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Increased Intracellular Survival of *Mycobacterium smegmatis* Containing the *Mycobacterium leprae* Thioredoxin-Thioredoxin Reductase Gene

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The thioredoxin (Trx) system of *Mycobacterium leprae* is expressed as a single hybrid protein containing thioredoxin reductase (TR) at its N terminus and Trx at its C terminus. This hybrid Trx system is unique to *M. leprae*, since in all other organisms studied to date, including other mycobacteria, both TR and Trx are expressed as two separate proteins. Because Trx has been shown to scavenge reactive oxygen species, we have investigated whether the TR-Trx gene product can inhibit oxygen-dependent killing of mycobacteria by human mononuclear phagocytes and as such could contribute to mycobacterial virulence. The gene encoding *M. leprae* TR-Trx was cloned into the apathogenic, fast-growing bacterium *Mycobacterium smegmatis*. Recombinant *M. smegmatis* containing the gene encoding TR-Trx was killed to a significantly lesser extent than *M. smegmatis* containing the identical vector with either no insert or a control *M. leprae* construct unrelated to TR-Trx. Upon phagocytosis, *M. smegmatis* was shown to be killed predominantly by oxygen-dependent macrophage-killing mechanisms. Coinfection of *M. smegmatis* expressing the gene encoding TR-Trx together with *Staphylococcus aureus*, which is known to be killed via oxygen-dependent microbicidal mechanisms, revealed that the TR-Trx gene product interferes with the intracellular killing of this bacterium. A similar coinfection with *Streptococcus pyogenes*, known to be killed by oxygen-independent mechanisms, showed that the TR-Trx gene product did not influence the oxygen-independent killing pathway. The data obtained in this study suggest that the Trx system of *M. leprae* can inhibit oxygen-dependent killing of intracellular bacteria and thus may represent one of the mechanisms by which *M. leprae* can deal with oxidative stress within human mononuclear phagocytes.

Mycobacterium leprae, the causative agent of leprosy, is an intracellular pathogen that resides in mononuclear phagocytes. The mechanisms by which *M. leprae*, and other mycobacteria, resists intracellular killing inside mononuclear phagocytes are poorly understood. Several mechanisms for resistance to intracellular killing have been proposed (19), one of which could be the scavenging of free radicals produced by mononuclear phagocytes upon infection. One protein with known free-radical scavenging activities is thioredoxin (Trx): Trx has been shown to protect cells from oxidative stress by scavenging harmful oxygen species (4). Furthermore, Trx can regenerate proteins that are inactivated as a result of stress (15). Trx forms an integrated redox-active complex with thioredoxin reductase (TR) and NADPH. This Trx system, present in all prokaryotic and eukaryotic cells studied to date, operates via redox-active disulfides and provides electrons for a wide variety of different metabolic processes (1, 8). We have recently identified the Trx system in *M. leprae* and showed that it is unique in that it is expressed as a single hybrid protein containing TR at the N terminus linked via a hydrophilic peptide spacer to Trx at the C terminus (25). In all other organisms studied to date, including other mycobacteria, TR and Trx are expressed from two separate genes encoding two separate proteins. Biochemical analysis showed that this TR-Trx hybrid protein is enzymatically active and thus has redox properties similar to those found for the separate components of other organisms. Comparison of the hybrid protein with separately expressed TR and Trx showed that especially at the low concentration level, the hybrid protein is more active (26). Here, we have studied whether the *M. leprae* TR-Trx hybrid gene product when expressed in a nonpathogenic mycobacterium can modulate intracellular killing by human mononuclear phagocytes.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and monocytes. The *Escherichia coli* strain XL-1 Blue (2) was cultured overnight at 37°C in LB medium or LB agar with 200 μg of hygromycin B (Boehringer GmbH, Mannheim, Germany) per ml as a selection marker for pOLYG. *Mycobacterium smegmatis* 1-2c (27) was cultured for 3 days at 37°C in Middlebrook 7H9 medium or on Middlebrook 7H11 agar plates supplemented with oleic acid, albumin, dextrose, and catalase (Difco Laboratories Inc., Detroit, Mich.) containing 50 μg of hygromycin B per ml. Mycobacteria were harvested, and clumps of mycobacteria were removed as described previously (6). *Staphylococcus aureus* (type 42D) and *Streptococcus pyogenes* (group A) were cultured overnight at 37°C in nutrient broth no. 2 (Oxoid Ltd., Basingstoke, United Kingdom) or on agar plates. The shuttle vector pOLYG (a kind gift of S. Barnini and D. B. Young, Department of Medical Microbiology, St. Mary's Hospital Medical School, London, United Kingdom) is a derivative of p16R (5). It was generated by insertion of the polylinker of pBluescript-II-SK (Statagene, La Jolla, Calif.) into the *Kpn*I site of p16R1. Monocytes were isolated from the buffy coats of 500 ml of peripheral venous blood of healthy human donors and used freshly without further culturing (13).

