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Phagocytosis of *Staphylococcus aureus* Induces a Selective Stress Response in Human Monocytes-Macrophages (Mϕ): Modulation by Mϕ Differentiation and by Iron

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Phagocytosis of microorganisms represents a stress not only for the phagocytosed agent but also for the host cell. We have investigated the stress response induced in human monocytes-macrophages (Mϕ) phagocytosing inactivated *Staphylococcus aureus*. Exposure of human Mϕ to *S. aureus* induced in these cells (i) a threefold increase in superoxide dismutase activity, (ii) a selective and differentiation-dependent induction of host heat shock protein synthesis (HSP70 but not HSP65), and (iii) de novo synthesis of heme oxygenase, but only when exogenous iron was added to the cultures. The coordinate upregulation of two scavenging enzymes and of HSP70 suggests that all three are part of cellular protective mechanisms against phagocytosis-related oxidative injury to host cells.

The synthesis of stress or heat shock proteins (HSPs) increases in all cells and organisms, from bacteria to humans, under stressful conditions (27). Heat shock proteins are classified into families according to their size: 100 to 110 kDa; 83 to 90 kDa, associated with steroid receptors; the 70-kDa family (66 to 78 kDa); the 60-kDa family; and the smaller HSPs (15 to 30 kDa), each of them having specific subcellular localizations and functions (27). A wide variety of stresses other than heat induce the so-called stress response, ranging from phases of the cell cycle, differentiation, and growth to inhibitors of energy metabolism and heavy metals (27). Increased expression of HSPs has also been observed in different pathological states associated with an increased oxidative burden, such as inflammation (reviewed in reference 36) and ischemia (28). Abnormal and degraded proteins are among the intracellular signals known to trigger the stress response (1, 31). The enzymes superoxide dismutase (SOD) and heme oxygenase are also part of the stress protein family: both SOD (38) and heme oxygenase (40) are induced by heat in certain species (bacteria and rat cells, respectively), and SOD is induced by tumor necrosis factor alpha (TNF-α) (46).

The known physiological functions of some stress proteins include their ability to bind other proteins, acting as carriers across membranes (molecular chaperones, reviewed in reference 23). Members of the HSP70 family have been found to be associated with resistance to further injury, indicating that they are involved in cellular protective mechanisms (24, 39). Exposure of cells of monocytic lineage to heat shock and induction of HSP70 protect these cells from subsequent oxidative injury by a calcium-independent mechanism (33); inhibition of HSP70 synthesis or activity during stress prevents the development of cellular tolerance to such stress (17, 39). HSPs have also been suggested to represent a nonimmunological mechanism for self-nonself discrimination (12), which may be of particular relevance during phagocytosis of pathogens.

On the other hand, immunodominant antigens from a wide variety of infectious agents (mycobacteria, other bacteria, and parasites) are HSPs (47). HSP65 in particular represents a major antigen in mycobacterial and other infections. Because stress proteins are also host cell constituents and because their structure is so highly conserved across species, molecular mimicry (30) between pathogen and host HSPs could lead to autoimmunity (21, 22). Central to the possibility that HSPs play a role in autoimmunity is the question whether host HSPs are expressed during bacterial infection. Mistry et al. recently reported that uptake of live or killed *Mycobacterium leprae* induces HSP70 synthesis in Schwann cells (26).

We investigated the effects of phagocytosis of another type of bacteria, *Staphylococcus aureus*, which are not associated with autoimmunity and usually induce acute and reversible diseases, on the stress response of human monocytes-macrophages (Mϕ). Our previous investigations on the stress response in Mϕ indicate that these cells synthesize stress proteins after exposure to exogenous H_2O_2 and during endogenous production of reactive oxygen metabolites secondary to the activation of the respiratory burst enzyme NADPH oxidase, associated with erythrophagocytosis (5). Besides the classical HSPs, phagocytosis of whole erythrocytes but not of red cell ghosts also induces a 32-kDa oxidation-specific protein, heme oxygenase, the induction of which is linked to the iron-catalyzed formation of hydroxyl radicals (5, 18). Here we report that (i) the interactions between Mϕ and *S. aureus* induced in host cells the synthesis of HSP70 but not of HSP65 and synthesis of the enzymes SOD and heme oxygenase; (ii) the level of induction of HSP70 was related to the differentiation state of the cells; and (iii) this response was modulated by iron. The coordinate upregulation of HSP70, SOD, and heme oxygenase suggests that the stress response may have autoprotective functions in human phagocytes.

