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Induction of a 23 kDa stress protein by oxidative and sulfhydryl-reactive agents in mouse peritoneal macrophages

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The synthesis of 23 kDa protein was enhanced when mouse peritoneal macrophages were exposed to oxidative agents such as hydrogen peroxide and menadione, or to sulfhydryl-reactive agents such as diethylmaleate, cadmium chloride and sodium arsenite. After 11 h exposure to these agents the 23 kDa protein was one of the actively synthesized proteins in the macrophages. Under similar conditions the 34 kDa protein previously identified as heme oxygenase, was induced and its synthesis preceded that of the 23 kDa protein. Neither the 23 kDa or the 34 kDa protein was induced by hyperthermia. Conversely, the various oxidative and sulfhydryl-reactive agents employed here did not induce the major heat shock proteins in the macrophages. When the macrophages were activated by bacterial lipopolysaccharide or other stimulants, many proteins are known to be induced, however, the 23 kDa and 34 kDa proteins were not induced. The 34 kDa protein, i.e., heme oxygenase, has been found to be stress-induced in various types of cell, but not the 23 kDa protein. This suggests that the 23 kDa protein is a stress protein predominantly expressed in macrophages.

Introduction

In eucaryotic cells exposure to various chemical and physical insults induces or enhances the synthesis of a number of proteins called stress or heat-shock proteins which have molecular masses ranging from 22 to 110 kDa [1,2]. Although it is suggested that these proteins play an important role in protecting cells against environmental insults, their precise function remains obscure. Recent studies on some heat-shock proteins, however, have shown that they take part in novel functions such as molecular chaperoning and translocation of proteins across subcellular membranes. These proteins modulate and promote protein folding, assembly and disassembly, and facilitate the degradation of malfolded polypeptides [3].

Recently it has been reported that in mammalian cells 32- or 34 kDa stress protein is induced by sodium arsenite, heavy metals, sulfhydryl-reactive agents [4], UVA irradiation and hydrogen peroxide [5]. This stress protein is identified as heme oxygenase [6]. We have also demonstrated that in mouse peritoneal macrophages both heme oxygenase activity and the 34 kDa stress protein are strongly induced upon exposure to sulfhydryl-reactive agents and have concluded that the macrophage 34 kDa stress protein and heme oxygenase are one and the same [7]. During the course of this study we have found another stress protein with a molecular mass of 23 kDa. In this paper details of the induction of this 23 kDa stress protein are described.

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Abbreviations: BCG, bacillus Culmette-Guerin; SDS, sodium dodecyl sulfate; LPS, lipopolysaccharide.

Materials and Methods

Preparation of macrophages

Macrophages were collected by peritoneal lavage from female C57BL/6N mice weighing 20–25 g, that had been given an intraperitoneal injection of 2 ml of 4% thioglycollate broth 4 days previously. In some experiments macrophages were collected from mice injected intraperitoneally 3–5 weeks previously with 2 mg/ml BCG[1] vaccine (Nippon BCG Seizo, Tokyo), and 4 days before harvest with 2 ml of 4% thioglycollate broth. The lavage medium was RPMI 1640 containing 10 unit/ml heparin. The cells were separated by centrifugation, washed once with RPMI 1640 medium, plated at 1×10^6cells/35-mm diameter culture dish in RPMI 1640 containing 10% fetal bovine serum, 50 unit/ml penicillin and 50 μg/ml strepto-

mycin, and were incubated at 37°C in 5% CO_2 in air. After 1 h the medium was replaced with the fresh medium in order to remove the nonadherent cells.

Stress condition and metabolic labeling

Macrophages prepared as described above were exposed to various injurious agents for a given time. Exposure of macrophages to hydrogen peroxide was carried out by incubating the cells with glucose oxidase which generates hydrogen peroxide by reaction with glucose in the culture medium. In heat-shock treatment, the macrophages were incubated for 30 min at 43°C and then incubated at normal temperature (37°C).

