

Superoxide Release in Human Fibroblasts upon Treatment with Culture Supernatants of the Arthritogenic Bacteria *Erysipelothrix rhusiopathiae* and *Mycoplasma arthritidis**

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Culture supernatants of the arthritogenic bacteria *Mycoplasma pneumonia*, *Mycoplasma arthritidis*, *Borrelia burgdorferi* and *Erysipelothrix rhusiopathiae* stimulated primary cultures of human fibroblasts to release reactive oxygen species into the environment, whereas cell walls and membranes of these bacteria had no effects. Lipopolysaccharides of various gram-negative bacteria and lipid A, the lipid moiety of endotoxins, also failed to stimulate the release of reactive oxygen species by fibroblasts.

The stimulatory fractions of the culture supernatants of *Mycoplasma arthritidis* and *Erysipelothrix rhusiopathiae* exhibited a molecular weight of about 9.5 kDa. After an induction period of 5 min the presence of the stimulant was not necessary any more. The primary radical released by the fibroblasts was the superoxide anion O_2^- . Radical formation took place continuously over some hours. Additionally, low-level chemiluminescence of fibroblasts was increased upon stimulation with culture supernatants of *Mycoplasma arthritidis* and *Erysipelothrix rhusiopathiae*. No irreversible injury of the fibroblast was caused upon stimulation and the cells exhibited normal proliferation pattern after replacing them to the culture medium.

Introduction

Rheumatoid arthritis finally leads to an irreversible destruction of joints and represents one of the serious problems of chronic disease, from which about 2% of the world population are suffering. In contrary to animals, where microorganisms (bacteria and virus) are postulated to be the causative agents (Schulz, 1980), the etiology of rheumatoid arthritis in man is still unknown, but several indications suggest that these forms of arthritis are also provoked by a primary infection (Wilkes and Meek, 1979).

We studied two different bacteria causing rheumatoid arthritis in animals, *Erysipelothrix rhusio-*

pathiae T28 and *Mycoplasma arthritidis* ISR1, which serve as a model for rheumatoid arthritis in man (Ajmal, 1970; Cole *et al.*, 1972; Drews, 1972; Schulz, 1980; Schulz *et al.*, 1974; Sikes, 1959). *Erysipelothrix rhusiopathiae* is a small, gram-positive non mobile coccobacillus causing a chronic arthritis in pigs and rats (Ward, 1922), which has an increasing interest due to its similarities to rheumatoid arthritis in man. Beside this pathogenicity it induces natural infections in a wide range of animals and men (Schulz, 1974; Ajmal, 1969).

Mycoplasma arthritidis is the causative agent of arthritis in rats and mice and as *Erysipelothrix arthritidis* represent animal models for rheumatoid arthritis in man (Cole *et al.*, 1971). Mycoplasma are small bacteria lacking a cell wall. Some species possess oligosaccharide chains bound to diglyceride (lipoglycan), which are soluble in organic solvents (Seid *et al.*, 1980). Like the lipopolysaccharides (LPS) of gram negative bacteria they have endotoxin-like activities (Razin, 1978). The membranes of *Mycoplasma arthritidis* are described to lack these lipoglycans (Razin, 1978). It is unknown whether lipoglycans or cell membranes of *Myco-*

Abbreviations: PMN, polymorphonucleare granulocytes; LPS, lipopolysaccharides; PBS, phosphate buffered saline.

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plasma arthritidis have the capacity to trigger the release of reactive oxygen species, as LPS do.

The earliest changes *Erysipelothrix rhusiopathiae* caused upon an experimental infection are those of systemic coagulopathy with widespread fibrinous exudation into connective tissue, endothel-injury with increased vascular permeability. After approximately 6 months, infected joints tend to become sterile and the bacteria move into the surrounding tissue where they are persistent for years (Hermanns *et al.*, 1981; Hermanns *et al.*, 1986).

Infection with *Mycoplasma arthritis* also leads to a rapidly developing arthritis. Already 2 days after infection histologically early lesions, as vascular changes and alteration in the lining cell layer of the synovium are seen, whereas the infiltration by polymorphonuclear neutrophils (PMNs) is observable not before 4 days after infection. Parallel with the infiltration the joint destruction by pannus-like granulating tissue is already initiated (Hermanns, 1983).

Infections with both bacteria lead to a rapid proliferating arthritis characterized by early initiated pannus production with bone and cartilage erosion, and histologically observable tissue alterations before leukocyte cell infiltration.