Construction of recombinant *M. smegmatis*. A 3.1-kb *Pst*I DNA fragment of a cosmid clone containing the gene encoding the TR-Trx protein (25) was subcloned in the *Pst*I site of the pOLYG shuttle vector to obtain pOLYG-TR-Trx. This construct produced in and isolated from *E. coli* XL-1 Blue was electroporated in *M. smegmatis* as described previously (20), creating the recombinant MS-TR-Trx. A similar approach was applied to construct the recombinant MS-43L, which contains the pOLYG shuttle vector with a 4.3-kb *Pst*I fragment encoding the 25-kDa protein 43L (23). This latter recombinant as well as the recombinant MS-pOLYG, containing the vector without an insert, were used as negative controls.

SDS-PAGE and immunoblotting. Five micrograms of mycobacterial lysates and 25 ng of purified TR-Trx hybrid protein (26) were analyzed by sodium

dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 12% gel (12). A prestained molecular weight marker was used as a size marker (Bio-Rad Laboratories, Richmond, Calif.). The proteins were transferred to polyscreen membranes (NEN Research Products, Boston, Mass.) with a Bio-Rad mini-transblot apparatus as described in the recommendations of the manufacturer (Bio-Rad). Incubation of the membranes was done as described previously (24) with rabbit antisera directed against purified Trx and TR of *M. leprae*. These antisera were obtained by standard immunization procedures (10), purified on protein A-Sepharose CL-4B, and affinity purified with purified TR and Trx protein immobilized on CNBr-activated Sepharose 4B as described in the recommendations of the manufacturer (Pharmacia LKB Biotech, Uppsala, Sweden).

Phagocytosis. Bacteria were preopsonized with 10% AB serum for 30 min as described before (7). The bacteria were subsequently labelled by incubating them with a fluorescent label (fluorescein isothiocyanate). After washing, 200 μl of fluorochrome-incubated bacteria ($10^7 \cdot ml^{-1}$) and 200 μl of mononuclear phagocytes ($10^7 \cdot ml^{-1}$) were incubated at 37°C with slow rotation (4 rpm) for 15 min. Phagocytosis was stopped by transferring aliquots of 100 μl of the mixture to 900 μl of ice-cold Hanks balanced salt solution containing 0.1% gelatin and 0.01% Tween (HBSS-GT). Bacteria not associated with cells were removed by washing and differential centrifugation. To permit the discrimination of bacteria that are phagocytosed from bacteria that are attached to the surface of the cells, we used fluorescence quenching (3). Phagocytosed bacteria were counted with a fluorescence microscope, and the numbers were expressed as a percentage of intracellular bacteria.

Intracellular killing of bacteria. The assay for intracellular killing was performed as described previously (22). In short, phagocytosis was stopped after 15 min by adding ice-cold HBSS-GT, and nonphagocytosed cells were removed by differential washing and centrifugation. The monocytes with cell-associated bacteria were resuspended in 1 ml of HBSS-GT containing 10% AB serum, of which 100 μl was directly used as time sample 0 min. The remaining 900 μl was divided into 300-μl portions and incubated at 37°C under slow rotation (4 rpm) for a given period of time. At indicated time intervals, the intracellular killing was stopped by adding ice-cold H_2O containing 0.01% bovine serum albumin to one of the aliquots and the monocytes were disrupted by vigorous shaking on a vortex mixer. After serial dilution, bacteria were plated onto agar plates, and after culturing, the number of viable cell-associated bacteria was determined. Intracellular killing was expressed as the decrease in the percentage of viable cell-associated bacteria.

