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MICROBIAL STRATEGIES TO PREVENT OXYGEN-DEPENDENT KILLING BY PHAGOCYTES

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Microorganisms which are taken up by professional phagocytic cells of a host organism (e.g., by macrophages and polymorphonuclear leukocytes) encounter a series of antimicrobial events including confrontation with toxic oxygen species, derived mainly from the superoxide radical produced by phagocytic NADPH oxidase after uptake of the microorganism. Many microbes are susceptible to the oxygen-dependent phagocytic stress and are efficiently killed. The strategies of some microorganisms to bypass an encounter with the phagocytes' reactive oxygen species, and biochemical systems contributing to the microbes' resistance to killing by reactive oxygen species are outlined.

KEY WORDS: Phagocytes, pathogenicity, intracellular lifestyle, superoxide dismutase, catalase, reactive oxygen species.

ABBREVIATIONS: CuZn SOD, copper plus zinc-containing SOD; FeSOD, iron-containing SOD; HP, hydroperoxidase; (k)Da, (kilo)Dalton; LD_{50}, lethal dose for 50% of the infected animals; MΦ, macrophage(s); MnSOD, manganese-dependent SOD; NADP(H), (reduced) nicotinamide adenine dinucleotide phosphate; O_2^-, superoxide radical; ·OH, hydroxyl radical; PMNL, polymorphonuclear leukocyte(s); ROS, reactive oxygen species; UV, ultraviolett; SOD, superoxide dismutase.

OXIDATIVE STRESS IN BACTERIA

Aerobic microorganisms constantly produce toxic oxygen derivatives by reduction of molecular oxygen during aerobic metabolism.[1,2] For example, thiol groups and flavins may reduce oxygen to result in the superoxide radical (O_2^-); glucose oxidase releases hydrogen peroxide (H_2O_2) as a product of the enzymatic reaction; xanthine oxidase produces superoxide as a side product. Superoxide may also be formed by UV- or γ-irradiation (for refs. and information on further sources of O_2^-, see[1-3]). Finally, redirection of the electrons within the mitochondrial respiratory chain may result in univalent reduction of dioxygen, and thus in the formation of O_2^- and O_2-derived reactive oxygen species (ROS).[4] Hydrogen peroxide and superoxide from these sources must be disposed of by the cells, as these ROS are toxic in several ways:

(i) Hydrogen peroxide oxidizes unsaturated (membrane) lipids and proteins,[1] and inhibits proton motive force-dependent and -independent membrane transport processes.[5] *E. coli* mutants devoid of catalase activities (see below) show enhanced rates of mutagensis.[6,7]

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TABLE I
Chemical reactions involving reactive oxygen-species

(1) Superoxide dismutase:	$2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2$
(2) Catalase:	$2H_2O_2 \longrightarrow 2H_2O + O_2$
(3) Peroxidase:	$H_2O_2 + RH_2 \longrightarrow 2H_2O + R$
(4) Glutathione peroxidase:	2 Glutathione (G-SH) + $H_2O_2 \longrightarrow$ G-S-S-G + $2H_2O$
(5) Glutathione reductase:	G-S-S-G + 2NADPH $\longrightarrow$ 2GSH + 2NADP
(6) Haber-Weiss-reaction:	$H_2O_2 + O_2^{\cdot-} \longrightarrow O_2 + OH^- + HO^{\cdot}$ (catalyzed by Fe^{2+})

(ii) Superoxide is deleterious to some enzymes *in vitro*, particularly those involved in biosynthesis of branched amino acids (e.g., α,β-dihydroxyisovalerate dehydratase, 6-phosphogluconate dehydratase,[8] and NADH-bound lactic dehydrogenase[9]). Indirect evidence for *in vivo* significance comes from the partial reversibility of growth inhibition of superoxide dismutase-negative, aerobically grown *E. coli* (see below) by the addition of the 20 proteinogenic amino acids to the culture minimal medium.[10] In addition, deficiency in the $O_2^{\cdot-}$-scavenging superoxide dismutases in *E. coli* increases the rate of spontaneous mutagenesis considerably,[10] more than does catalase deficiency.[6].

(iii) Hydroxyl radicals ($^{\cdot}OH$) can be formed in different ways from H_2O_2 and $O_2^{\cdot-}$, particularly in iron-catalyzed reactions (Table I, 6.). Due to its extreme reactivity, $^{\cdot}OH$ is highly toxic to cells, reacting with all biological macromolecules including DNA.[1,4] Chemical modification of several amino acid residues by $HO^{\cdot}$ *in vitro* has been established. Hydroxyl radicals can be scavenged *in vitro* by mannitol, benzoate or ethanol.[11] $HO^{\cdot}$-Production may be at least partially responsible for the toxic effects seen in catalase and superoxide dismutase-deficient *E. coli* mutants. (For a compilation of further ROS which might be important for oxidative stress-phenomena, see ref. 1).

Besides this "endogenous" oxidative stress, bacteria may also encouter "exogenous" oxidative stress. This type of stress may occur rather rarely when the cells are living within soil and water habitats (e.g., *Pseudomonas fluorescens* produces a superoxide-generating agent, pyocyanin, which may force other bacteria to produce $O_2^{\cdot-}$; ref. 4). A frequent type of exogenous oxidative stress is part of the defense and digestive system of mammalian phagocytic cells and of phagocytic protozoa (such as *Acanthamoeba castelanii*[12]). Here, we will discuss phagocytic cells of the immune system, whose production of ROS is a major factor in the killing of microbes. Furthermore, some microbial strategies to avoid confrontation with ROS-attack or to survive it will be presented.

PHAGOCYTES AND KILLING OF MICROORGANISMS

"Professional" phagocytes [particularly macrophages (MΦ) and polymorphonuclear phagocytes (PMNL)] are a part of the defense system designed to ingest foreign material, to kill it, if it is alive, and to digest it. Phagocytes in principle employ two different types of systems using oxygen-dependent and oxygen-independent mechanisms.