MATERIALS AND METHODS

Cells and media. Human peripheral blood monocytes were isolated by gradient centrifugation and purified by adherence as described previously (5). Alveolar macrophages (AM) were obtained by ex vivo lavage of excised human lungs as

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described before (9). All cells were cultured in RPMI 1640 (GIBCO, Paisley, Scotland) supplemented with 10% fetal calf serum (GIBCO) and 1% glutamine (GIBCO). One percent penicillin-streptomycin was added to AM cultures. For labeling experiments, cells were cultured in RPMI without methionine (GIBCO) throughout the experiments. All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

Reagents. 1,25-Dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] was kindly provided by U. Fisher (Hoffmann-La Roche, Basel, Switzerland). Electrophoresis-purity reagents were from Bio-Rad Laboratories (Richmond, Calif.). Phorbol myristate acetate (PMA) was from Sigma (St. Louis, Mo.). All other reagents were from Merck (Darmstadt, Germany). Radiolabeled nucleotides [α-^{32}P]dCTP (≈3,000 Ci/mmol) and [α-^{32}P]UTP (≈800 Ci/mmol) were from Amersham (Buckinghamshire, England).

Bacteria. *S. aureus* Wood USA 46 (kindly provided by P. D. Lew, Division of Infectious Diseases, Geneva) was grown overnight in 10 ml of Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) and washed three times with phosphate-buffered saline (PBS; GIBCO) by centrifugation at 1,860 × *g* for 10 min. Bacteria were resuspended in PBS and inactivated either with 0.05% Formalin for 30 min at room temperature or, for the SOD experiments, by heating at 85°C for 60 min in the presence of 7% NaCl. It has been reported previously that heating bacteria under these conditions inhibits bacterial SOD (3). The bacteria were washed four times with PBS by centrifugation (1,860 × *g* for 10 min each), adjusted to 3×10^8 bacteria per ml in PBS, and kept at −20°C until use. Prior to the phagocytosis assay, bacteria were opsonized with 10% pooled human serum for 30 min at 37°C under agitation and then washed once. Bacteria were added to the cells at a ratio of 200:1, after initial experiments showed maximal respiratory burst activation at this ratio (see Results and Fig. 1). The phagocytosis assay was done for 3 h in all experiments.

Exposure to heat. Monocytes or AM in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered medium were exposed for 20 min to 44 or 45°C, respectively, in a water bath and allowed to recover for 2 h at 37°C, as described previously (5).

Superoxide determination. O_2^- production was measured by the SOD-inhibitable reduction of ferricytochrome *c*, as described before (16), after 30 min of stimulation with PMA (100 ng/ml) or various amounts of inactivated *S. aureus*.

SOD activity. (i) Preparation of extracts. Monocytes were allowed to phagocytose *S. aureus* for 3 h. The cells were then scraped from culture dishes and washed three times with PBS by centrifugation (300 × *g* for 10 min each). Pellets were resuspended at 10^6 cells per 100 μl in 50 mM sodium phosphate buffer (pH 7.8) containing 0.1 mM EDTA. The cell suspension was sonicated twice for 30 s each in a Bronson Sonifier. The sonicates were centrifuged at 9,390 × *g* for 10 min.

(ii) Enzyme assay. SOD activity was measured in crude extracts with xanthine-xanthine oxidase, an O_2^--generating system, by the method described by Crapo et al. (8). Briefly, the reaction mixture contained, in a 1-ml final volume, phosphate-EDTA buffer, 10^{-5} M cytochrome *c* (Sigma), and 50 μM xanthine (Sigma). The system was initiated by addition of an appropriate amount of xanthine oxidase (Sigma) to obtain an A_{550} of 0.025. The cell extract was standardized to 5 μg of protein, and bovine SOD (Sigma) was used as a standard. One unit of SOD activity was defined as the amount of enzyme which inhibited cytochrome *c* reduction by 50%. Total protein content was determined by the method of Bradford (2) with bovine serum albumin as the standard.