DPI assay. Monocytes were preincubated for 30 min with 5 μM diphenyleniodonium (DPI; a generous gift of A. R. Cross, Department of Biochemistry, University of Bristol, Bristol, United Kingdom). During subsequent phagocytosis, 5 μM DPI was also added. The intracellular killing of the recombinant *M. smegmatis* containing the empty pOLYG shuttle vector was determined by using monocytes preexposed to DPI and exposed to DPI during phagocytosis or monocytes not treated with DPI. Intracellular killing of *M. smegmatis* was determined as described above.

Intracellular killing of *S. aureus* and *S. pyogenes* coinfected with recombinant *M. smegmatis*. The influence of recombinant *M. smegmatis* on the intracellular killing of *S. aureus* or *S. pyogenes* was determined as described above. For these experiments, 10^7 monocytes ml^{-1} were mixed together with 10^7 *M. smegmatis* bacteria ml^{-1}. After 10 min of phagocytosis, 10^7 *S. aureus* or *S. pyogenes* bacteria ml^{-1} was added and phagocytosis was continued for another 5 min. After removal of the extracellular bacteria, the number of viable cell-associated bacteria was determined at given intervals, as described above.

Analysis of data. Results are expressed as the mean ± standard error of the mean, and the number of experiments is indicated as n = x. Differences between various groups were assessed by use of Student's paired t test. The level of significance was set at 0.05.

RESULTS

Immunoblotting of recombinant *M. smegmatis* containing the *M. leprae* TR-Trx gene. Antisera directed against TR, which is a 35-kDa protein, and Trx, which is a 14-kDa protein, of *M. leprae* were used to analyze the expression of the TR-Trx gene product in the recombinant *M. smegmatis*. The antiserum directed against Trx is specific for *M. leprae* Trx and does not recognize *M. smegmatis* Trx (Fig. 1A), while the antiserum directed against TR does recognize *M. smegmatis* TR (Fig. 1B). Figure 1 shows that the TR-Trx hybrid protein, which is a protein of approximately 100 kDa consisting of two subunits of 49 kDa, is expressed in the recombinant *M. smegmatis* containing the gene encoding the TR-Trx hybrid protein (MS-TR-Trx). We have described previously that *M. leprae* expresses a single Trx protein besides the TR-Trx hybrid protein (25). This was also found for MS-TR-Trx, which strengthens our hypothesis that the single Trx protein is encoded from the same gene

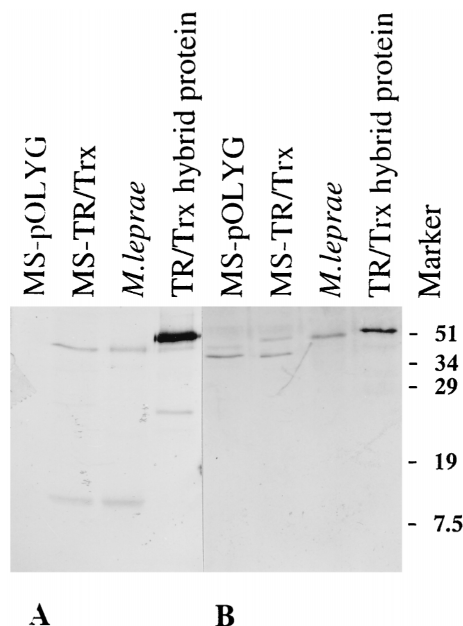

FIG. 1. Immunoblot of recombinant *M. smegmatis*. Mycobacterial lysates and purified TR-Trx hybrid protein were probed with an antiserum directed against *M. leprae* Trx (A) and *M. leprae* TR (B). Molecular masses are indicated in kilodaltons.

as the TR-Trx hybrid protein. The single Trx protein is not a cleavage product of the hybrid TR-Trx protein since no single TR product was observed in the *M. leprae* lysate (Fig. 1B, lane 3). As a positive control, we have included the purified *M. leprae* TR-Trx hybrid protein overexpressed in *E. coli*. Since this purified product contains a tag for affinity purification, it appears as a protein with a slightly higher molecular weight.

