) fibroblasts stimulated with 290 nmol of TNF/l, (*c*) unstimulated fibroblasts. The fibroblasts were incubated with 200 μl of test buffer containing 50 mmol of DMPO/l for 30 min. Test conditions are described in detail in the Materials and methods section.

whereas the addition of heat-inactivated SOD (1 μmol/l) had no effect on the formation of the radical adducts. It is concluded that the primary radical induced was $O_2^{\cdot-}$ and the DMPO-OH adduct was due to a decomposition of the DMPO-OOH adduct [25].

Determination of ethane production

After incubation with TNF or IL-1 (2.8 to 2800 nmol/l) for 30 min at 37 °C, the fibroblasts released ethane into the gas space at a concentration of up to 0.8 pmol/10^6 cells. In non-stimulated cells, no ethane production was observed.

Low-level chemiluminescence

Fibroblasts stimulated with IL-1 or TNF showed a spontaneous chemiluminescence (Fig. 6). The addition of azide up to 1 mmol/l had no effect on light emission. Because azide is a quencher of singlet oxygen, the photoemissive species may be excited carbonyl compounds rather than singlet oxygen. This was supported by the spectral characteristics; cut-off filters of > 620 nm completely absorbed the light emitted by the fibroblasts, so that the photoemission is in the visible spectral region attributable to excited carbonyls. Chemiluminescence is caused by excited carbonyls in the form of a radical chain reaction; at a sufficient concentration of 'start radicals', one 'start radical' initiates the formation of several

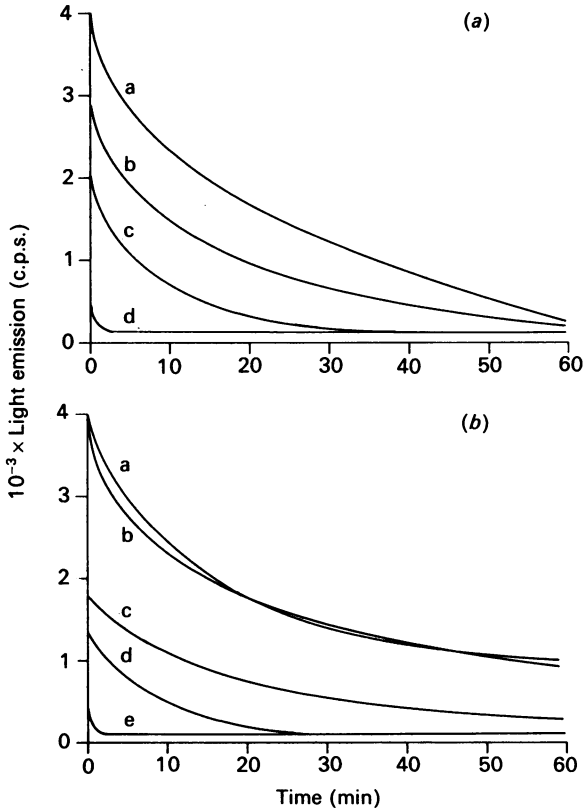

Fig. 6. Low-level-chemiluminescence

Stimulation was with (*a*) various concentrations of IL-1 (nmol/l): a, 28; b, 5.6; c, 2.8; d, zero; or (*b*) various concentrations of TNF (nmol/l): a, 28; b, 14; c, 2.8; d, 1.4; e, zero. Each graph shows the mean values from three different experiments.

carbonyls. Thus the concentration of the start radicals is not directly correlated to the chemiluminescence. High concentrations of radicals may even decrease the spontaneous chemiluminescence. The concentration of excited carbonyls decreased with time, due to an oxidation of membrane compounds. As a result, a time-independent chemiluminescence over 4 h, in accordance with the kinetics of $O_2^{\cdot-}$ formation cannot be expected.

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) and allopurinol (100 μmol/l) did not alter oxygen radical production, indicating that xanthine oxidase is an unlikely source.

Likewise, azide (1–100 μmol/l), cyanide (1–100 μmol/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), $O_2^{\cdot-}$ production increased 6-fold. As both substrates are thought to be unable to penetrate through the cell membrane, the radical-generating system might be localized in the outer cell membrane, like the NADPH oxidase system [28]. NAD(P)H does not induce the formation of excited oxygen species in unstimulated fibroblasts.

DISCUSSION

Human fibroblasts produce reactive oxygen species upon stimulation with the cytokines IL-1 or TNF. So far, oxygen radical production has been described only for polymorphonuclear granulocytes, monocytes and monocyte-like cells such as Kupffer cells, and for lymphocytes, mesangial cells and platelets. Cytokine-stimulated oxygen radical production was described for polymorphonuclear granulocytes and monocytes [6,18,19–21] and for glomerular mesangial cells [29].