Protein synthesis analysis. After exposure to *S. aureus* or to heat, cells were labeled with 9 μCi of L-[^{35}S]methionine (specific activity, >1,000 Ci/mmol; Amersham Laboratories) per ml for 90 min at 37°C, recovered, and washed twice with PBS. The cells were then lysed in sodium dodecyl sulfate (SDS) buffer by the method of Laemmli (20). Proteins from aliquots corresponding to equal cell numbers were analyzed by one- or two-dimensional gel electrophoresis as described by Laemmli and O'Farrell (29), respectively. Bacteria were inactivated in order to distinguish between host and pathogen proteins and to prevent monocyte death. Indeed, preliminary experiments with live bacteria showed that synthesis of proteins by monocytes disappeared at a bacterium/monocyte ratio of 40:1 and up and was replaced by the exclusive synthesis of bacterial proteins.

Immunoblotting analysis. Proteins were electrotransferred to nitrocellulose membranes (41). The membranes were saturated with casein-containing buffer for 2 h and then hybridized with appropriate antibodies: monoclonal anti-HSP70, specific for the inducible form of the protein (StressGen, San Francisco, Calif.) (45); N27, a monoclonal antibody against constitutive HSP72 (a gift from W. J. Welch, San Francisco); or anti-mycobacterial HSP65 (ML-30; a kind gift from Y. Ivanyi, Medical Research Council Unit for Tuberculosis and Related Infections, Hammersmith Hospital, London), which cross-reacts with human HSP65 (10). HSP70 and HSP65 were revealed with AuroProbe BL plus goat anti-mouse immunoglobulin (Janssen Pharmaceutica, Brussels, Belgium) or with AuroProbe BL plus immunogold reagent and intense BL silver enhancement system (Janssen Pharmaceutica).

RNA extraction and Northern (RNA blot) analysis. Total RNA was isolated from 10^7 monocytes by acid guanidium thiocyanate-phenol-chloroform extraction as described by Chomczyski and Sacchi (4). For Northern blots, 5 μg of total RNA was denatured and fractionated by electrophoresis on a 1% agarose–5% formaldehyde gel in MOPS buffer [20 mM 3-(morpholino)-propanesulfonic acid (pH 7), 5 mM sodium acetate, 0.1 mM EDTA] by the method of Maniatis et al. (25). Following electrophoresis, RNA was transferred onto a Biodyne membrane in 20× SSC (1× SSC is 0.15 M NaCl, 0.015 M trisodium citrate [pH 7]), and the membranes were baked for 2 h at 80°C. For analysis of heme oxygenase mRNA, the membranes were prehybridized for 6 h in 5× SSC–10 mM $NaPO_4$–50% formamide–5× Denhardt's solution (1× Denhardt's solution is 0.02% polyvinylpyrrolidone, 0.02% Ficoll, and 0.02% bovine serum albumin)–0.1% (SDS)–250 μg of salmon sperm DNA per ml and hybridized for 16 h in the same buffer at 42°C to a [^{32}P]dCTP-labeled DNA probe (1×10^6 to 2×10^6 cpm/ml). This probe was prepared by random-primed synthesis of a 500-bp restriction fragment of the cDNA of the human heme oxygenase gene, a kind gift from R. M. Tyrrell (19), with the Multiprime labeling kit from Amersham. After hybridization, the filters were washed at 45°C once with 2× SSC, once with 1× SSC, and twice with 0.5× SSC containing 0.1% SDS. They were then subjected to autoradiography on Hyperfilm (Amersham) at −70°C with intensifying screens.