After exposure to various stresses, the cell proteins were radiolabeled by incubating macrophages for 30 min in 1 ml of methionine- and cysteine-free RPMI 1640 containing 10% dialyzed fetal bovine serum and about 50 μCi ^{35}S-protein labeling mixture (EXPRE^{35}S^{35}S Protein Mix from Du Pont-New England Nuclear, specific activity > 1000 Ci/mmol, containing > 77% L-[^{35}S]methionine and 18% L-[^{35}S]cysteine). The radioactive medium was then removed, and the cells were rinsed three times with phosphate-buffered saline and lysed with 0.2 ml of SDS sample buffer (2% SDS, 10% glycerol, 3% 2-mercaptoethanol, 50 mM Tris-HCl (pH 6.8) and 0.1 mM phenylmethylsulfonyl fluoride).

Gel electrophoresis and fluorography

For the analysis of cellular proteins, the cell lysate was heated to 100°C for 3 min and the samples containing equal amounts of radioactivity were loaded on 12.5% SDS-polyacrylamide gel. After the electrophoresis run, the gels were fixed, stained with Coomassie brilliant blue G-250, impregnated with EN^{3}HANCE (Du Pont-New England Nuclear) and dried. Fluorography was carried out using Kodak XAR-5 X-ray film.

Two-dimensional electrophoresis was performed using the technique of O'Farrell [8] with a slight modification. 5 μl of the cell lysate were mixed with 40 μl of urea solution (9.2 M urea, 2% NP-40, 5% ampholine and 5% 2-mercaptoethanol) and the mixture was loaded on the isoelectric focusing gel containing pH 3.5–10 ampholine. Focusing was accomplished for a total of 10000 Vh using 0.2 M NaOH as the cathode solution and 0.01 M phosphoric acid as the anode solution.

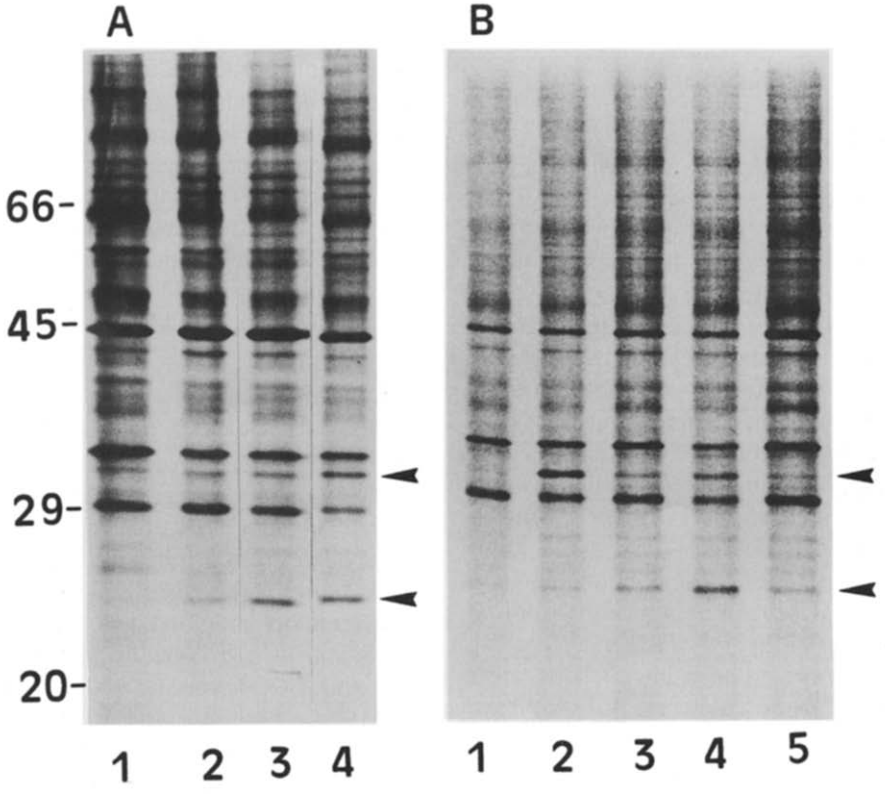

Fig. 1. Induction of 23 kDa protein in mouse peritoneal macrophages by exposure to oxidative or sulfhydryl-reactive agents. (A) Lane 1, control cells (with no treatment); lane 2, cells incubated for 11 h without any insults; lane 3, cells incubated for 11 h with 5 mU/ml glucose oxidase; lane 4, cells incubated for 11 h with 5 μM menadione. (B) Lane 1, cells incubated for 11 h without any insults; lane 2, cells incubated for 11 h with 2.5 μM sodium arsenite; lane 3, cells incubated for 11 h with 10 μM cadmium chloride; lane 4, cells incubated for 11 h with 100 μM diethyl maleate; lane 5, cells incubated for 11 h with 100 μM zinc chloride. The arrows indicate 23 and 34 kDa proteins.