In the chronic state of the arthritis *Erysipelothrix rhusiopathiae* is not present in the joint with chronical destructive inflammation anymore, but moves into the surrounding connective tissue, of periost, perichondrium and tendons, which histologically shows no remarkable inflammation. So in the chronical state a direct interaction between the inflammatory cells and the persistent antigen is excluded. However, low molecular weight compounds released by the bacteria could diffuse from the surrounding tissues into the joint and might function as inducible and regulatory factors. Moreover the connective tissue cells could release diffusible compounds upon stimulation by the bacteria themselves or compounds released by the bacteria which also could have the function of "second messengers" and both could participate in the self-perpetuating and self-accelerating mechanism of destruction in rheumatoid arthritis, finally leading to an irreversible destruction of the joint.

The persistence of *Mycoplasma arthritis* in the chronical state is unknown, as these bacteria are not detectable by histological techniques, and the

attempts are not convincing to reculture the bacteria in the chronical state of the infection out of the synovia.

The marked tendency to pannus formation and the longevity of the arthritis make these diseases interesting, experimental models of chronic poly-arthritis, especially as they have been induced by LPS free microorganisms.

Gram-negative bacteria, which carry LPS in the cell wall are also the causative agents of several chronic infections. Certain symptoms observed after a bacterial induced inflammation are associated with a heat-stable toxin which is firmly bound to cell walls of gram negative-bacteria. These toxic substances were termed endotoxin, to distinguish them from the actively secreted heat-labile exotoxins (Brade, 1988), and identified as high molecular weight protein-lipid-polysaccharide complexes. The biologically active principle was identified as the lipid moiety of endotoxin, which is termed lipid A.

Bacterial LPS and lipid A are stimulatory substances non-specifically enhancing the bactericidal or tumoricidal activity of PMNs and macrophages, triggering the release of reactive oxygen species. However the reports are contradictory, as some laboratories could not demonstrate the release of reactive oxygen species upon stimulation with endotoxins. This might be due to differences in preparations, as rough-forms (R-form) preparations and lipid A were potent stimulants, while smooth-forms (S-form) preparations only activated PMNs at high concentrations and to a lower extent (Kapp, 1987). Moreover the LPS coated to bovine serum albumin may increase the availability for the PMNs and macrophages and influence the results.

In contrary to leukocytes LPS failed to stimulate superoxide release from endothelial cells (Matsubara, 1986).

Histological observations indicated the participation of tissue cells in inflammatory process caused by *Erysipelothrix rhusiopathiae* and *Mycoplasma arthritis*. Recently the release of reactive oxygen species by fibroblasts upon stimulation with various agents was described (Meier *et al.*, 1989; Murell *et al.*, 1990; Meier *et al.*, 1989, 1991) which might exert regulatory functions in physiological processes. So low amounts of reactive oxygen species induced proliferation (Murell *et al.*,

1990) in fibroblasts and triggered chemotactic movements in fibroblasts (Wach, 1987) or PMNs (Martin *et al.*, 1985). Moreover they trigger the translocation of the nuclear transcription factor kappaB from the cytoplasm into the nucleus and thus induce the formation of several pro-inflammatory cytokines (Schreck and Baeuerle, 1991; Schmidt *et al.*, 1995).

Previously we demonstrated a stimulated release of O₂⁻ in PMNs upon contact with culture supernatant of *Mycoplasma arthritidis* (Meier *et al.*, 1990). Thus our objective was to study whether bacterial LPS might stimulate the release of reactive oxygen species by fibroblasts in comparison to non-LPS possessing arthritogenic bacteria and their culture supernatants. For comparison we studied the release of reactive oxygen species by human leukocytes upon stimulation with culture supernatants or cell walls of these bacteria or with membranes in the case of *Mycoplasma*.