RESULTS

***S. aureus*-stimulated superoxide production in human monocytes.** We first established under what conditions *S.*

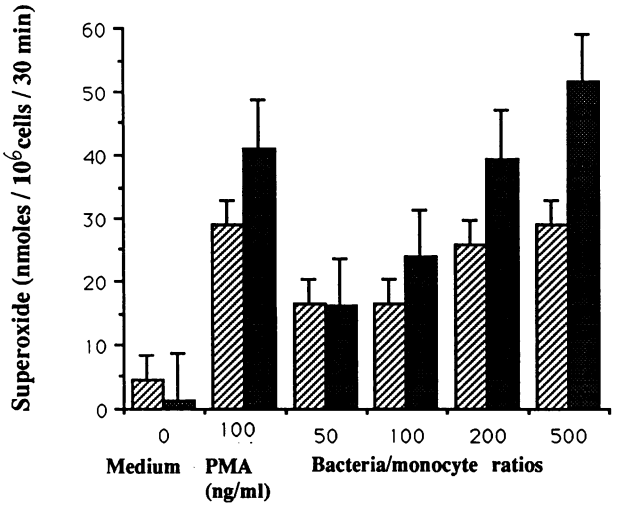

FIG. 1. Superoxide production by peripheral blood monocytes activated by PMA or *S. aureus*. Human peripheral blood monocytes from healthy donors were isolated, purified, and cultured as described under Materials and Methods. Monocytes were incubated for 48 h with (shaded bars) or without (hatched bars) 1,25-$(OH)_2D_3$ (10 ng/ml). O_2^- release was measured after 30 min of stimulation of 10^6 cells with either PMA (100 ng/ml) or various amounts of Formalin-inactivated bacteria. Values shown are means ± standard error of the mean (n = 3).

aureus would induce maximal production of O_2^- by human monocytes. Cells were incubated for 48 h with and without the steroid hormone 1,25-$(OH)_2D_3$ (10 ng/ml), which induces monocyte differentiation (42) and increases the production of reactive oxygen species and the synthesis of HSPs by these cells (6, 34). Monocytes (10^6 cells per ml) were then stimulated with increasing amounts (from 50 to 500 bacteria per monocyte) of Formalin-inactivated and opsonized *S. aureus* for 30 min, and O_2^- release was measured, with PMA as a positive control. O_2^- was generated in a dose-dependent manner that depended on the number of bacteria (Fig. 1). At the ratio of 200 bacteria per monocyte, O_2^- production was identical to PMA-stimulated O_2^- production; this ratio was therefore used in subsequent experiments. As expected, previous differentiation of the monocytes by preincubation with 1,25-$(OH)_2D_3$ (10 ng/m) for 48 h (Fig. 1) significantly increased O_2^- production after either PMA or phagocytic stimulation.

SOD activity in phagocytic cells. SOD activity in monocytes from four separate experiments (four distinct donors) is shown in Fig. 2. Monocytes were maintained in culture for 48 h, and enzyme activity was determined in control cells or after exposure to heat-inactivated *S. aureus* (200 bacteria per monocyte) for 3 h. Bacteria were heated for 60 min at 85°C in the presence of 7% NaCl to inactivate the bacterial enzyme before SOD determination (3). Under these conditions, there was no detectable bacterial SOD activity (not shown). Bacterial phagocytosis induced a two- to fourfold increase in SOD activity (Fig. 2). In contrast, whereas heat shock induces SOD in bacteria (38), it had little effect on SOD activity in human Mϕ (39a).

Stress response during phagocytosis of *S. aureus*: modulation by Mϕ differentiation. Protein synthesis in monocytes was analyzed by one- and two-dimensional gel electrophoresis. HSP70 was induced (Fig. 3, panel 2, arrow) in phagocytic monocytes but not in the control cells (Fig. 3, panel 1),

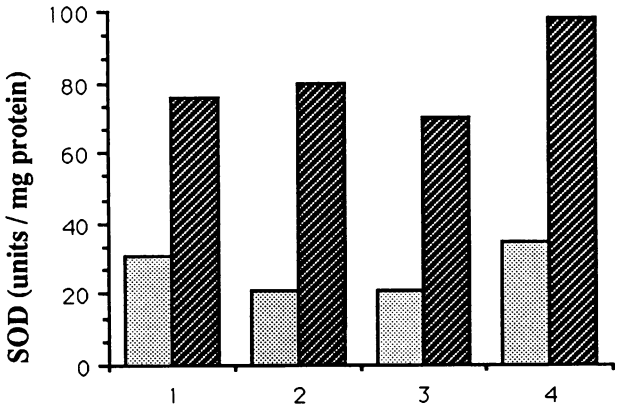

FIG. 2. Effect of phagocytosis of *S. aureus* on SOD activity in peripheral blood monocytes. SOD activity was measured in control cells (shaded bars) or after 3 h of phagocytosis of heat-inactivated bacteria (hatched bars). Results are from four separate experiments (n = 2 for each experiment).