The Oxygen-Dependent Microbicidal System

When a phagocyte encounters a foreign particle, the latter is surrounded by a portion of the phagocyte's membrane, and is ingested within this "inside-out-vesicle", phagosome. Simultaneously, the phagocyte increases oxygen uptake up to 30-fold the resting rate.[13] Furthermore, the production of NADPH by the hexose monophosphate shunt is considerably increased,[14] and NADPH oxidase[15] is integrated into the phagosomal membrane. Following to ingestion-response regulatory mechanisms, molecular oxygen is reduced by NADPH oxidase, and superoxide is released into the phagosomal space.[16] The pH in the ingestion vacuole drops within a few minutes,[17] probably by the action of a proton-pumping protein,[18] favoring spontaneous dismutation of $O_2^{\cdot-}$ to H_2O_2 (with maximal rate at pH 4.8; the rate of formation decreases by a factor of 10 for each unit increase in pH above 4.8[3]). It has been calculated that H_2O_2 concentrations within the phagosome may reach levels as high as 100 mM.[15] Such intraphagosomal H_2O_2 may partially stem from other sources than NADPH oxidase,[16,19] and might in some cases even be supplied by the ingested bacteria.[20] Thus, several types of ROS can be found within the phagosome which probably work in concert with the $O_2^{\cdot-}$ production commencing within just minutes from the beginning of the phagocytic process.[17] In addition, further phagocytic ROS exist: (i) PMNL but not MΦ contain an unusual peroxidase, myeloperoxidase,[15,16] capable of oxidizing Cl^- to the highly microbicidal OCl^-.[13] This enzyme may account for up to 5% of the dry weight of PMNL in mammals but seems to be absent from avian neutrophils.[15] (ii) Singlet oxygen ($^1O_2$) is a high-energy form of molecular oxygen which can produce DNA strand breaks *in vitro*.[21] However, its biological importance and its role in phagocytic killing is not yet understood.[16,17] (iii) Whether or not hydroxyl radicals are produced *in vivo* by phagocytes is still controversial, particularly as iron is required for the Fenton reaction, but is bound *in vivo* by phagocytic lactoferrin. In this form it probably does not act as a catalyst in $^{\cdot}OH$-formation.[16,22]

During the phagocytic events, heat shock protein-synthesis can be initiated in both microorganisms and host phagocytes;[23,24] in the latter case, the levels of CuZnSOD and heme oxygenase can also be increased.[24]

The Oxygen-Independent Microbicidal System

Some minutes after phagocytosis, phagosomes fuse with other, granulous and membrane-surrounded vesicles. These vesicles contain, depending upon the type of phagocytic cell and the "activation" state,[25] various proteins, mainly hydrolases: acid hydrolases, lysozyme, neutral proteases, (deoxy)ribonucleases, iron-complexing lactoferrin and cationic antibacterial proteins.[26] Neutrophilic granules may also contain the cobalamin-binding protein and collagenase.[17] These proteins are probably responsible for the decomposition of killed microorganisms. Also, secretion by the activated phagocyte of some hydrolytic factors has been described, e.g., for alkaline phosphodiesterase, nucleotidase, and amino peptidase (cited after ref. 26).

STRATEGIES OF SOME PATHOGENS TO BYPASS ENCOUNTER WITH ROS AND TO AVOID NON-OXIDATIVE KILLING

There are several lines of defense employed by pathogenic microorganism used singly

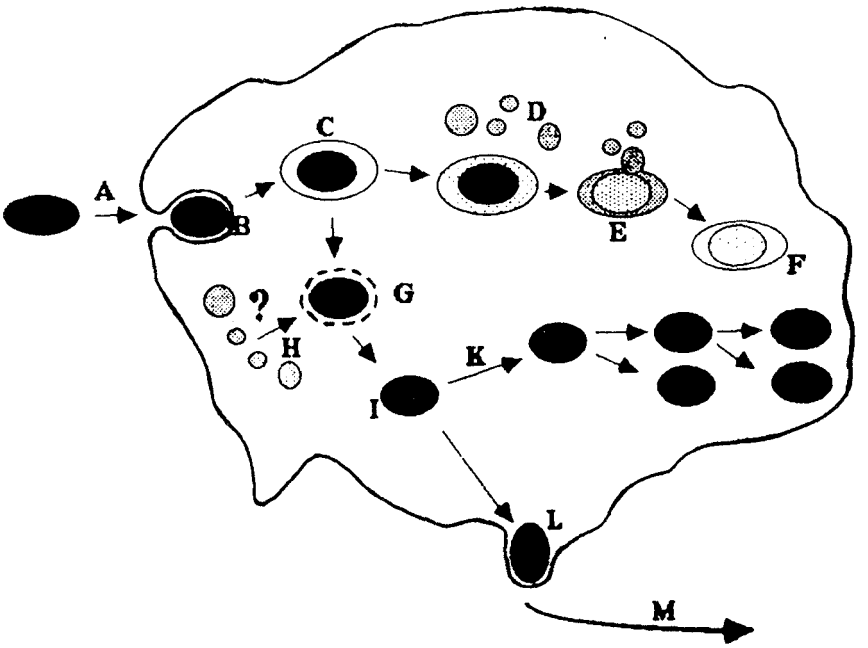

FIGURE 1 Course of events after ingestion of a microorganism by a macrophage. (A) A microorganism gets in contact with the phagocyte. This process may be triggered by binding to the phagocyte via phagocytal recognition of bound host antibodies or complement or by microbial attachment factors. (B) Ingestion occurs by invagination of the phagocyte membrane. An intracellular vesicle which contains the microbe ("phagosome") is formed. (C) During phagocytosis, processes are initiated which lead to the production of ROS and a release of O_2^- into the phagosomal space. (D) Lysosomes which contain an arsenal of bacteriostatic and bacteriocidal proteins fuse with the phagosome and release their contents (E). (F) The microorganism is killed and digested.