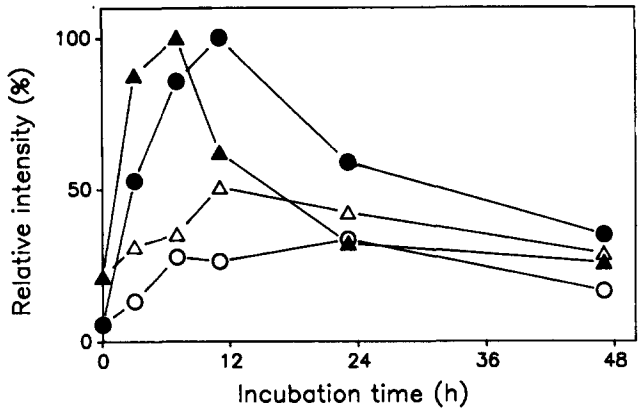

Fig. 2. Time course of synthesis of 23 and 34 kDa proteins after addition of diethyl maleate in mouse peritoneal macrophages. Macrophages were incubated with or without 100 μM diethyl maleate and at the time indicated, the cells were then labeled and the proteins were analyzed. The synthesis of 23 and 34 kDa proteins were quantitated by scanning densitometry of the fluorographs. Data were expressed as a percent of the maximum synthesis of 23 and 34 kDa proteins, i.e., those of cells incubated for 11 h and 7 h with diethyl maleate, respectively. ●, Synthesis of 23 kDa protein with diethyl maleate; ○, without diethyl maleate; ▲, synthesis of 34 kDa protein with diethyl maleate; △, without diethyl maleate.

Results

Induction of 23 kDa protein in mouse peritoneal macrophages by oxidative and sulfhydryl-reactive agents

In the freshly prepared macrophages, the synthesis of 23 kDa protein was very weak (Fig. 1A). However, it was significantly enhanced in the cells incubated for 11 h without any injurious agents. In the cells incubated for 11 h with glucose oxidase or menadione the synthesis of 23 kDa protein was enhanced more prominently. Glucose oxidase produces hydrogen peroxide by catalyzing the reaction of glucose and oxygen in the culture medium, and menadione produces active oxygen species. Thus, the 23 kDa protein seems to be induced by the oxidative stress. The enhanced synthesis of the 23 kDa protein in the cells incubated for 11 h without the oxidative agents may be explained by the fact that, under routine culture conditions, atmospheric oxygen itself constitutes an oxidative stress for the macrophages isolated from mouse abdominal cavity; oxygen tension in the peritoneal fluid is much lower than that in the culture medium equilibrated with air [9]. The effect of some sulfhydryl-reactive agents on the induction of 23 kDa protein was investigated (Fig. 1B). Treatment of the macrophages with diethylmaleate for 11 h strongly enhanced the synthesis of 23 kDa protein. The synthesis of 23 kDa protein was enhanced by exposure to cadmium chloride, sodium arsenite, or zinc chloride for 11 h. The synthesis of 34 kDa protein was also enhanced by these agents. However the relative intensity of the 34 kDa and 23 kDa protein bands was variable; hydrogen peroxide strongly induced the 23 kDa protein, but only slightly the 34 kDa protein, whereas sodium arsenite strongly induced the 34 kDa but not so the 23 kDa protein. Fig. 2 shows the time course of 23 kDa protein synthesis in macrophages exposed to diethylmaleate. Elevated synthesis of 23 kDa protein was detected 3 h after the addition of diethylmaleate and reached a maximum after 11 h. The peak of the synthesis of 23 kDa protein was preceded by that of 34 kDa protein. A similar result was obtained when the cells were exposed to hydrogen peroxide or sodium arsenite (data not shown).