and was detected in undifferentiated monocytes only by two-dimensional gel electrophoresis. This induction was increased when cells were preincubated with 1,25-$(OH)_2D_3$ (Fig. 3, panel 4) and even more so when bacteria were phagocytosed by fully differentiated tissue macrophages, i.e., AM (Fig. 3, panel 3), although in this case, total cellular metabolic activity also appeared to have increased. The effects of differentiation on HSP expression were further assessed by Western blotting. The constitutive and heat-inducible expression of HSC70 and HSP70 was analyzed in

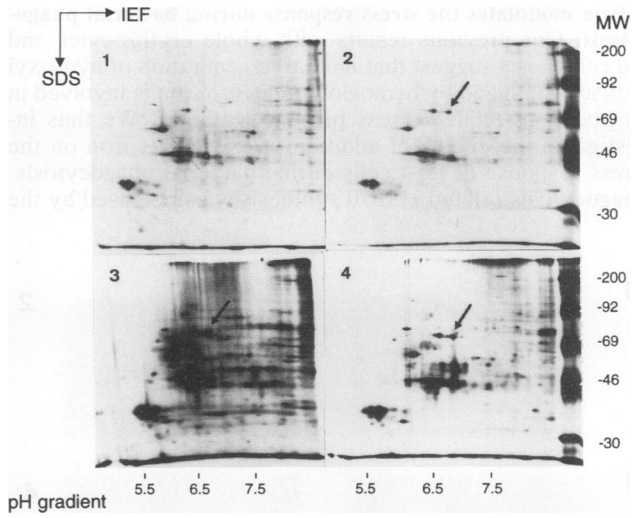

FIG. 3. Two-dimensional gel electrophoresis of monocytes and AM after phagocytosis of *S. aureus*. Peripheral blood monocytes and AM were cultured for 48 and 24 h, respectively, in RPMI medium without methionine and with (panel 4) or without (panels 1, 2, and 3) 1,25-$(OH)_2D_3$ (10 ng/ml). Phagocytosis of heat-inactivated *S. aureus* was for 3 h (panels 2, 3, and 4). The cells were then metabolically labeled with 9 μCi of [^{35}S]methionine per ml for 90 min. Samples corresponding to equal cell numbers were analyzed by two-dimensional gel electrophoresis. Panel 1, unstressed monocytes; panel 2, monocytes after phagocytosis of *S. aureus*; panel 3, AM after phagocytosis of *S. aureus*; panel 4, 1,25-$(OH)_2D_3$-treated monocytes after phagocytosis of *S. aureus*. Arrows indicate induced HSP70. Sizes are shown in kilodaltons. IEF, isoelectric focusing.

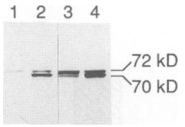

FIG. 4. Detection of constitutive HSP70 in monocytes and AM. The antibody N27 recognizes both the constitutive and inducible forms of HSP70. Monocytes (lanes 1 and 2) or AM (lanes 3 and 4) were maintained under control conditions (37°C) or exposed either to 44°C (lane 2) or 45°C (lane 4). Higher expression of both forms of the protein was found in AM (lanes 3 and 4 versus lanes 1 and 2).

monocytes and AM with monoclonal antibody N27, which recognizes both forms (Fig. 4). The levels of both the constitutive and inducible forms of the protein were higher in AM than in monocytes under basal conditions as well as after heat shock.

We also compared the effects of bacterial phagocytosis of *S. aureus* with those of exposure to heat shock (45°C, 20 min, 2 h recovery) and to the heavy metal cadmium ($CdSO_4$; 25 μM for 3 h) by Western blotting after two-dimensional gel electrophoresis with the antibody against the inducible form of HSP70 (SPA801, which is specific for the inducible form) (Fig. 5). In AM, phagocytosis of *S. aureus* (Fig. 5, panel 4) and heat shock (Fig. 5, panel 3) or cadmium treatment (Fig. 5, panel 2) led to similar expression of multiple isoforms of the inducible HSP70.

In contrast to HSP70, we did not detect the induction of HSP65 synthesis during bacterial phagocytosis even under conditions of maximal HSP70 induction by any of the methods used, i.e., one- or two-dimensional gel electrophoresis as well as Western blotting with monoclonal antibody ML-30 (not shown).