Some pathogenic microorganisms (e.g. *L. monocytogenes* or *S. flexneri*) disrupt the phagosomal membrane by secretion of protein toxins (G) and thus are released into the host cell's cytoplasm (I). It is not clear whether phagosome-lysosome-fusion occurs in those cases, in which these bacteria survive the phagocytic attack (H). The pathogen multiplies within the cytoplasm (K), eventually disrupting the host cell membrane, or it is transferred with its descendants from one MΦ to another by cell-to-cell contact without being presented to the environment (M).

or in concert by a given microorganism, rendering it difficult to assign the role of a single defense mechanism to survival in phagocytes. It seems as if nearly any "reasonable" strategy to avoid encounter with or damage by phagocytic ROS has been realized. The microbial strategies discussed below correspond to the different antimicrobial events presented in Figure 1. It should be emphasized that the data mentioned stem partially from *in vivo* results obtained, e.g., with purposely or randomly mutated bacteria, and partially from indirect evidence (e.g., comparison of enzymatic activities).

Killing of the Phagocyte

The microbe kills the phagocytic cell by (protein) toxins, before it can be killed itself. This strategy is realized by streptococci via streptolyins and *Staphylococcus aureus* via leukocidin.[14]

Resistance to Uptake by Phagocytes

The microorganism is not recognized as a target by the phagocyte. Bacterial (carbon hydrate) capsules contribute to this phenomenon by conferring on the bacteria hydrophobic surfaces which are not readily detected by the host's antibodies and complement factors.[27] Examples are meningitis-causing *Neisseria meningititis* and *Escherichia coli*, and pneumococci.[27] Some pathogenic streptococci produce "M-proteins", presented on the surface of these bacteria conferring antiphagocytic properties.[27]

Inhibition of Acidification

Some microorganisms secrete factors which inhibit acidification of the phagocytic vacuole.[18] Acidification itself is only rarely bactericidal, e.g., with pneumococci;[17] however, as mentioned above, spontaneous dismutation of O_2^- within the phagosome is maximal at a pH of 4.8. Furthermore, the hydrolases within the lysosome operate best at pH 4–5.[17] Blocking acidification might thus decrease both the oxidative stress and the effect of digesting factors acting on the microorganism. Phagosome-lysosome fusions seem to be enhanced at acidic phagosomal pH. Thus, blocking of acidification may as well play a role in this process. Inhibition of acidification has been described for pneumonia-causing intracellular *Legionella pneumophila*[28] and for *Toxoplasma gondii*.[29]

Inhibition of an Oxidative Burst and/or ROS-Production

L. pneumophila secretes a product of a molecular weight higher than 10 kDa inhibiting the oxidative burst of PMNL.[33] *Leishmania donovani*[34] and *Legionella micdadei*[35] produce extracellular and/or peripheric acidic phosphatases that block superoxide generation *in vitro*. The corresponding inhibitory mechanism has not yet been studied in detail, but it might be possible that these acidic phosphatases interact with NADPH which is being used in the phagosomal membrane for the reduction of molecular oxygen by NADPH oxidase; thus, O_2^- production might be decreased or stopped.

T. gondii does not trigger the respiratory burst by macrophages *in vitro*, but, on the other hand, also does not inhibit H_2O_2 release which had been triggered by *Candida albicans* cells.[36,37] When a respiratory burst occurred in *T. gondii*-infected macrophages, it did not affect intracellular survival of the protozoan, possibly due to its relatively high contents of superoxide dismutase and catalase.[29] *Chlamydia trachomatis* inhibits the neutrophilic NADPH oxidase-system *in vitro*.[38] Although the mechanisms and the *in vivo* significance of this observation have still to be determined, inhibition of superoxide generation at its source seems to be an appealing and direct survival strategy.

Inhibition of Phagosome-Lysosome-Fusions and Resistance to Non-Oxidative Killing Mechanisms

Most phagocytosed microorganisms which have encountered an attack by ROS are more susceptible to enzymatic attack by the hydrolytic enzymes contained in the lysosomes of macrophages (MΦ) and, in particular, polymorphonuclear leukocytes (PMNL). Thus, ROS can, in many cases, be regarded as the major agents for microbial killing, whereas the protein factors may mainly contribute to the digestion

of ROS-damaged microorganisms. Inhibition of phagosome-lysosome fusion by some mycobacteria,[39] by *T. gondii*,[39] the facultative intracellular bacterium *L. pneumophila*,[28] by *Chlamydia*,[39] and *Salmonella typhimurium*[40] might contribute to their pathogenicity. Resistance to non-oxidative killing is only poorly understood; e.g., it has been recently shown that *S. typhimurium* mutated in the *pagC*-locus is less resistant towards non-oxidative damage by MΦ than the parental strain.[40] *Mycobacterium leprae* resists lysosomal enzymes since it is protected by a wax-like surface layer.[39]

Escape from the Phago(lyso)some

Microorganisms which escape from the phagosome by the secretion of membrane-damaging cytolysins might effectively avoid contact with ROS and lysosomal contents. The intracellular bacterium *Listeria monocytogenes*[30] (causes listeriosis), the dysentery-causing *Shigella flexneri*,[31] and the protozoan *Trypanosoma cruzi*[32] (causing Chagas' disease) seem to use this strategy.

MICROBIAL ANTI-REACTIVE-OXYGEN-SPECIES SYSTEMS

Non-Enzymatic Resistance to ROS

It has been shown recently that the phenolic glycolipid of *Mycobacterium leprae* and the lipophosphogylycan of *Leishmania donovani* are highly effective in scavenging hydroxyl radicals and superoxide anions and thus might play a role in the pathogenicity of these microorganisms.[42] These results are in agreement with the earlier observations that (i) the lipophosphoglycan seems to be necessary for the survival in macrophages of *Leishmania major* and its pathogenicity in mice, that (ii) binding *M. leprae* phenolic glycolipid I to *Staphylococcus aureus* significantly protected these bacteria from the microbicidal mechanisms of activated human monocytes, and (iii) that washing of a major portion of glycolipids from viable *M. leprae* rendered them susceptible to *in vitro*-killing by the superoxide-producing xanthine–xanthine oxidase system, but not by H_2O_2, generated by a glucose–glucose oxidase-system (references for (i)–(iii) are compiled in ref. 42). Recently, it has been reported that the catalase-negative *Haemophilus somnus* (a cattle-pathogen) degraded H_2O_2 independent of catalase, a process which required the presence of a utilizable carbon-source.[43]