Phagocytosis of different recombinant mycobacteria by human mononuclear phagocytes. To exclude that the heterologous expression of *M. leprae* genes in *M. smegmatis* strains would influence phagocytosis, fluorescence quenching phagocytosis assays were performed. This technique is based upon the quenching of fluorochrome present on extracellular bacteria by exogenously added ethidium bromide. Counting bacteria under a fluorescence microscope before the addition of ethidium bromide reveals the number of intracellularly and extracellularly attached bacteria. The number of fluorescent bacteria remaining after ethidium bromide addition represents the number of phagocytosed bacilli. By using the fluorescence quenching phagocytosis assay, optimal phagocytosis of the bacteria was found after 15 min of incubation of mycobacteria with monocytes. The average number of bacteria per cell was approximately 2 to 4. At this time point, the total numbers of MS-pOLYG, MS-43L, and MS-TR-Trx attached to monocytes were similar (data not shown). Approximately 50% of these bacteria were found intracellularly, and no difference between the three *M. smegmatis* recombinants was found (Fig. 2).

Prolonged phagocytosis resulted in differences in the numbers of intracellular bacteria between the various *M. smegmatis* recombinant strains, which is probably due to the onset of intracellular killing. When a microbiological method to study the percentage of phagocytosis was used by which no distinction between intracellular and cell-attached bacteria can be made, no differences between MS-pOLYG, MS-43L, and MS-TR-Trx were observed either, which is in line with the fluorescence quenching assay.

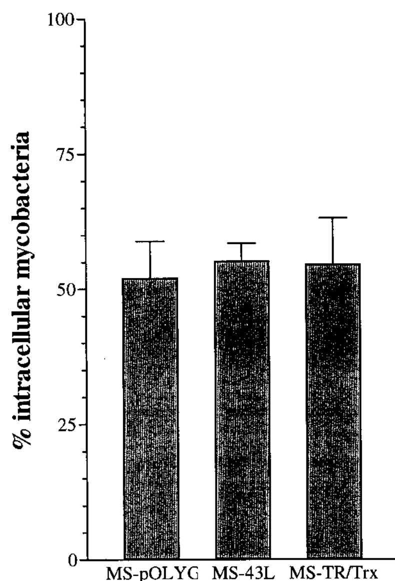

FIG. 2. Phagocytosis of different recombinant *M. smegmatis* strains. The amount of intracellular mycobacteria was determined by use of the fluorescence quenching technique. Results are expressed as means ± standard errors of the means of five experiments.

Effect of the TR-Trx gene product on the intracellular killing of recombinant *M. smegmatis* by human mononuclear phagocytes. A bacterial killing assay was performed to assess the effect of the TR-Trx hybrid gene in *M. smegmatis* on intracellular killing. MS-pOLYG, containing the vector without an insert, and MS-43L, containing a control construct, were chosen as negative controls in these experiments. Figure 3 shows a representative experiment demonstrating that MS-TR-Trx is killed to a significantly lesser extent than the controls. In three

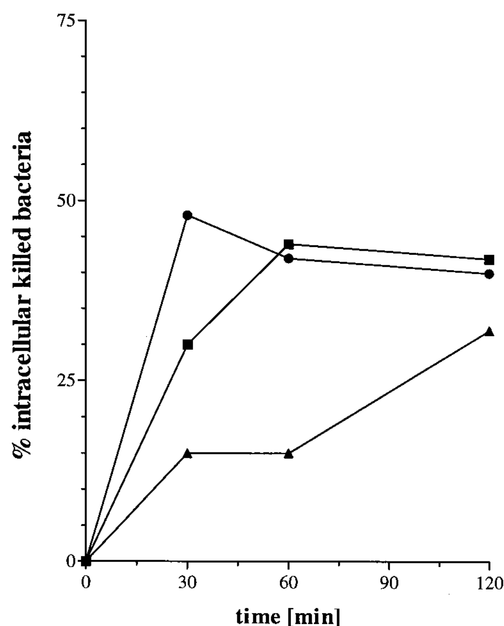

FIG. 3. Intracellular killing of different *M. smegmatis* recombinants. The effect of the TR-Trx-encoding gene (▲) on intracellular killing of *M. smegmatis* was determined, with MS-pOLYG (■) and MS-43L (●) as negative controls. Results from one representative experiment are shown.