Materials and Methods

Alpha-MEM medium and trypsin/EDTA (0.125%/0.01%; w/v) were obtained by Gibco BRL (Eggenstein, FRG), fetal calf serum (charge no. 4K03; FCS) by Biochrom (Berlin, FRG), steril plastic material for the cell cultures was from Nunc (Wiesbaden, FRG.) and the plastic cover slips 22 x 60 mm and 4-well multiplates from Lux (Newbury Park, California, USA). The culture media for the bacteria were obtained from Difco Laboratories (Detroit, Michigan, USA). The lipopolysaccharides from *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella minnesota*, *Salmonella typhimurium*, *Shigella flexneri*, *Escherichia coli* EH100 (Ra mutant), *Salmonella minnesota* R7 (Rd mutant), *Salmonella minnesota* Re595 (Re mutant), *Salmonella typhimurium* TV119 (Ra mutant), and *Shigella flexneri* (Re mutant), as well as lipid A, peroxidase type III, cytochrome c grade III, and Percoll 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). Penicillin, bovine serum albumin (BSA) and phosphate buffered saline (PBS) were from Boehringer (Mannheim, FRG). Cu-Zn-superoxide dismutase (Peroxinorm) was a gift of Grünenthal AG.,

Aachen, FRG. All other chemicals used were from Merck (Darmstadt, FRG) of the highest purity available.

Methods

Human fibroblast cell culture

Primary cultures of human fibroblasts were established by the method of explantate-culture as described (Meier *et al.*, 1989). To avoid contaminations by macrophages, only cells of the 10th to 20th passage were used for the experiments.

Culture conditions

Cells were cultured in alpha-MEM medium, supplemented with 10% heat-inactivated fetal calf serum in 5% CO₂ in air at 37 °C (Meier *et al.*, 1989).

To determine the O₂⁻ and H₂O₂ production fibroblasts were cultured on all four sides of glass cuvettes to confluency (5x10⁴ cells/cm²), on glass sliders or in 96 well chambers or on plastic slips to determine ultraweak spontaneous light emission.

Isolation of polymorphonuclear neutrophils and monocytes from human blood

PMNs and monocytes were isolated from buffy coats of heparinized blood, obtained from a single donor by venous puncture, by sedimentation in a discontinuous density gradient of Percoll, consisting of 80%, 65%, 60%, 50%, 45% and 30% Percoll. After centrifugation for 40 min at 700xg at 22 °C separated in distinct bands: Lymphocytes and monocytes between 30% and 40%, PMNs between 50% and 60%, and erythrocytes between 65 and 80%. The cell layers were collected, washed three times with PBS and microscopically checked for homogeneity and viability with the trypan-blue exclusion assay. The cells showed homogeneity and viability of more than 95%. The cell concentration was adjusted to 10⁵ cells/ml and 100 µl were added to each well. The cells were sedimented by centrifugation of the microtiter plates.

Bacterial cultures

Erhysipelothrix rhusiopathiae strain T 28 was grown in standard-I-bouillon (25 g/l) at 37 °C for 24 h. The cells were removed by centrifugation at 7000xg for 15 min.

Mycoplasma arthritidis strain *ISR1* (Laber *et al.*, 1975) was propagated at 37 °C in modified Friis medium (Friis, 1975) or 48 h. The mycoplasma were sedimented (30 min at 17000×g) and the culture supernatants were collected and lyophilized.

The bacterial cells were broken by ultrasonication, membranes purified by ultracentrifugation (1 h at 105000×g), washed with PBS and resedimented by ultracentrifugation.

Determination of superoxide formation in fibroblasts

Fibroblasts were cultured in cuvettes as described (Meier, 1989). The activity tests were performed in 2 ml phosphate buffer (50 mmol/l, pH 7.2) supplemented with NaCl (150 mmol/l), MgCl₂ (1 mmol/l), CaCl₂ (0.6 mmol/l) and glucose (10 mmol/l) (Test buffer) at 37 °C. O₂⁻ radical formation was determined photometrically (UVI-KON 820, Kontron, Hannover, FRG) at 550 nm by the reduction of cytochrome *c* (50 µmol/l) (30) or nitroblue-tetrazolium (100 µmol/l) to the blue formazan for photographic documentation. Upon addition of superoxide dismutase (100 nmol/l) it was confirmed that the reduction of cytochrome *c* and nitro blue tetrazolium was completely caused by O₂⁻.

The cuvettes were pre-incubated for 5 min with the bacterial cell walls, respectively membranes, or the bacterial culture supernatants and washed afterwards with the test buffer. In other experiments these substances were directly added to the test system.