Glutathione and ROS

Glutathione, in combination with glutathione peroxidase and glutathione reductase is able to scavenge ROS, especially H_2O_2[44,45] (Table I, 4.). In contrast, it has been shown for *E. coli* that glutathione probably does not play a role in resistance towards H_2O_2-mediated stress; glutathione reductase-negative *E. coli* were even somewhat more resistant towards exogenous H_2O_2 and γ-radiation that the isogenic parental strain,[46] probably due to the lack of glutathione peroxidase in *E. coli*. However, a recent study[47] showed that proteins which are immunologically related to a *Proteus mirabilis* glutathione reductase are also present in at least one strain of *Enterobacter cloacae* and *E. coli* (both enterobacteriaceae like *Proteus*). But non-cross-reacting glutathione transferases were detected in other strains of *E. coli*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa* and mammalian samples (all ref. 47). These data suggest that

there are several types of bacterial glutathione-metabolizing proteins and that the distribution may even vary within one species.

Superoxide Dismutases

Superoxide dismutase (SOD) is a superoxide-converting enzyme found in almost all microorganisms assayed for its presence[2] even in obligately anaerobic bacteria[48] which normally should not encounter highly oxidizing environments. The enzyme is most probably responsible for short periods of survival within microaerobic niches. *Lactobacillus plantarum* (a non-pathogen) has been shown to contain 20 mM manganese ions, which can substitute for SOD function.[2]

Superoxide dismutases exist in several isozyme-types, depending on the metal ions: There are iron-dependent (FeSODs), manganese-dependent (MnSODs), cambialistic (MnSODs/FeSODs), and copper–zinc SODs (CuZnSODs). The first three species are most commonly found in bacteria, whereas the latter predominates in higher organisms. Most SODs are cytoplasmically located, although a few are secreted through the cytoplasmic membrane (see, e.g., ref. 49). For a detailed compilation of data on the distribution and biochemistry of SODs, see ref. 2. The molecular genetics of SODs and regulation of their genes have recently been reviewed by Touati[50] and Hassan,[51] respectively.

Catalases/Peroxidases

Nearly all aerobic microorganisms possess at least one catalase and/or peroxidase-isozyme (for the reactions they catalyze, see Table I, 2. and 3.). Knowledge on the biochemistry and genetics of bacterial catalases has increased considerably only in the last few years (although catalase was one of the first enzymes described). It became evident that several types of bacterial catalases exist: (i) Typical catalases of the mammalian catalase type (prototype bovine liver catalase). Such enzymes have been detected in *Micrococcus luteus*, *Rhodopseudomonas sphaeroides*, and *Listeria seeligeri* (for biochemical details, see e.g., refs. 52, 53, 54). (ii) Catalase-peroxidases (prototype HPI of *E. coli*): These enzymes not only possess a catalase activity (which is lower than in typical catalases) but also a broad-range peroxidase activity. Catalase-peroxidases from *Escherichia coli*, *S. typhimurium*, *Rhodopseudomonas capsulata*, and *Chromatium vinosum* serve as examples.[52,55,56] (iii) "Unusual" catalases, which differ from (i) and (ii) usually in more than one important biochemical characteristic have also been described, e.g., the *Klebsiella pneumoniae* catalase KpA.[52]

There are both single and multiple catalases or SODs per microorganism, and isozymes within the same organism may belong to different types (e.g., mycobacterial catalases[56] and *E. coli* SODs[51]). As microbial SODs and catalases can detoxify ROS, they are possible pathogenicity-supporting factors, particularly of intracellular microorganisms.

DNA repair mechanisms certainly also constitute one line of microbial defense against ROS, which are of particular importance, once that damage has occurred.[1,4] Inducible systems corresponding to the *E. coli* SOS-regulon or the SoxR- and the OxyR-response, which both regulate DNA modifying enzymes, may play some role *in vivo*,[57] as well as the DNA recombination systems.[58]

STRATEGIES OF PATHOGENIC MICROORGANISMS TO DETOXIFY PHAGOCYTIC REACTIVE OXYGEN SPECIES

These are microbes which survive within the phago(lyso)some or which escape from it but may nevertheless encounter confrontation with ROS that is initiated within a few seconds. It should be emphasized that probably not all microorganisms of a given species which enter the host during an infective process must be equally efficient in detoxifying ROS. Some may die after confrontation with ROS and others may not. In these cases, it would be critical how much of the infectious dose remains alive after encounter with phagocytic ROS. Furthermore, only some strains of a given species may apply certain pathogenicity factors, as has been shown for virulence factors from several microorganisms.

Escherichia coli/Staphylococcus aureus

In 1974, Yost and Fridovich reported in one of the first studies on confrontation of microorganisms with phagocyte ROS that *E. coli* is more resistant to exogenously added ROS and killing mechanisms of leukocytes *in vitro* when grown in iron-rich medium (compared to iron-deficient *E. coli*); they have shown that this resistance was probably due to increased capacity of FeSOD.[59] From today's point of view it seems possible that regulatory effects of the high iron concentration during cultivation might also have contributed to this effect (for a review on iron regulation in *E. coli* see ref. 60). However, an iron overload can also be deleterious to cells when they encounter high ROS concentrations: iron-loaded cells of the gram-positive *Staphylococcus aureus* were not more susceptible to human PMNL-killing than iron-deprived cells, but were more efficiently killed in human monocytes.[61] Iron-loaded staphylococci were also much more susceptible to $H_2O_2^-$ mediated killing *in vitro*,[62] probably due to $^{\cdot}OH$-formation. In a survey of 15 staphylococci, there was a good correlation between catalase activity and mouse lethality, but no correlation between SOD activity and virulence.[63]