Iron modulates the stress response during bacterial phagocytosis. Our previous results with whole erythrocytes and red cell ghosts suggest that increased generation of hydroxyl radicals catalyzed by hemoglobin-derived iron is involved in phagocytosis-related stress protein synthesis. We thus investigated the effects of addition of exogenous iron on the stress response of host cells during bacterial phagocytosis. Phagocytosis-related HSP70 synthesis was increased by the addition of iron (500 μM $FeSO_4$) to the cultures, with a dose-dependent response to bacteria (Fig. 6, lanes 5 and 7). Phagocytosis in the presence of iron induced another protein of approximately 15 to 16 kDa (Fig. 6, lanes 5 and 7); whether this protein is a small heat shock protein or a cytokine remains to be clarified. Iron alone failed to induce a stress response (Fig. 6, lane 2). Inactivated bacteria did not incorporate methionine (Fig. 6, lane 8), indicating that the new proteins observed during phagocytosis were indeed produced by Mϕ.

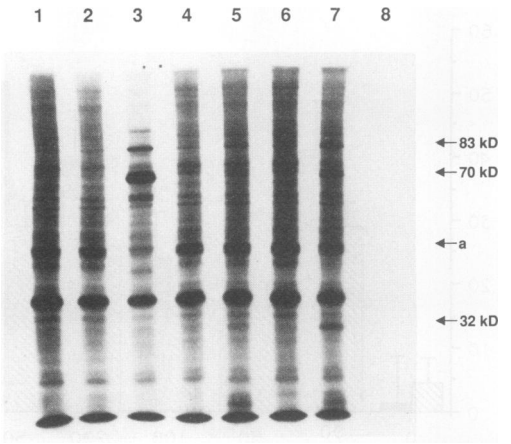

FIG. 6. Effect of iron on *S. aureus*-induced stress response in human monocytes. Monocytes were exposed to 500 μM $FeSO_4$ (lane 2), to 44°C for 20 min and then 2 h of recovery at 37°C (lane 3), or to *S. aureus* for 3 h in the presence (lanes 5 and 7) or absence (lanes 4 and 6) of 500 μM $FeSO_4$. The ratio of bacteria to monocytes was varied from 25:1 (lanes 4 and 5) to 100:1 (lanes 6 and 7). Unstressed cells are shown in lane 1, and bacteria alone are shown in lane 8. a, actin. [^{35}S]methionine-labeled proteins were resolved by SDS-PAGE on a 10% acrylamide gel.

The samples from lanes 1, 3, 6, and 7 in Fig. 6 were further separated by two-dimensional gel electrophoresis, and the results are shown in Fig. 7. HSP70 induction was higher during phagocytosis of *S. aureus* in the presence of iron (Fig. 7, panel 4) than in its absence (Fig. 7, panel 3), but lower than in cells exposed to 44°C for 20 min (Fig. 7, panel 2). In unstressed cells, only the constitutive form of HSP70 was

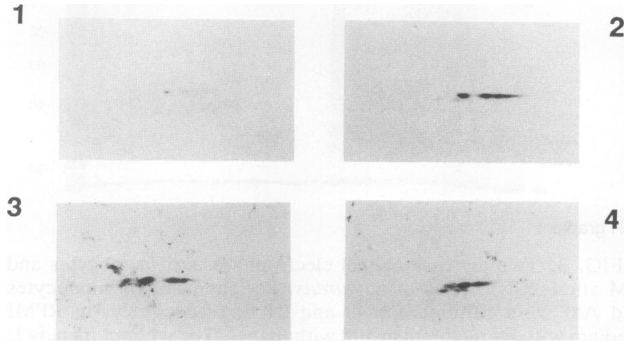

FIG. 5. HSP70 induction in AM. AM were exposed to the HSP inducer $CdSO_4$ (25 μM) (panel 2), to 45°C for 20 min (panel 3), or to *S. aureus* for 3 h (panel 4) before metabolic labeling with [^{35}S]methionine (9 μCi). Panel 1 is an untreated AM control. The pattern of protein synthesis was analyzed by two-dimensional gel electrophoresis, and HSP70 was characterized by immunoblotting with an anti-HSP70 monoclonal antibody. Blots were cut around the dots, revealing the multiple isoforms of HSP70 (70 kDa; isoelectric point between pI 6.5 and 7.5).