Additionally radical formation was measured by ESR-spin trapping with dimethylpyrroline-N-oxide (DMPO). DMPO was purified by filtration through charcoal (Finkelstein, 1980). 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). The cells were cultured in macrowells, washed twice with the test buffer and 200 µl final volume of the test buffer including 50 mmol/l DMPO and the test samples were added. The cells were incubated at room temperature for 15 min to 1 hour with the test solution, scraped, transferred into a flat cell and measured under following conditions: amplitude 100 kHz; field modulation, 0.5 mT; microwave power, 10 mW; receiver gain, 4 × 10⁶; recording

time 2000s with a response time of 0.5 s; field center 0.342 T; sweep width 20 mT. Only 10 mT around the center were recorded. The magnetic field was measured with a N. M. R. oscillator.

Determination of hydrogen peroxide formation

Hydrogen peroxide was determined fluorometrically (SFM 23, Kontron, Hannover, FRG) by the peroxidase mediated oxidation of scopoletin (Root, 1975). Scopoletin (40 nmol/l) and peroxidase (1 µmol/l) were added to 2 ml test buffer, and the decrease of fluorescence (excitation wavelength 381 nm, emission wavelength 436 nm) was recorded. Calibration was done with hydrogen peroxide.

Low-level chemiluminescence

Chemiluminescence of fibroblast monolayer cultures on plastic slides was measured with a photon counter according to (Cadenas, 1984) supplemented with a red sensitive photo multiplier cooled to -25 °C by a thermostatic 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 x 4.5 cm x 0.7 cm, with the cell covered side towards the photomultiplier. The assays were carried out in test buffer at 37 °C.

Separation of culture supernatants by molecular weight exclusion filtration

The lyophilized culture supernatants of *Erysipelothrix rhusiopathiae* and *Mycoplasma arthritidis* dissolved in the test buffer without glucose (500 g/l) and 3 ml of this solution were separated by molecular weight filtration on a Sephadex G-10 column (2 x 35 cm), a Sephadex G-15 (2 x 35 cm), a TSK HW-40 column (2 x 35 cm) or a TSK HW-55 column (2 x 35 cm). Fractions of 2 ml were collected when an absorption at 280 nm was observable.

Results

Fibroblasts pre-stimulated or stimulated with lyophilized culture supernatants (1 g/l and 10 g/l) of *Mycoplasma arthritidis* *ISR-1* or released O₂⁻ into the environment as visualized by the reduction of nitroblue-tetrazolium to the blue formazan

(not shown) The distribution of the blue formazan particles was rather homogeneous over the plasma membrane and could not be correlated to a special intracellular compartment. Without stimulation NBT was not reduced; the stimulated reduction of NBT was inhibited in the presence of 1 $\mu\text{mol/l}$ superoxide dismutase. Neither upon pre-stimulation with the bacteria or with the bacterial cell walls (10 mg/l), nor upon pre-stimulation with LPS (up to 100 mg/l) of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella minnesota*, *Salmonella typhimurium*, *Shigella flexneri*, *Escherichia coli* EH100 (Ra mutant), *Salmonella minnesota* R7 (Rd mutant), *Salmonella minnesota* Re595 (Re mutant), *Salmonella typhimurium* TV119 (Ra mutant), and *Shigella flexneri* (Re mutant) or lipid A (up to 10 mg/l in the presence of absence of bovine serum albumin) a reduction of NBT was observable. Over a period of 4 hours the cells were visually intact, as tested by the exclusion of trypan blue.

PMNs and monocytes isolated from blood also released superoxide upon stimulation with the culture supernatants (10 mg/l) and reduced NBT to the blue formazan. The reduction was inhibited in the presence of superoxide dismutase (100 nmol/l).

Quantitative determination of the stimulated O₂⁻ release by fibroblasts was performed by reduction of cytochrome c. Only the part of cytochrome c reduction which would be inhibited by the addition of superoxide dismutase was correlated to O₂⁻ radical (Fig. 1a). The basic production was below the determination limit of the assay system. Cytochrome c reduction was completely inhibited by the addition of 100 nmol/l superoxide dismutase. The bacterial cell walls or LPS (up to 100 mg/l) of the *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella minnesota*, *Salmonella typhimurium*, *Shigella flexneri*, *Escherichia coli* EH100 (Ra mutant), *Salmonella minnesota* R7 (Rd mutant), *Salmonella minnesota* Re595 (Re mutant), *Salmonella typhimurium* TV119 (Ra mutant), or *Shigella*