Bloch and coworkers constructed *sodA* mutants ($MnSOD^-$) of pathogenic *E. coli* by site-directed mutagenesis.[64] As MnSOD is the most strongly induced *E. coli* protein during transition from anaerobic to aerobic growth conditions *in vitro*,[65] the authors hypothesized that this enzyme might be crucial after the entry of the bacterium from the gastrointenstinal tract into the bloodstream. The pathogenic potential of these mutants was not diminished in a bacteremia-rat-model.[64] It should be mentioned, however, that (i) MnSOD might be far less important for *in vivo* resistance to ROS than FeSOD, as the latter is constitutively expressed, whereas MnSOD is present only in aerobic cells[51] (see *S. flexneri*, below). (ii) The bacteria may encounter only slight oxidative stress in this model. (iii) *E. coli per se* is very sensitive to ROS of host cells.[59,66,67] Thus, the loss of an SOD activity might not further increase the microbes' sensitivity.

These data are important, as they clearly indicate that a lack in SOD activity does not generally reduce survival of microorganism in a host.

Shigella flexneri

An eminent role of *S. flexneri* FeSOD in resisting phagocytic killing has recently been proven using an *sodB* strain ($FeSOD^-$). *S. fexneri* is an enterobacterium and a

medically important (dysentery-causing) intracellular microorganism: *sodB* cells were efficiently destroyed when confronted with phagocytes *in vitro*: Within a few minutes after contact the rate of surviving bacteria was reduced by at least 3 log units, compared with isogenic *sodB*$^+$ cells.[68] Thus, the authors stated that "this gene is most critical for virulence".[68] Loss in catalase activity also decreased viability to a smaller extent: In phagocyte-assays with *katFkatG* double mutants (devoid of HPI and HPII), the rate of surviving bacteria was reduced by approximately two log units compared to isogenic *katF*$^+$*katG*$^+$. The susceptibility towards phagocytic attack was well correlated with the patterns of histological destruction in a rabbit ileal loop-model.[68] These authors elegantly mutated the *katFkatG*-genes by using P1-transduction from *E. coli* cells transposon-mutated in the corresponding genes (*E. coli* and *S. flexneri* are phylogenetically closely related). No mutation in the *S. flexneri sodA* gene (coding for MnSOD) could be obtained this way; this may have been due to a lack of sufficient nucleotide sequence homology to *E. coli sodA*. Nevertheless, it can be suggested that FeSOD is more critical *in vivo*, as MnSOD is probably not expressed during the first infection steps which mainly take place in the anaerobic gut, whereas FeSOD should be (analogous to SOD regulation in *E. coli*[51]).

The results on the survival of these *S. flexneri* mutants within phagocytes are somewhat surprising and difficult to interpret. (i) *S. flexneri* produces a contact hemolysin which enables it to escape from the phago(lyso)some;[31] once cytoplasmically located, the bacterium should be protected against ROS and one would not expect further killing; (ii) in infected PMNL, *S. flexneri* had been shown to produce five- to tenfold less chemiluminescence than *S. aureus*;[68] (iii) it has been shown that O_2^- crosses biological membranes only rather poorly due to its negative charge,[14] whereas H_2O_2 crosses membranes quite well. But here, SOD was more critical for survival than catalase and peroxidase. (iv) In addition, O_2^- has been suggested to be less cytotoxic *in vitro* than hydrogen peroxide, and hydrogen peroxide is actually formed in the phagosome from superoxide at acidic pH[3], and consequently [also considering (iii)] one would expect catalase to be more important for survival than SODs. (v) Catalases and SODs seem not to be critical at all for *in vivo*-survival of the intracellular bacterium *Salmonella typhimurium*, which is closely related to *S. flexneri* (but which probably uses other ROS-detoxifying mechanisms, see below).[61] (vi) Most interestingly, the respective *S. flexneri* mutants were very efficiently killed by MΦ and PMNL, although they had been cultured aerobically *in vitro* before infection.[68] Under these circumstances, both the FeSOD and the MnSOD are present in high enzymatic activity in *Shigella*; nevertheless absence of FeSOD was lethal. This is surprising as there is no difference in subcellular location of the SOD isozymes in the closely related *E. coli*,[70] and both enzymes catalyze the dismutation of superoxide with similar efficiency. FeSOD is even more susceptible to inactivation by its reaction product, H_2O_2, than is MnSOD.[2] One would expect that functional interchange between MnSODs and FeSODs should be readily possible, yet it is not. Again, it is worth noting that survival of *E. coli* in blood serum may also partially depend upon the level of FeSOD activity.[59]

Nocardia asteroides

This gram-positive bacterium produces an unusual SOD which contains Mn, Fe, and Zn, and which is located in the cell periphery and also is partially secreted into the medium.[71] Anti-nocardial SOD-monoclonal antibodies were successfully used to

demonstrate that virulent *N. asteroides* which otherwise survive well within mice were cleared much faster from the murine organs when they had been incubated with the anti-SOD-antibody. In contrast, a control monoclonal antibody against another surface-associated antigen did not have an effect on survival.[72] This indicates that nocardial SOD is critical for virulence *in vivo*. Catalase is possibly also of some importance, as virulence and resistance to PMNL of *N. asteroides* correlate well with their catalase activities measured *in vitro*.[26] There was a direct correlation between the growth phase (influencing chemical composition of the cell envelope and catalase activities) and the virulence of *N. asteroides*; nocardiae might further use glycolipids and mycolic acids as ROS scavengers, similar to mycobacteria.[26]

Mycobacterium tuberculosis

Catalase has been suggested to be a virulence factor of *M. turberculosis*, as low virulence was associated with low resistance to exogenously added H_2O_2 and partly correlated with catalase content.[73] No correlation was found between SOD levels and virulence, and even weakly virulent mycobacteria were quite resistant to O_2^- *in vitro*. In another study, a virulent *M. tuberculosis* was highly resistant towards several types of ROS.[74] These observations might now be explained by the presence of ROS-scavenging glycolipids on the surface of some mycobacteria;[42] the same may be true for the pathogenic fungus *Candida tropicalis*, which is also resistant to ROS and which contains abundant lipids on its cell surface.[74] It is remarkable that *M. lepraemurium* contains the highest concentration of intracellular SOD reported thus far for any organism, accounting for at least 7% of the total protein in cell extract.[2]