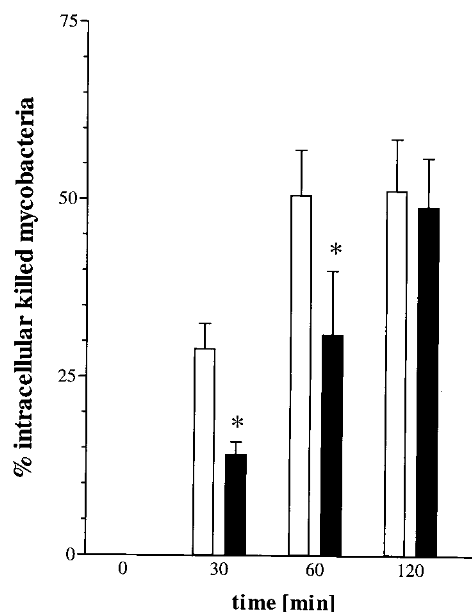

FIG. 4. Effect of the TR-Trx-encoding gene on intracellular killing of *M. smegmatis*. The intracellular killing was monitored over time and expressed as the decrease in the percentage of viable intracellular plus cell-associated bacteria, reflecting directly the increase in the percentage of killed mycobacteria. Symbols: open bars, killed MS-pOLYG; closed bars, killed MS-TR-Trx. Results are expressed as means ± standard errors of the means of 10 experiments. *, $P < 0.05$.

additional separate experiments, the killing of MS-43L was found to be similar to that of MS-pOLYG, demonstrating that the difference in intracellular killing found between MS-pOLYG and MS-TR-Trx was not due to the presence of a given foreign gene in *M. smegmatis* per se. Further experiments using only MS-pOLYG as a control were therefore performed.

Figure 4 shows the mean value of the percentage of intracellularly killed MS-pOLYG and MS-TR-Trx from 10 individual experiments. Although the ability of mononuclear phagocytes to clear intracellular bacteria differed between the various blood donors, in each experiment, MS-TR-Trx was killed to a lesser extent than MS-pOLYG. The differences in intracellular killing were significant after 30 and 60 min (P = 0.0018 and 0.026, respectively), while after 120 min, a significant difference was no longer found (P = 0.42). These in vitro experiments show that the presence of the gene encoding TR-Trx enhances the capacity of *M. smegmatis* to resist intracellular killing in the initial phase of infection.

Contribution of oxygen-dependent microbicidal mechanisms on the intracellular killing of *M. smegmatis* by mononuclear phagocytes. To assess whether the intracellular killing of *M. smegmatis* was mediated by oxygen-dependent or oxygen-independent microbicidal mechanisms, DPI was employed to inhibit the oxygen-dependent pathways. This compound inactivates NADPH oxidase and NO synthetase, thereby inhibiting the production of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) (9, 21). Addition of DPI appeared to decrease the intracellular killing of MS-pOLYG (Fig. 5). A significant decrease in the intracellular killing of MS-pOLYG incubated with DPI compared to the killing of MS-pOLYG by mononuclear phagocytes not incubated with DPI was observed after 60 and 120 min (P = 0.047 0.0047, respectively). These findings indicate that the killing of *M. smegmatis* is therefore mediated to a large extent by oxygen-dependent microbicidal mechanisms.