The 23 kDa protein induced by diethylmaleate was

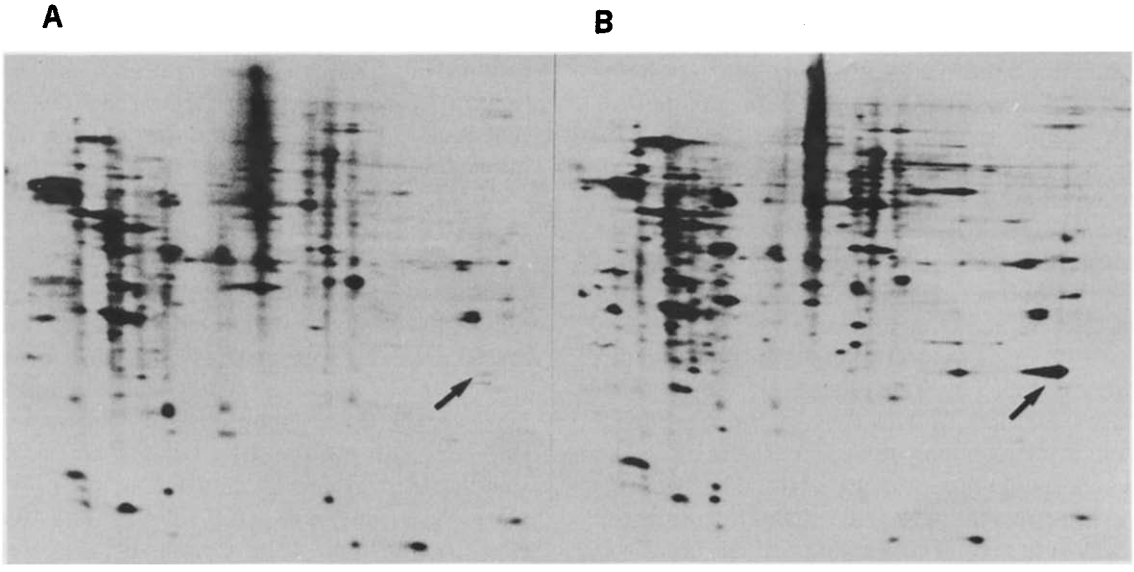

Fig. 3. Two-dimensional gel electrophoresis of 23 kDa protein. Proteins from the control cells (with no treatment) (A) and those from cells incubated for 11 h with 100 μM diethyl maleate (B) were analyzed by two-dimensional gel electrophoresis. The basic end of each gel is to the right. The arrow indicates 23 kDa protein.

analyzed by two-dimensional electrophoresis (Fig. 3). The fluorographs show that a polypeptide with a molecular mass of 23 kDa and an isoelectric point of about 8.5 was induced by the diethylmaleate treatment.

Induction of 23 kDa protein was investigated in other cells exposed to diethylmaleate (100 μM) for 11 h. The stress-inducible protein of a molecular mass of about 23 kDa was found in mouse resident peritoneal macrophages (data not shown). However, it was not detected in monocytes, neutrophils and lymphocytes obtained from human peripheral blood, primary cultured rat brain cells from embryonic cerebrum, and cultured human fibroblasts derived from fetal lung (data not shown).

Glutathione is known to protect cells against the oxidative and sulfhydryl-reactive agents described above. The subunits of glutathione *S*-transferase and glutathione peroxidase have molecular masses of about 23 kDa [10–12]. The activities of glutathione *S*-transferase and glutathione peroxidase in the untreated cells or in the cells exposed for 11 h to diethylmaleate were measured using 1-chloro-2,4-dinitrobenzene and *t*-butyl hydroperoxide as substrates, respectively. However, we could not find any significant difference in the activities between the untreated cells and the cells exposed for 11 h to diethylmaleate (data not shown).

Induction of 23 kDa protein by other stress

The effect of heat shock on the protein synthesis was investigated in macrophages. Fig. 4 shows the presence of typical 90 and 70 kDa heat-shock proteins after a short time, i.e., 2.5 h of heat shock. The 23 kDa protein, however, was not induced 2.5 h after heat shock. The heat-shock protein bands almost disappeared in 10.5 h after heat shock. It is of interest to know whether the oxidative or sulfhydryl-reactive agents can induce heat-shock proteins in macrophages. We have examined the synthesis of heat-shock proteins in macrophages exposed to hydrogen peroxide, diethylmaleate or sodium arsenite for various time (1–11 h). However, in no case were the major heat-shock proteins detected (data not shown).