Recently, SOD genes from *M. tuberculosis*[75] and *M. leprae*[76] have been isolated by screening recombinant *E. coli* clones for the presence of antigens which reacted with monoclonal antibodies against major antigenic proteins (23 kDa and 28 kDa-antigens, respectively); later, nucleotide sequence analysis revealed the identity of these antigens with mycobacterial SODs. This finding suggests some role of SODs during mycobacterial infection. A similar approach has been used for the isolation of a *Brucella abortus* gene which was identified to encode a CuZnSOD.[77]

It had been reported that virulent *M. tuberculosis* secrete SOD into the culture fluid *in vitro*[75,78] (and *Brucella abortus* into the periplasic space[77]). In contrast, amino acid sequence analysis did not reveal an indication for a myobacterial transport-mediating signal sequence.[78] SOD release is probably *not* due to cell lysis.[75] As also the *L. monocytogenes* SOD may be partially secreted,[79] and as we did not find a typical leader sequence either,[80] one must consider the possibility that there may be specialized (SOD)-transport pathways in some bacteria. The availability of the cloned mycobacterial SOD genes may permit the construction of site-specific *in vivo* insertional *sod*-mutants of these bacteria, for which the virulence and thus the role of SOD can be determined.

Listeria monocytogenes

It has been suggested that SOD and/or catalase might be important for the survival of the intracellular *L. monocytogenes*.[30,79,81,82] Nevertheless, it has been shown with transposon-mutated *L. monocytogenes* that catalase is not absolutely required for survival within mice.[83] On the other hand, catalase-negative *L. monocytogenes*, obtained either by transposon-mutagenesis, or isolated from patient or environmental

TABLE II
Listeria species and their susceptibility to oxidative stress

Strain	Catalase-activity[1]	SOD-activity[2]	Inhibition by H_2O_2[3]	Pathog.[4]
L. monocytogenes Sv. 4b	156 U/mg	n.d.	n.d.	++
L. monocytogenes Sv. 3a	48 U/mg	n.d.	n.d.	+
L. monocytogenes Mack[5]	75 U/mg	+	n.d.	++
L. monocytogenes EGD[6]	543 U/mg	++	1.5	++
L. ivanovii	141 U/mg	++	4.0	+[7]
L. grayi	170 U/mg	++	2.5	−
L. seeligeri	264 U/mg	+++	2.5	−
L. innocua	469 U/mg	++	0.5	−

n.d., not determined

[1]Specific units of catalase activity per mg protein as determined in.[54]

[2]SOD activity as determined from SOD electropherograms; a "+" corresponds to app. *E. coli* SOD activity.

[3]Determined in a disc growth-inhibition experiment as described in,[86] but using brain heart infusion media instead of LB media (indicated is the radius in mm from the border of the disc when 600 μg H_2O_2 was applied per disc); using the O_2^- generating agent paraquat, we could not find any sensitivity towards this agent, even at very high concentrations (300 μg/disc; probably due to a lacking uptake[89]).

[4]Pathogenic strains are marked by a +, very pathogenic by ++, non pathogenic by a −.

[5]*L. monocytogenes* serovar. 1/2a Mackaness

[6]*L. monocytogenes* serovar. 1/2a EGD

[7]*L. ivanovii* is pathogenic to a limited number of animals, including sheep and mice. Its LD_{50} is two to three log units higher in mice than that for virulent *L. monocytogenes*.

samples, all show considerably increased SOD levels[79,81,83] which could compensate for the loss in catalase activity; similarly, very high SOD activities may compensate for a lack of catalase and peroxidase activities in *Streptococcus sanguis*,[84] or high catalase activities may compensate for a lack in SOD activity in some strains of *Neisseria gonorrhoeae*.[85] Most of *L. monocytogenes* SOD was reported to be associated with the cell periphery.[79]

To determine the role of these enzymes in the pathogenesis of listeriosis, we have isolated the SOD genes from the pathogenic species *L. monocytogenes*[80] and *L. ivanovii*.[86] Amino acid sequence comparison of these and other SODs showed that they are most probably Mn-dependent SODs.[80] We have also isolated the gene coding for catalase from *L. seeligeri*, an apathogenic *Listeria* species[54] closely related to *L. monocytogenes*. *Listeria* spp. most probably contain single SOD and catalase genes (refs. 54, 86 and unpublished data). Thus, it is relatively easy to mutagenize the single genes and their combination and create mutants which completely lack these enzymes. So far *Listeria* is the only genus besides *Escherichia* for which both the catalase and superoxide dismutase genes have been isolated. Concerning the distribution of catalase and SOD activities within the genus *Listeria*, we did not find a direct correlation between pathogenicity and levels of enzymatic activity (Table II).

Listeria is an interesting model from another point of view: As mentioned above, catalase-negative *L. monocytogenes* produce significantly higher SOD activities (refs. 79, 83 and our own observations). One might expect, therefore, that *L. monocytogenes* (or other listeriae) which have high catalase activity are relatively low in SOD activity. This is, however, not the case (Table II), in accordance with observations from others.[79,87] In addition, catalase activities are considerably higher in logarithmic-phase *L. monocytogenes* than in stationary phase-cells,[82] and growth phase correlates with the *in vitro* resistance to phagocyte ROS.[82] Catalase levels determine the *in vitro*

susceptibility of listeriae to stress mediated by exogenously added H_2O_2 (Table II), which is not true for all microorganisms, e.g., gonococci.[88] Finally, SOD activity is dependent on the partial pressure of oxygen within the culture medium.[89]