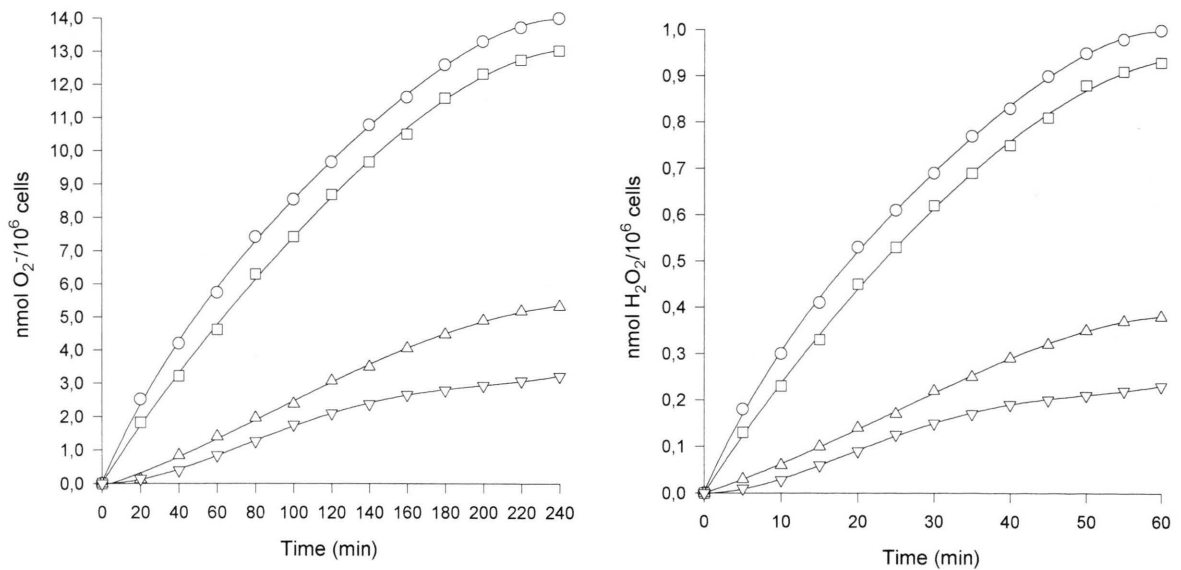


Fig. 1. Superoxide and hydrogen peroxide formation upon stimulation with culture supernatants of *Erysipelothrix rhusiopathiae* or *Mycoplasma arthritidis*.

a) The fibroblasts were pre-incubated with the culture supernatante in medium for 5 min, washed twice with the test buffer and superoxide release was determined by the reduction of cytochrome c. Culture supernatants of *Erysipelothrix rhusiopathiae* ○ (10 g/l) respectively ◇ (1 g/l) or *Mycoplasma arthritidis* △ (10 g/l) respectively ▽ (1 g/l) were used for the stimulation.

b) The fibroblasts were pre-incubated with the bacterial culture supernatantes in medium for 5 min, washed twice with test buffer and hydrogen peroxide release was determined with scopoletin. Culture supernatants of *Erysipelothrix rhusiopathiae* ○ (10 g/l) respectively ◇ (1 g/l) or *Mycoplasma arthritidis* △ (10 g/l) respectively ▽ (1 g/l) were used for the stimulation.

Controls without stimulants were below the detection limit. Each graph represents the mean values of 6 different experiments.

flexneri (Re mutant) or lipid A (up to 10 mg/l in the presence or absence of bovine serum albumin) did not trigger the release of O₂⁻ by fibroblasts. Also a pre-incubation of LPS or lipid A did not lead to a release of O₂⁻ radicals by fibroblasts.

The nature of the radicals released by fibroblasts upon stimulation was determined with ESR-spin trapping. Upon treatment of the fibroblasts with culture supernatants of *Erysipelothrix rhusiopathiae* signals in the g=2 region were observable mainly due to the DMPO-OH adduct and to a minor case to the DMPO-OOH adduct. Treatment with culture supernatants of *Mycoplasma arthritidis* only led to weak signals which could not be identified. The signals disappeared in the presence of 100 nmol/l superoxide dismutase, whereas the addition of catalase (1 µmol/l) was without effect. This led to the conclusion that in both cases the primary radical released was O₂⁻ and the DMPO-OH adduct observable resulted from the DMPO-OOH adduct (Finkelstein, 1980).