Macrophages are known to be activated by various stimuli, including LPS, with the synthesis of some proteins [13–14]. We investigated the induction of 23 kDa protein in cells treated with LPS or zymosan. The cells exposed to LPS or zymosan did not express enhanced levels of 23 kDa protein synthesis (Fig. 5A). Macrophages are activated in vivo by intraperitoneal injection of BCG to obtain tumoricidal activity in the presence of a small amount of LPS [16]. The synthesis of 23 kDa protein was investigated in these macrophages (Fig. 5B). The 23 kDa protein band was very faint in the BCG-activated macrophages with no further treatment, as it was in macrophages not activated by BCG (Fig. 1A). This means that the synthesis

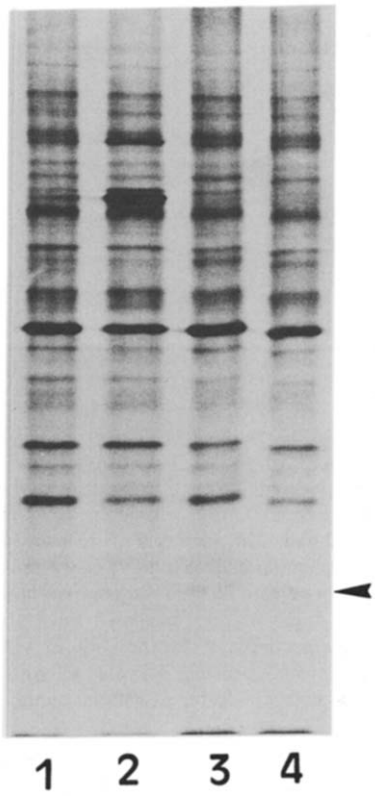

Fig. 4. Synthesis of 23 kDa protein in mouse peritoneal macrophages treated with heat-shock. Lane 1, cells incubated for 3 h without heat-shock; lane 2, cells incubated for 2.5 h after heat shock (43°C, 30 min) treatment (total 3 h in incubation); lane 3, cells incubated for 11 h without heat-shock; lane 4, cells incubated for 10.5 h after heat-shock (43°C, 30 min) treatment (total 11 h in culture). The arrow indicates 23 kDa protein.

of 23 kDa protein was not induced during the activation by BCG in vivo. After 11 h of incubation, a distinct induction of 23 kDa protein was observed in these BCG-activated macrophages. The incubation for 11 h with diethylmaleate led to a further induction of this protein, but LPS had no effect on the enhancement of the synthesis of 23 kDa protein. These results suggest that the 23 kDa protein was different from the proteins induced during the activating process of macrophages.

Discussion

We have previously shown that 34 kDa stress protein, identified as heme oxygenase, is induced in macrophages by various stress including sodium arsenite, cadmium chloride, diethylmaleate and hydrogen peroxide [7]. Both sodium arsenite and cadmium chloride are sulfhydryl-reactive agents known to induce some stress proteins in mammalian cells [4]. Arsenite particularly interacts with vicinal sulfhydryl groups whereas transition series metals such as cadmium and zinc are capable of affecting generally a redox-sensitive pathway. These studies and the present results indicate that both 34 and 23 kDa proteins were induced in the

macrophages by oxidative stress and sulfhydryl-reactive agents. However, the expression of these proteins seems to be regulated by different mechanisms. The 23 kDa protein was induced several hours later than the 34 kDa protein and the susceptibility to the stress, e.g., sodium arsenite, in the induction of 23 kDa protein is different from that of 34 kDa protein. It is suggested that the 34 kDa protein (heme oxygenase) has a protective function for cells by providing increased levels of the putative antioxidant bilirubin and by reducing the concentrations of potentially toxic heme compounds [6]. It is likely that 23 kDa protein also plays a role in protective mechanisms directed against oxidative stress.

The low-molecular-weight heat-shock proteins have been well studied in *Drosophila*, and some of these proteins have the molecular mass of about 23 kDa [17]. However, they are different from the 23 kDa protein described here in that the macrophage 23 kDa protein is not induced by heat shock and has a more basic isoelectric point than those of the *Drosophila* 23 kDa heat-shock proteins [18]. In chicken embryo cells the synthesis of the protein with a molecular mass of about 23 kDa was enhanced by several transition series metals and sodium arsenite [19]. Although it is unknown whether this avian protein is induced by the oxidative stress, it might be an analogue to the 23 kDa protein shown here in mammalian cells.