In summary, ROS-stress response by listeriae seems to be an interesting subject for regulatory studies, particularly as these results suggest that even high catalase concentrations (app. 5 times that of *E. coli* HPI plus HPII) are increased under normal *in vitro* cultivation conditions when SOD is absent. Interestingly, most of the *L. monocytogenes* are killed within the first three minutes after contact with rat peritoneal MΦ.[90] Furthermore, reduction of the partial pressure of oxygen in a MΦ/*Listeria*-mixture results in an inhibition of listericidal and anti-*Pseudomonas*-activity *in vitro* (cited after[25]). This finding underscores the role of ROS in killing of *Listeria*, as the non-oxidative killing mechanisms set in only later, suggesting that pathogenic listeriae leave the phagosome early after phagocytosis is completed in order to efficiently infect a host. ROS might also influence the efficiency of escape from the phagosome by inactivation of the secreted toxin, as has been shown for pneumolysin from *Streptococcus pyogenes*, a toxin which is structurally very related to LLO. Killing of *Listeria* may be oxygen-dependent as well as oxygen-independent, depending on the type of assay and the host cell type used,[91,92] similar to the results obtained with *T. gondii*.[16]

Salmonella typhimurium

In 1986, Fields and coworkers[69] published a comprehensive study on the contribution of certain genes to intracellular survival: Approximately 10,000 transposon mutants were screened *in vitro* for survival within macrophages, and 83 mutants were isolated which had a diminished capacity for intracellular survival. It is interesting to note that three of these mutants, whose LD_{50} (lethal dose in 50% of the mice) was raised by 3 to 4 log units, showed altered sensitivity towards various prooxidants *in vitro*. Surprisingly, none of the mutations screened this way showed increased activities of either SOD, catalase, or glutathione-linked enzymes, as determined by activity gel analyses. In fact, one out of 3,700 mutants which were tested for production of oxygen from H_2O_2 was completely deficient in HPII synthesis, but survived normally in macrophages *in vitro* and was virulent *in vivo*. Finally, *S. typhimurium* inactivated in the gene encoding OxyR, a positive regulator of H_2O_2-induced genes, are less virulent than the isogenic parental strains.[69] How can these data be interpreted with regard to the data obtained with SOD and catalase mutants of the very closely related *S. flexneri*, which are highly sensitive towards phagocytic killing (see above)? One possible answer is that virulent salmonellae probably inhibit the oxidative burst of phagocytes,[6,93] whereas oxidative bursts within *Shigella flexneri*-infected phagocytes are rather normal.[68] Nevertheless, similar phenomena have been observed with *E. coli*: *In vitro*, OxyR is, under certain circumstances, more important for resistance towards exogenously added H_2O_2 than the *E. coli* catalases are.[57] Recently, a transposon-adhering gene has been isolated from two apathogenic transposon-mutants of *S. typhimurium*, screened for virulence *in vivo*.[94] It was shown that these mutations mapped in the *htrA*-locus which seems to be necessary for resistance to oxidizing agents and which might encode a protease (the *E. coli*-homologue is a heat shock gene). Thus, the major role of this gene in intracellular survival might not be the resistance to oxidative stress but a general heat shock protein-like function, in this

case probably co-responsible for degradation of aberrant (e.g., ROS-damaged) proteins. This is in accordance with the demonstration that two of the major proteins produced by *S. typhimurium* during infection of macrophages are the heat shock-proteins GroEL and DnaK.[23]

It is important to note that many *auxotrophic* transposon-mutants of *S. typhimurium* have been characterized as avirulent (LD_{50} up to 5 log units higher than in the parental strain). On the other hand, no auxotrophic *Listeria monocytogenes*-mutant has been found using a very similar approach.[95] This again indicates that a given factor may play a key role during pathogenesis of one intracellular bacterium but may be unnecessary during pathogenesis of another; the negative selection of auxotrophic salmonellae probably occurs, because these pathogens grow and multiply within the phagocytic vacuole to form large inclusion bodies with many bacteria per phagosome.[40] In this environment, it might be necessary for the virulent salmonellae to synthesize most physiologic constituents from nutrients which are present in the phagosomal fluid. In contrast, successful *L. monocytogenes* leave the phagosome by lysis of the phagosomal membrane by a protein toxin[30] and thus are released into the rather growth-permissive cytoplasm. Although not all salmonellae found in an infected tissue are intracellular,[39] complete cure requires eradication of intracellular salmonellae.

LESSONS FROM "HOST MUTANTS"

Chronic Granulomatous Disease (CGD)

This inheritable disease is characterized by the lack of superoxide production by phagocytes, and has recently been shown to be in most cases due to the inability to produce a cytochrome b-constituent of the NADPH oxidase complex.[16] Affected persons suffer from frequent infections, particularly with opportunistic pathogens which, in normal phagosomes, are rapidly killed by ROS: *E. coli*, *Klebsiella pneumoniae* and streptococci may serve as examples.[14] The illness can lead to early death of the patients.[14] In many cases, microorganisms may contribute to their own destruction by secreting H_2O_2 and/or O_2^- into the phagosome.[15,26,96] Mutant strains of pneumococci and staphylococci which do not produce H_2O_2 are consequently not killed by CGD leukocytes, whereas catalase-positive (non-isogenic), H_2O_2-producing bacteria of the same species are.[14,26]

Glucose 6-Phosphate Dehydrogenase-(G6-PD)-Deficiency

G6-PD is necessary to supply the NADPH oxidase with reducing equivalents from the hexosemonophosphate shunt. Patients with severe G6-PD deficiency will not efficiently produce and release ROS, thus these patients suffer from a similar, albeit less severe, clinical picture like CGD patients.[17]

Myeloperoxidase (MPO)-Deficiency

Patients show delayed killing of bacteria in polymorphs but are normally resistant to most bacterial infections,[14] demonstrating that MPO is not absolutely required for an efficient *in vivo*-clearance of all microbes.