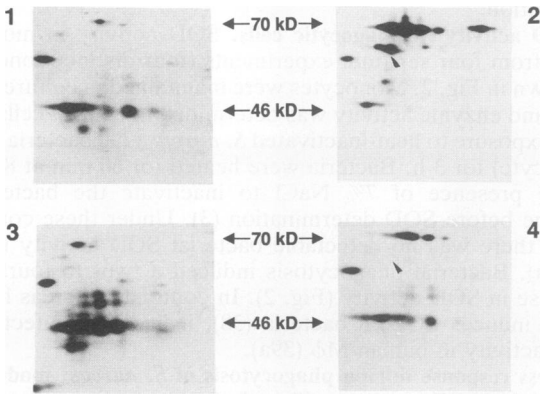

FIG. 7. Two-dimensional gel electrophoresis of induced proteins. Samples from lanes 1 (panel 1), 3 (panel 2), 6 (panel 3), and 7 (panel 4) of Fig. 5 were further analyzed by two-dimensional gel electrophoresis. Arrowheads indicate HSP65.

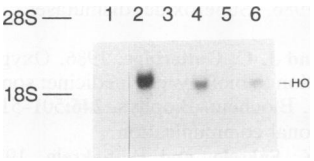

FIG. 8. Northern blot analysis of heme oxygenase expression. Monocytes were maintained in standard conditions (lane 1) or exposed to 20 μM $CdSO_4$ (lane 2) or to heat-inactivated *S. aureus* without (lane 3) or with (lane 4) 500 μM $FeSO_4$ for 90 min. Lanes 5 and 6, Formalin-inactivated *S. aureus*. The filters were exposed to Hyperfilm for 40 h. Phagocytosis of *S. aureus* alone did not induce heme oxygenase (HO) mRNA (lanes 3 and 5), but both heat- and formalin-inactivated bacteria did so in the presence of iron (lanes 4 and 6).

observed (Fig. 7, panel 1). Even when iron was present, and as mentioned above, we observed no induction of HSP65 during phagocytosis of *S. aureus* (Fig. 7, panel 4, arrowhead, versus HSP65 induced by heat shock, arrowhead on panel 2), indicating a differential regulation of HSP70 and HSP65.

Effect of exogenous iron on heme oxygenase gene activation during phagocytosis of *S. aureus*. In addition to HSP70, exogenous iron added during phagocytosis also induced a 32-kDa protein (Fig. 6, lane 7), which was identified as heme oxygenase by comparison, on one- and two-dimensional gels (data not shown), with the 32-kDa protein induced during erythrophagocytosis (5) and by Northern blotting. For these experiments, human monocytes were allowed to phagocytose heat- or Formalin-inactivated *S. aureus* (200 bacteria per monocyte) with or without 500 μM $FeSO_4$. $CdSO_4$ (20 μM), a well-known inducer of heme oxygenase, was used as a positive control. The cells were exposed to the different agents for 90 min, and RNAs were extracted and hybridized as described under Materials and Methods.

Figure 8 shows a representative example of the resulting Northern blot. Heme oxygenase mRNA was detected during phagocytosis of *S. aureus* only when iron was supplied (Fig. 8, lanes 4 versus 3 and 6 versus 5) whether bacteria were heat (5) or formalin (6) inactivated. Unstressed monocytes are shown in lane 1 and Cd-induced heme oxygenase mRNA is shown in lane 2 of Fig. 8. Iron alone did not induce heme oxygenase mRNA (not shown).

DISCUSSION

Here we report that phagocytosis of *S. aureus* by human Mϕ induced in these cells the coordinate upregulation of HSP70, heme oxygenase, and SOD. The induction of these three members of the stress protein families was, however, differentially regulated.

First, phagocytosis of *S. aureus* induced a threefold increase in SOD activity, one of the members of the stress protein families and a classical radical scavenger and protective enzyme (13). Experiments performed with 1,25-$(OH)_2D_3$-differentiated U937 cells indicate that phagocytosis of *S. aureus* selectively increases the manganous (and not the copper-zinc) isoform of SOD (18a). The increase in SOD appears to be dependent, at least in part, on the production of TNF-α (46) during bacterial phagocytosis, since it was partially prevented by anti-TNF-α antibodies (our unpublished data). TNF-α may also participate in the induction of HSP70 (11).