The cytokine concentrations required to induce formation of reactive oxygen species in fibroblasts were in the physiological range of the receptor binding constant [6,30]. $O_2^{\cdot-}$ and H_2O_2 were released continuously for at least 4 h, in contrast to an oxidative burst as observed in neutrophils and macrophages. Whereas the high amounts of reactive oxygen species generated in phagocytes rapidly destroy these cells [31,32], the fibroblasts remained visibly intact for up to 4 h after simulation. One of the reasons for the survival of the fibroblasts is probably the relatively low amount of reactive radicals produced in comparison with neutrophils. Another could be their antioxidant capacity.

A continuous production of superoxide was observed only in platelets stimulated by thrombin, collagen, fluoride or an ionophore after a short oxidative burst [33,34]. The amount of $O_2^{\cdot-}$ produced is considerably lower (about 0.5 pmol of $O_2^{\cdot-}$/15 min per 10^6 cells), and like fibroblasts these cells were not destroyed by the radicals. In platelets, the continuous liberation was regarded as a by-product of cell metabolism. This seems not to be the case for fibroblasts, because the amount of superoxide production is 2500-fold higher than in platelets, and in non-stimulated fibroblasts no formation of excited oxygen species is observable.

An indirect indication of $O_2^{\cdot-}$ formation by normal tissue cells was given by the observation that TNFα, TNFβ, IL-1α and IL-1β specifically induce Mn-SOD and Mn-SOD mRNA in a variety of cell lines and normal cells *in vitro* and in various organs of mice *in vivo*, whereas other antioxidant or mitochondrial enzymes were not altered [35]. Other reagents which induce radical formation in monocytes and polymorphonuclear cells, such as interferon-α, -β, and -γ, transforming growth factor-β, IL-6, IL-2, or a combination of phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharides did not induce Mn-SOD, which suggests a specific regulation of the induction, possibly by induction of $O_2^{\cdot-}$ formation. These observations support our observations that some potent stimulants for cells of the immune system (such as PMA, some lipopolysaccharides, *N*-fMetLeuPhe leukotriene Bu, C3b) were rather weak stimulants, or even unable to induce excited oxygen species in fibroblasts (B. Meier, S. Selle, H. H. Radeke, K. Resch, G. G. Habermehl & H. Sies, unpublished work).

The nature of the radical-producing system in fibroblasts is unknown. Neither the xanthine oxidase system nor the mitochondrial respiration chain seems to be involved. An increase of $O_2^{\cdot-}$ production was observable after the addition of either NADH or NADPH only, which cannot penetrate the cell membrane. So either the radical-generating system is located in the outer cell membrane, like NADPH oxidase [28], with binding capacity towards the surface, or the cell membrane of some cells was already damaged by lipid peroxidation, although the cells were visibly intact.

The primary radical species produced upon stimulation with IL-1 or TNF was $O_2^{\cdot-}$, as confirmed by e.s.r. spin trapping. One of the major mechanisms by which $O_2^{\cdot-}$ could lead to cellular and tissue damage is the initiation of a radical-chain peroxidation of polyunsaturated fatty acids in phospholipids of functional membranes. In fact, lipid peroxidation upon stimulation of the fibroblasts with IL-1 or TNF is suggested to occur because of an increase in low-level chemiluminescence [27] in the visible region, and because of the observed ethane production [36].

The cytokines IL-1 and TNF are directly involved in inflammatory processes after binding to a high-affinity receptor molecule expressed on the surface of cell types including fibroblasts, chondrocytes and synovial cells [4,5,12,13,15,16]. Fibroblasts in particular are well known to participate in joint-damaging processes in rheumatoid arthritis. Cytokine-induced prostaglandin and collagenase production followed by an unphysiological collagen release might enhance 'pannus' formation and lead to the observed loss of joint function. Beside a direct damaging effect due to the $O_2^{\cdot-}$ released by the fibroblasts, continuous $O_2^{\cdot-}$ production might have a function in the regulation of inflammatory processes either by the liberation of arachidonic acid metabolities or by a direct stimulation of lymphocytes [33,37,38]. Whether other normal tissue cells which are possibly involved in inflammatory processes are capable of releasing excited oxygen species after stimulation is a worthy subject for further investigations.

This work was supported by Deutsche Forschungsgemeinschaft grants Ha 457.38-1 and SFB 244/B1/B11. We appreciate the opportunity to perform our cell cultures at the laboratories of Professor E. Lamprecht (Hannover), Prof-

essor W. Leibold (Hannover) and Professor D. Beyersmann (Bremen), and we are grateful to the University of Bremen for providing the e.s.r. spectrometer. Moreover, we wish to thank Dr. C. Baumstark-Khan (Bonn) for providing us with primary cultures of fibroblasts and Mrs. C. Bovenkerk and Mrs. E. Wöbbecke for technical assistance. We are grateful to Mr. H. Scholich for help in the chemiluminescence assays.