Blood leukocytes were separated as described and the fractions containing PMNs or monocytes and lymphocytes were incubated for 15 min with the lyophilized culture supernatants (10 g/l) of *Erysipelothrix rhusiopathiae* or *Mycoplasma arthritidis* or cell walls, respectively the membranes of these bacteria (10 mg/l) in test buffer and the superoxide dismutase (100 nmol/l) inhibited part of the cytochrome c reduction was determined. Upon incubation with the culture supernatants of both bacteria PMNs released about 33.1 nmol O₂⁻/15 min x 10⁶ cells. An incubation of the fractions containing monocytes and lymphocytes with culture supernatants of *Erysipelothrix rhusiopathiae* triggered the release of about 7.3 nmol O₂⁻/15 min x 10⁶ cells while the incubation with culture supernatants of *Mycoplasma arthritidis* led to a release of about 4.1 nmol O₂⁻/15 min x 10⁶ cells. The cell walls, respectively membranes of both bacteria did not induce the release of O₂⁻ by PMNs and only a low amount in mononuclear cells. The spontaneous production was below 1 nmol O₂⁻/15 min x 10⁶ cells.

Determination of hydrogen peroxide formation

H₂O₂ formation was determined fluorimetrically by the peroxidase mediated oxidation of scopoletin (Fig. 1b). Untreated fibroblasts did not

alter the fluorescence of scopoletin and the oxidation of scopoletin upon pre-stimulation for 5 min with 1 or 10 g/l lyophilized culture supernatants was completely inhibited by the addition of catalase (10 µmol/l). The bacterial cell walls, LPS of the various bacteria or lipid A did not cause the release of H₂O₂ in fibroblasts. The direct addition of the lyophilized culture supernatants to the fibroblasts or the addition of albumin and LPS or lipid A was not possible due to the high fluorescence of these substances.

Low-level chemiluminescence

Pre-incubation for 5 min of the fibroblasts with the culture supernatants of the arthritogenic bacteria led to a spontaneous ultraweak light emission (Fig. 2), whereas the bacterial cell walls or LPS of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella minnesota*, *Salmonella typhimurium*, *Shigella flexneri*, *Escherichia coli* EH100 (Ra mutant), *Salmonella minnesota* R7 (Rd mutant), *Salmonella minnesota* Re595 (Re mutant), *Salmonella*

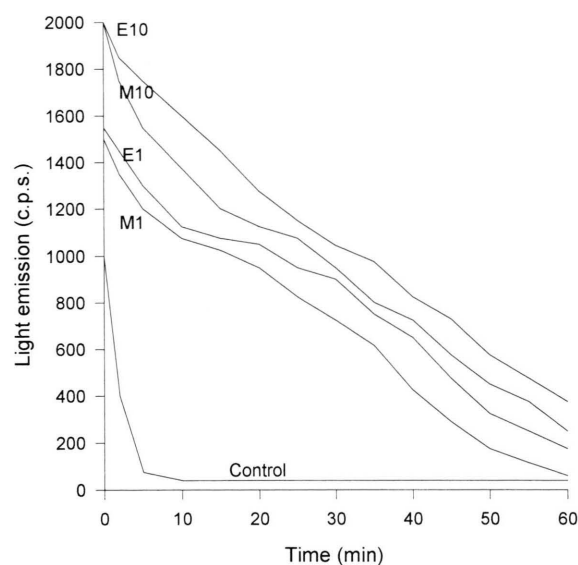


Fig. 2. Low level chemiluminescence upon stimulation with culture supernatants of *Erysipelothrix rhusiopathiae* or *Mycoplasma arthritidis*.

Spontaneous low level chemiluminescence of the fibroblasts upon stimulation with culture supernatants of *Erysipelothrix rhusiopathiae* or *Mycoplasma arthritidis*. Culture supernatants of *Erysipelothrix rhusiopathiae* (E10) (10 g/l) respectively (E1) (1 g/l) or *Mycoplasma arthritidis* (M10 = 10 g/l) respectively (M1 = 1 g/l) were used for the stimulation.

typhimurium TV119 (Ra mutant), and *Shigella flexneri* (Re mutant) or lipid A were without effect.

Separation of the culture supernatants by molecular weight filtration

To determine whether the stimulation of the fibroblast, blood PMNs and monocytes to release reactive oxygen species was triggered by similarly or different compounds the culture supernatants of *Mycoplasma arthritidis* and *Erysipelothrix rhusiopathiae* were separated by molecular weight exclusion membranes of 1000 kDa, 500 kDa, 100 kDa, 50 kDa, 30 kDa and 10 kDa. Fibroblasts cultured in microtiter plates were incubated for 5 min with 10% of the filtrates or remaining residues, washed with PBS and incubated for 4 hours with the test buffer and NBT (100 µmol/l). To PMNs or mononuclear cells the fractions were directly added in a 1:10 dilution and the cells were incubated for 30 min respectively 2 hours. The filtrate of the 10 kDa fraction triggered the release of reactive oxygen species in all cells tested.