Second, phagocytosis of *S. aureus* induced HSP70 but not HSP65. This selective upregulation of HSP70 is in agreement with the recently reported induction of HSP70 (and not HSP65) during uptake of *M. leprae* by Schwann cells (26) and indicates differential regulation of HSP synthesis. For both these pathogens, HSP70 induction was independent of the metabolic activity of the bacteria. The timing of induction was, however, different (3 h of phagocytosis for *S. aureus*, 24 h for *M. leprae*). These results suggest that oxidative metabolism (which is induced rapidly during phagocytosis of *S. aureus*) is involved in HSP70 induction during bacterial phagocytosis. Differential expression of HSPs has been reported during other situations of acute O_2^- generation and oxidative stress, such as ischemia and reperfusion injury (14a). During reperfusion injury, HSP70 upregulation has been suggested to represent an autoprotective mechanism (7, 28). Whereas HSP65 has been central to the proposed role of HSPs in autoimmunity (21, 43), the possibility that HSP70 plays a role similar to that of HSP65 in induction of autoimmunity has yet to be established (26). Indeed, HSP65-specific T cells are usually directed against conserved determinants (22), whereas HSP70-specific T cells or antibodies are raised against nonconserved epitopes (35). We therefore favor in this case a protective rather than an deleterious role for HSP induction during bacterial phagocytosis.

In our study, HSP70 induction was dependent upon Mϕ differentiation and modulated by the presence of iron. 1,25-$(OH)_2D_3$ induces monocyte differentiation, increases the synthesis of HSPs in U937 cells, and protects these cells from thermal injury (34). The hormone also induces (in U937 [37]) or increases (in monocytes [6]) the generation of reactive oxygen species to various stimuli. This was the case in this study as well, since preincubation with 1,25-$(OH)_2D_3$ increased O_2^- production by monocytes exposed to *S. aureus*. We have suggested previously (33) that in cells treated with 1,25-$(OH)_2D_3$, the increased oxidative burden leads to the upregulation of protective mechanisms, including HSPs. The increased expression of stress proteins in AM may relate both to the state of differentiation of these tissue Mϕ and to their increased exposure to oxidative burden even under basal conditions.

Iron plays a key role in oxidative injury because it catalyzes the formation, from superoxide anions, of the highly reactive hydroxyl radical (Fenton reaction) (14). Our previous experiments in the erythrophagocytosis model indicated that the iron-driven generation of hydroxyl radicals is important in stress protein induction during phagocytosis, and our current observations further support this possibility. Although HSP70 was detectable in the absence of iron, its expression was increased in the presence of the metal, whereas heme oxygenase induction was strictly dependent upon the presence of iron during phagocytosis of *S. aureus*. These results are in agreement with our former observations on the differential downregulation of HSP70 and heme oxygenase by the flavonoids quercetin and kaempferol, both of which are radical scavengers and iron chelators (18). These studies indicated that whereas oxygen-reactive species partially regulate HSP70 expression, they are essential for heme oxygenase induction. One more argument in favor of a role for hydroxyl radicals (and the phagocytosed material) in stress protein induction is the observation that O_2^- production is insufficient by itself for stress protein induction: PMA, at concentrations leading to a similar activation of NADPH oxidase as caused by *S. aureus* phagocytosis (Fig. 1), does not induce significant stress protein synthesis in Mϕ (36). Phagocytosis of opsonized zymosan, another

classical activator of the respiratory burst in Mϕ, also leads to a different pattern (31a, 36).

Phagocytosis represents a considerable stress not only for the pathogenic microorganism but also for the phagocyte, which produces, during phagocytosis, toxic oxygen metabolites, proteases, lipid mediators of inflammation, and cytokines, all products to which it is itself exposed. Neutrophils are often lysed during this process; along these lines, we found no induction of stress proteins in neutrophils during identical conditions of phagocytosis of *S. aureus* (unpublished). In contrast, the longer-lived Mϕ survive. Since HSPs, particularly HSP70, exert protective effects against oxidative injury (33), against TNF-α (15), and against cell injury in general (44), we propose that HSP70, heme oxygenase, and SOD are coinvolved in protection of Mϕ against the toxic factors they produce during phagocytosis of defined microorganisms.

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