REGULATION OF THE BACTERIAL DEFENSE AGAINST ROS

Regulation of bacterial defense mechanisms against ROS is only poorly understood, except for *E. coli* and *S. typhimurium*. There are recent excellent reviews on this topic.[4,50,51,97] Here, we will merely give a brief overview. The most important regulons in an answer to ROS detected thus far are the oxygen-inducible regulon, OxyR;[98] the superoxide-inducible regulon, SoxR;[99,100] and the aerobic respiration control, Arc.[101] OxyR responds mainly to hydrogen peroxide, SoxR to superoxide stress, *katE* is probably a sigma-factor in DNA transcription,[97] which responds to cell starvation (and which regulates *katE* and *xthA*[97]), and Arc reacts to a change between anaerobic and aerobic lifestyles. Some genes are regulated by more than one of these regulatory factors and may also be induced by the heat shock response (HspR).[97] For example, it has been shown for *E. coli* MnSOD gene that it is regulated by activated SoxR,[100,101] Arc[102] and Fur,[103] but not by OxyR[104] or HspR.[105] *E. coli* HPI is induced by activated OxyR, but not by SoxR.[97] OxyR is the best characterized regulon which responds to ROS, and its eminent role, function and molecular structure have been demonstrated in some excellent recent studies.[98,106,107] Arc may belong to the class of two component sensory-transmitter-proteins.[101] Recently, a genetic locus has been isolated from *Bacillus subtilis* which mediates resistance to *B. subtilis* towards high, but not low concentrations of H_2O_2 (*hpr* locus[108]).

KILLING BY NITRIC OXIDE

Mouse macrophages produce toxic nitric oxide radicals at acidic pH.[109,110] Thus, NO· might be involved in phagocytic defense mechanisms.[111,112] The production of nitric oxide by phagocytes is triggered by various bacteria to a different extent; it seems as if considerably more gram-positive bacteria are necessary to initiate nitric oxide production *in vitro* than gram-negatives.[110]

CRITICAL EXPERIMENTAL ISSUES

(i) The choice of the phagocytic cell line for the assays: It has been shown that some *S. typhimurium* mutants which survived well in splenic and bone-marrow MΦ survived only poorly in peritoneal MΦ.[113] The activation state of the phagocytes[14,25] may also be crucial in a particular experiment, as might be the choice of the type of phagocyte (MΦ or PMNL) and the production or non-production of certain oxygen metabolites (see, e.g., NO·).

(ii) Microbial mutants for pathogenicity tests should be obtained by a defined mutagenicity procedure. Results obtained with bacteria carrying chemically induced mutations or with mutants isolated from natural habitats are quite difficult to interpret: they may carry multiple independent mutations, or a mutation may be located in a regulatory region which downregulates not only the factors under study, but also other sets of proteins. Similarly, simply comparing the level of activities of ROS-metabolizing enzymes from different species of a genus is not sufficient,[74] as minute SOD or catalase activities may detoxify ROS to a large extent (see, e.g., *S. flexneri*[68]). Furthermore, the activities of ROS-metabolizing enzymes must not correlate with the microbes' ROS susceptibility (e.g., in gonococci[88]). Finally,

TABLE III
Genes coding for superoxide dismutase, catalase or peroxidase which have been isolated from pathogenic microorganisms.

Source	Illness	Type of enzyme	Ref.
Bacteroides gingivalis[1]	periodontal disease	cambialistic SOD	116, 117
Brucella abortus	brucellosis	CuZnSOD	77
Coxiella burnetii	Q fever	MnSOD or FeSOD	118
Escherichia coli	urogenital infections, wound infections meningitis, septicaemia	FeSOD	119, 120
Escherichia coli	see above	MnSOD	121, 122
Escherichia coli	see above	catalase-peroxidase (HPI)	123
Escherichia coli	see above	catalase (HPII)	124
Listeria ivanovii	listeriosis	MnSOD	86
Listeria monocytogenes	listeriosis	MnSOD	80
Listeria seeligeri	apathogenic[2]	catalase	54
Mycobacterium leprae	leprosis	MnSOD	76
Mycobacterium tuberculosis	tuberculosis	FeSOD	75
Salmonella typhimurium	typhoid fever in mice	catalase-peroxidase (HPI)	55

[1]Identical with the species *Porphyromonas gingivalis*
[2]*L. seeligeri* is a non-pathogenic species, but is closely related to the pathogenic listeriae *L. monocytogenes* and *L. ivanovii*, which contain a single, closely related catalase gene, respectively.

pathogenicity-mediating genes may be strongly induced *in vivo* but not *in vitro*, as has been demonstrated for the *S. typhimurium* heat shock genes *groEL* and *dnaK*.[23]

Several genes for ROS-defending factors are available from pathogenic microorganisms (Table III). Thus, it is now feasible to construct specifically mutated strains from several (intracellular) pathogens. Using a site-specific procedure, we have constructed an SOD-negative mutant of *L. monocytogenes* using the cloned gene (K. Brehm *et al.*, unpublished data). In addition to the factors mentioned in Table III, it would be interesting to design regulatory mutants of, e.g., *S. flexneri* using further cloned genes from *E. coli* and test them for virulence (mutagenesis by transduction, like in[68]). Examples for such candidates are *E. coli oxyR*,[97,114] *soxR*,[100] *soxS*,[100] *arc*[101] and *fur*.[60]

(iii) The *in vivo* effect of ROS may, in some cases, be ascribed to a ROS-independent killing. For example, it has been shown that the protein-toxin pneumolysin from *Streptococcus pneumoniae* is inactivated by PMNL-derived myeloperoxidase via modification of the critical cysteine residue.[115] *Clostridium difficile* cytotoxin and *C. perfringens* phospholipase C were rapidly inactivated by neutrophil cell sonicates.[16] Thus, the release of some pathogens into the growth-permissive phagocytic cytoplasm by secretion of a protein toxin may be decreased by ROS/toxin-interactions.

(iv) Finally, it has been described for some intracellular bacteria that they are found within phagolysosomes after infection of phagocytes *in vitro* or *in vivo*. This does not necessarily mean that these microorganisms can withstand lysosomal attack, but it may also be interpreted in terms of failure of some bacteria of the infectious dose to survive, while others escape from the phagosome before fusion, or they inhibit phagosome-lysosome fusion.

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