For a more precise determination the 10 kDa filtrate was lyophilized, redissolved in a minimal volume of distilled water and separated by molecular weight filtration using a Sephadex G-10 column (<1 kDa), a Sephadex G 15 column (<1.5 kDa), a TSK HW-40 column (<10 kDa), and a TSK HW-55 (1–1000 kDa). 100 µl of the separated fractions and 100 µl of the test buffer containing NBT (100 µmol/l) were mixed and added to the fibroblasts cultured in microtiter chambers, to PMNs (10⁴ cells per well) or mononuclear cells (10⁴ cells per well) isolated from blood as described. The cells were incubated for 4 hours at 37 °C and superoxide release led to the formation of blue insoluble formazan. The superoxide release in fibroblasts and mononuclear cells was stimulated by a single fraction in the case of *Mycoplasma arthritidis* culture supernatant possessing a molecular weight about 9.5 kDa. In *Erysipelothrix rhusiopathiae* culture supernatant besides a fraction with a molecular weight around 9.5 kDa, a second fraction causing a weaker stimulation of lower molecular weight about 8 kDa was eluted. Superoxide formation by fibroblasts treated with culture supernatants of both bacteria separated on a TSK-HW40 column is shown (Fig. 3a). Fractions

of the TSK HW-55 column added to the fibroblasts did not lead to visible blue color shift, possible due to the higher dilution of the activating substances. After separation on Sephadex G-15 or G-10 columns the activating substances were completely eluted in the exclusion volume.

The granulocytes were activated by fractions of both culture supernatants showing broad a molecular weight distribution between 1 kDa and 3 kDa (Fig. 3b).

Tests for survival of the fibroblasts after treatment with the culture supernatants

The confluent fibroblasts were incubated for 24 h with 1 and 10 g/l lyophilized culture supernatant of *Erysipelothrix rhusiopathiae* or *Mycoplasma arthritidis*. Afterwards the cells were washed twice with 37 °C PBS, trypsinized, diluted and recultured in an equal cell density for 3 weeks to form single colonies. The cells pretreated with the culture supernatants exhibited normal proliferation pattern in comparison to untreated fibroblasts.

Discussion

The observations reported may elucidate a basic mechanism of chronic inflammation. Both bacteria release low molecular weight substances into the culture supernatant triggering a respiratory burst in PMNs and mononuclear cells and stimulate fibroblasts to a long lasting release of reactive oxygen species over several hours.

The fibroblasts functionally can respond towards specific stimuli, p. e. to circulating pathophysiological substances, with the release of reactive oxygen species without necessarily producing irreversible cell damage. In contrast to phagocytes, where the release of high amounts of reactive oxygen species in form of a burst is part of the host defense against infectious agents, the continuous release of low amounts of reactive oxygen species by fibroblasts has regulatory functions in cell activation and cell – cell interaction. Activated phagocytes are destructed by their own radicals and in this respect an acute inflammation is terminated, whereas an activation of fibroblasts may lead to a permanent disregulation. So superoxide in low concentrations as released by fibroblasts upon stimulation induces proliferation of fibroblasts (Murell *et al.*, 1990) and superoxide