REFERENCES

1. Akahoshi, T., Oppenheim, J. J. & Matsushima, K. (1988) J. Clin. Invest. **82**, 1219–1226
2. Chouaib, S., Bertologio, J., Blay, M. F. & Fradelizi, D. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 6875–6880
3. Dinarello, C. A. (1985) J. Clin. Immunol. **5**, 287–297
4. Krakauer, T. (1986) CRC Crit. Rev. Immunol. **6**, 213–244
5. Eastgate, J. A., Wood, N. G., DiGiovine, F. S., Symons, J. A., Grinlinton, F. M. & Duff, G. W. (1988) Lancet **i**, 706–709
6. Dower, S. K.. & Urdal, D. L. (1987) Immunol. Today **8**, 46–51
7. Carswell, E. A., Old, J. L., Kassel, R. L., Green, S., Fiore, N. & Williamson, B. (1975) Proc. Natl. Acad. Sci. U.S.A. **72**, 3666–3670
8. Collins, T., Lappierre, L. A., Fiers, W., Strominger, J. L. & Pober J. S. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 446–450
9. Pfitzenmaier, K., Scheurich, P., Schluter, C. & Kronke, M. (1987) J. Immunol. **138**, 975–980
10. Palombella, V. J., Yamashiro, D. J., Maxfield, F. R., Decker, S. J. & Vilcek, J. (1987) J. Biol. Chem. **262**, 1950–1954
11. Matthews, N., Neale, M. L., Jackson, S. K. & Stark, J. M. (1987) Immunology **62**, 153–155
12. Beutler, B. & Cerami, A. (1987) N. Eng. J. Med. **316**, 379–385
13. Billingham, M. E. J. (1987) Br. Med. Bull. **43**, 415–421
14. Marx, J. L. (1988) Science **239**, 257–258
15. Tracey, K. J., Beutler, B., Lowrey, S. F. & Faher, T. J. (1986) Science **234**, 470–474
16. Tracey, K. J., Lowry, S. F., & Faher, T. J. (1986) Surg. Gynecol. Obstet. **164**, 415–422
17. Beutler, B. & Cerami, A. (1986) Nature (London) **320**, 584–588
18. Clark, I. A., Chaudhri, G. & Cowden, W. B. (1988) UCLA Symp. Mol. Cell. Biol. New Ser. **82**, 53–60
19. Ferrante, A., Mades, M., Bates, E. J. D., Goh, D. H. B. & Beard, L. J. (1988) Immunology **63**, 507–512
20. Klempner, M. S., Dinarello, W. R., Henderson, W. R. & Gallin, J. I. (1979) J. Clin. Invest. **64**, 996–1002
21. Tsujimoto, M., Yokota, S., Vilcek, J. & Weissmann, G. (1986) Biochem. Biophys. Res. Commun. **137**, 1094–1100
22. Baumstark-Khan, C. (1988) Symposium molekulare und zelluläre Mechanismen der biologischen Strahlenwirkung, GSF München Neuherberg, Germany
23. McCord, J. M. & Fridovich, I. (1969) J. Biol. Chem. **244**, 6049–6055
24. Beauchamp, C. & Fridovich, I. (1971) Anal. Biochem. **4**, 276–287
25. Finkelstein, E., Rosen, G. M. & Rauckman, E. J. (1980) Arch. Biochem. Biophys. **200**, 1–16
26. Root, R. K., Metcalf, J., Oshino, N. & Chance, B. (1975) J. Clin. Invest. **55**, 945–955
27. Cadenas, E. & Sies, H. (1984) Methods Enzymol. **105**, 221–231
28. Babior, B. M., Curnutte, J. T. & Okamura, N. (1988) in Oxygen Radicals and Tissue Injury (Halliwell, B., ed.), pp. 43–48, FASEB, Bethesda
29. Radeke, H., Meier, B., Topley, N., Flöge, J., Habermehl, G. G. & Resch, K. (1989) Kidney Int., in the press
30. Tsujimoto, M., Yip, Y. K. & Vilcek, J. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 7626–7630
31. Babior, B. M. (1978) N. Engl. J. Med. **298**, 659–668
32. Babior, B. M. (1978) N. Engl. J. Med. **298**, 721–725
33. Marcus, A. J. (1978) J. Lipid Res. **19**, 793–826
34. Marcus, A. J. (1979) Sem. Hematol. **16**, 188–195
35. Wong, G. H. W. & Goeddel, D. V. (1988) Science **242**, 941–944
36. Kappus, H. (1985) in Oxidative Stress (Sies, H., ed.), pp. 273–310, Academic Press, London and New York
37. Fidelius, R. K. (1987) Cell. Immunol. **113**, 175–182
38. Vol'sky, N. N., Kashloskova, N. V. & Kozlov, V. A. (1988) Tsitologiya **30**, 898–903

Received 23 February 1989/22 May 1989; accepted 30 May 1989