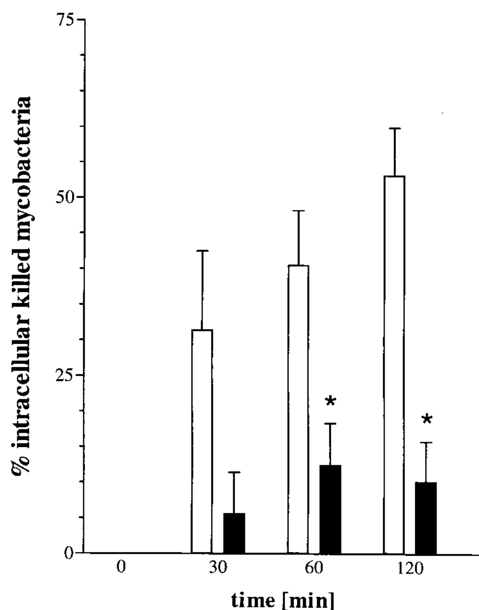

FIG. 5. Effect of DPI on the intracellular killing of *M. smegmatis*. Macrophages were incubated with (closed bars) or without (open bars) DPI. The intracellular killing was monitored over time and expressed as the percentage of killed mycobacteria. Results are expressed as means ± standard errors of the means of six experiments. *, $P < 0.05$.

Effect of MS-TR-Trx on the intracellular killing of *S. aureus* or *S. pyogenes* by mononuclear phagocytes. To obtain further and more direct evidence that the decrease in intracellular killing of MS-TR-Trx relative to that of MS-pOLYG could indeed be attributed to interference of the hybrid gene product with oxygen-dependent microbicidal mechanisms, we coinfected mononuclear phagocytes with MS-TR-Trx and *S. aureus* or *S. pyogenes*. The killing of *S. aureus* by human phagocytes has been demonstrated to occur mainly via oxygen-dependent microbial mechanisms, whereas the killing of *S. pyogenes* is largely mediated by oxygen-independent mechanisms (7).

The numbers of bacteria at time zero ($t = 0$) were similar, indicating that infection of monocytes with MS-TR-Trx does not impair phagocytosis of *S. aureus* or *S. pyogenes* relative to monocytes infected with MS-pOLYG. The results shown in Fig. 6 show that the intracellular killing of *S. aureus* coinfected with MS-TR-Trx is decreased relative to the coinfection with MS-pOLYG. The decrease in intracellular killing is significant for all three time points ($P = 0.0036$, 0.034, and 0.047 for $t =$ 15, 30, and 60 min, respectively); however, the effects are minor. In contrast, coinfection of *S. pyogenes* with MS-TR-Trx does not affect the intracellular killing of *S. pyogenes*. Although the effects on intracellular killing of *S. aureus* are minor, these data indicate that the gene encoding TR-Trx in *M. smegmatis* can also modulate the killing of bacteria that are coinfected with this recombinant. Because MS-TR-Trx affects only the intracellular killing of *S. aureus*, the gene product most likely interferes with oxygen-dependent microbicidal mechanisms.

DISCUSSION

A variety of mechanisms employed by mycobacteria to escape from the antimicrobial actions of mononuclear phagocytes have been reported (19). Possible mechanisms include inhibition of phagosome-lysosome fusion, inhibition of acidification of the phagosome, and scavenging or inhibition of bactericidal agents, such as reactive radicals. Studies regarding the ability of *M. leprae* to survive within mononuclear phagocytes have been hampered by the inability to culture *M. leprae* in vitro. As a consequence, relatively little is understood about the mechanisms that enable *M. leprae* to survive intracellularly. It has been demonstrated previously that *M. leprae* is susceptible to the actions of the cell-free myeloperoxidase-H_2O_2 system which generates large amounts of ROI (11). Our results show that the *M. leprae* TR-Trx hybrid gene product can inhibit the oxygen-dependent antimicrobial mechanisms of human mononuclear phagocytes, thus significantly limiting the killing