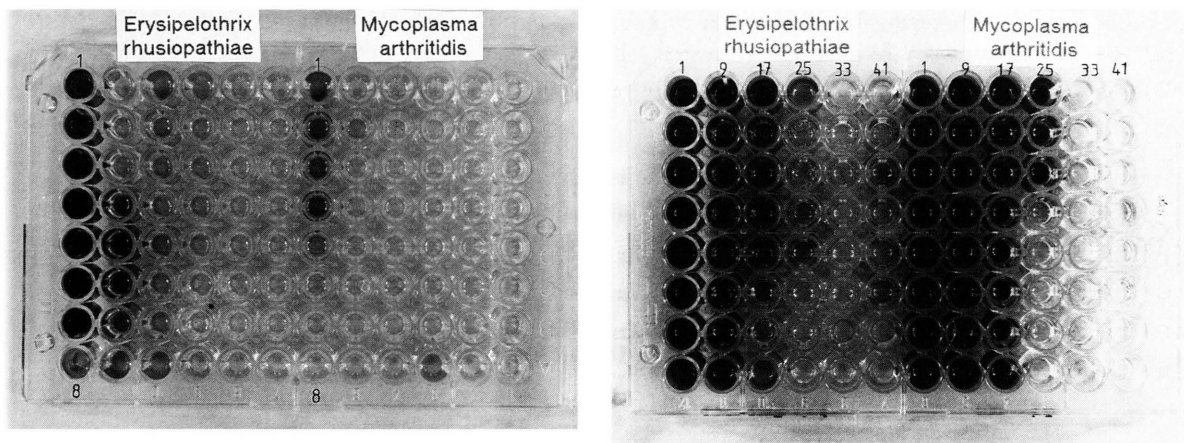


Fig. 3. Superoxide dependent reduction of nitroblue-tetrazolium by fibroblasts or PMNs stimulated with culture supernatants of *Erysipelothrix rhusiopathiae* or *Mycoplasma arthritis* after gel permeability chromatography. a) Culture supernatants were separated by chromatography on a TSK HW-40 column as described in Material and Methods and 100 μ l of the separated fractions and 100 μ l of the test buffer containing nitroblue-tetrazolium (100 μ mol/l) were mixed and added to the fibroblasts cultured in microtiter chambers. The cells were incubated for 4 hours at 37 °C and superoxide release led to the formation of blue insoluble formazan (black wells). The black wells represent the fractions of the column which stimulated the cells to release O₂⁻ into the solution. b) Culture supernatants were separated by chromatography on a Sephadex G-15 column as described in Material and Methods and 100 μ l of the separated fractions and 100 μ l of the test buffer containing nitroblue-tetrazolium (100 μ mol/l) were mixed and added to the PMNs (10⁴ cells per well) in microtiter chambers. The cells were incubated for 30 min at 37 °C and superoxide release led to the formation of blue insoluble formazan (black wells). The black wells represent the fractions of the column which stimulated the cells to release O₂⁻ into the solution.

radicals are involved in chemotaxis (Wach *et al.*, 1987). Moreover the translocation of nF-kappaB, which is induced by reactive oxygen intermediates triggers the formation of several pro-inflammatory cytokines, like IL-1 or TNF which stimulated the release of superoxide in fibroblasts (Meier *et al.*, 1989).

Some other bacteria p.e. *Escherichia coli* (Yoshida *et al.*, 1975) and *Propionibacterium acnes*, which recently was described as the causative agent of a rheumatoid arthritis (Kooijmans *et al.*, 1989), also releases low molecular weight substances into the culture medium, which induce a respiratory burst in PMNs and increased chemotactic mobility (Pulverer *et al.*, 1988), but if additionally an activation of fibroblasts is induced, is unknown.

An activation of tissue cells, and possibly other cell types must not be restricted to the near environment of the bacteria, as substances exhibiting a low molecular weight may pass the membranes, which in intact joints function as a barrier against infectious agents. Moreover they can be distrib-

uted by the blood- and lymph-tract and in this way interfere in regulation pattern in sensitive cells.

In respect to rheumatoid arthritis these substances may misregulate cellular metabolism in an early state without involvement of PMNs as observed by histological studies. Induction of proliferation (Meier *et al.*, 1991), and additionally a chemotactic movement of cells (Wach *et al.*, 1987) tightening the tissues could lead to a rapid growing cell accumulation leading to pannus formation. A collapse of this pannus which is insufficient supplied with nutritive substances additionally may trigger immigration of PMNs and macrophages beside a direct activation of these cells. So the physiological useful mechanism in wound healing, that superoxide released by inflammatory cells induces chemotaxis of fibroblasts and proliferation of the surrounding tissue cells, which moreover is selfmaintaining by superoxide radical release by these cells till the fibroblasts reach contact inhibition, leading to a determination of superoxide release in the G₀ phase (unpublished), is disturbed by a permanent stimulated superoxide release by

fibroblasts. Also triggering the formation of several pro-inflammatory cytokines may lead to a self maintaining mechanism, which gets finally independent from the presence of the infectious agents.

In these means a continuously released of activating low molecular substances by persistent bacteria may contribute to the persistence of a chronic inflammatory and auto-immune-diseases.

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