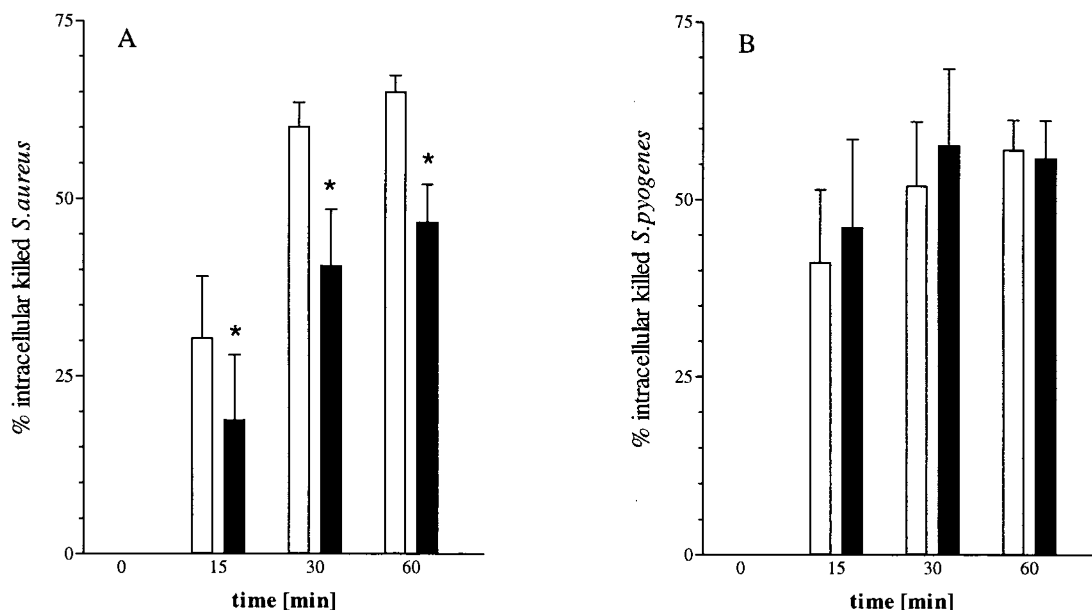

FIG. 6. Effect of MS-TR-Trx on the intracellular killing of *S. aureus* (A) and *S. pyogenes* (B). The percentage of killed *S. aureus* or killed *S. pyogenes* in the presence of MS-pOLYG (open bars) or MS-TR-Trx (bars) was monitored over time. Results are expressed as means ± standard errors of the means of six different experiments. *, $P < 0.05$.

of bacteria at least in the initial phase of infection. The TR-Trx hybrid protein is enzymatically active by itself (26), and it is feasible that this *M. leprae*-specific product acts by scavenging free radicals, thus partially protecting the bacteria against these toxic products. This is further supported by our findings that the presence of MS-TR-Trx inhibits the killing of *S. aureus*, an organism whose killing is mainly mediated by oxygen-dependent antimicrobial mechanisms, whereas the killing of *S. pyogenes*, which is mainly killed by oxygen-independent mechanisms, is not affected.

Our data suggest that the TR-Trx gene product is involved mainly in the enhanced survival of *M. leprae* in the initial phase of the infection of mononuclear phagocytes. After a certain time period, the differences in the numbers of MS-TR-Trx and MS-pOLYG are no longer significant. This might, however, be an in vitro phenomenon. Given these data, one could speculate that the TR-Trx gene product serves mainly to limit the toxic effects of ROI that are produced by monocytes during or immediately after phagocytosis. RNI are produced in a later stage of the infection, after several hours (16), which makes it unlikely that the TR-Trx gene product is involved in the limitation of the actions of RNI.

Besides TR-Trx, another *M. leprae*-specific moiety, phenolic glycolipid PGL-1, has been demonstrated to scavenge ROI (17). Moreover, *M. leprae* also contains superoxide dismutase, which can scavenge O_2^- (14). *M. leprae* thus has several potentially powerful mechanisms to withstand killing by ROI produced by human monocytes. Besides inhibiting the toxic actions of ROI, *M. leprae* might also possess several other mechanisms to resist killing by human monocytes. Recently, at least three different *M. leprae* genetic elements that might be involved in the intracellular survival of *M. leprae* have been described (18).

The Trx system is a ubiquitous redox-active system present in all organisms studied to date. The unique feature of the *M. leprae* redox system is that TR and Trx are expressed as a single hybrid protein. We have shown previously that in *M. leprae* lysates, the single Trx subunit is present in addition to the TR-Trx hybrid protein. This single Trx is most likely expressed from the same messenger as the hybrid protein, because there is only one copy of the gene present on the *M. leprae* genome (25). This Trx is not a cleavage product of the hybrid protein since the single TR subunit is not present in *M. leprae* lysates. Thus, it is quite possible that the effects on intracellular killing observed with MS-TR-Trx are mainly the result of overexpressing the Trx subunit of TR-Trx, either attached to its enzyme TR or separately translated as a free protein.

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