We have investigated the induction of 23 kDa protein in cells other than macrophages and the synthesis of proteins with molecular mass of approx. 23 kDa was not enhanced by diethylmaleate. Zatloukal et al. reported the induction of 34 kDa protein by sodium arsenite in short-term cultured mouse hepatocytes [20], but the induction of 23 kDa protein was not reported. The induction of 32 or 34 kDa proteins (both are identical with heme oxygenase) was reported in various cultured mammalian cell lines, including Chinese hamster ovary cells [21], murine and human melanoma [4] and human skin fibroblasts [5]. In any case induction of a protein of about 23 kDa was not reported. It seems that the 23 kDa protein is predominantly induced in macrophages by oxidative stress and sulfhydryl-reactive agents.

In macrophages and neutrophils there is evidence showing the induction of the synthesis of particular proteins during their activation. In mouse peritoneal macrophages elicited with proteose-peptone, induction of novel proteins by LPS has been investigated by two-dimensional electrophoresis [22]. Under these conditions a protein with a molecular mass of 23.5 kDa and an isoelectric point of 6.0 is induced and that the

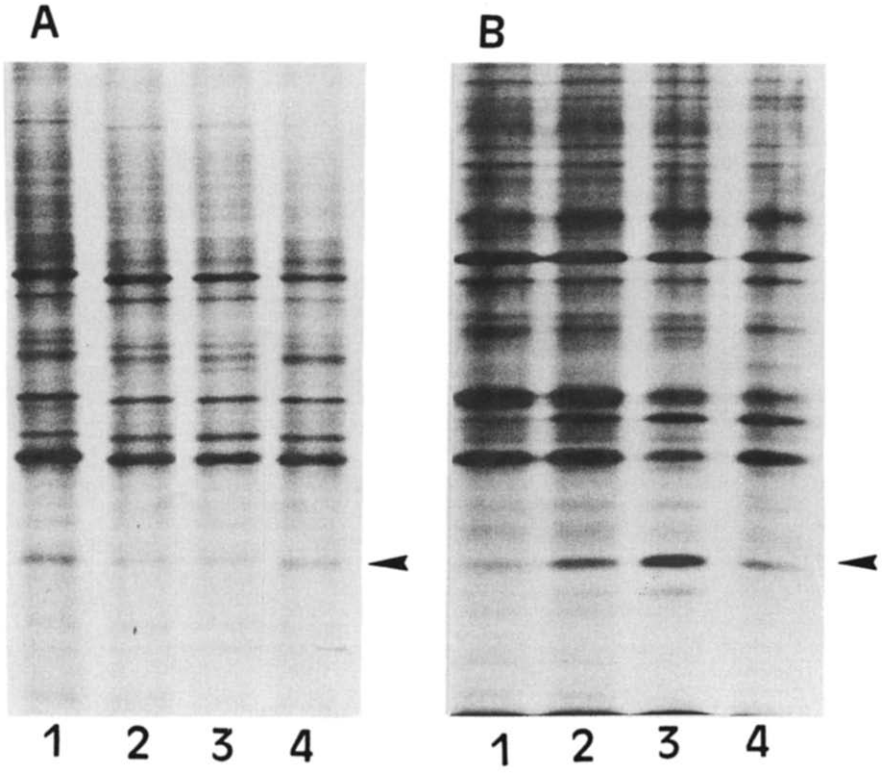

Fig. 5. Synthesis of 23 kDa protein in mouse peritoneal macrophages activated in vitro and in vivo. (A) Lane 1, the macrophages incubated for 11 h with no stimulants; lane 2, cells incubated for 11 h with 1 μg/ml LPS; lane 3, cells incubated for 11 h with 10 μg/ml LPS; lane 4, the cells incubated for 11 h with 50 μg/ml zymosan. (B) Macrophages activated in vivo with BCG as described in Experimental Procedures were treated as follows: lane 1, cells with no treatment; lane 2, cells incubated for 11 h without LPS; lane 3, cells incubated for 11 h with 100 μM diethyl maleate; lane 4, cells incubated for 11 h with 1 μg/ml LPS. The arrow indicates 23 kDa protein.

induction of this protein correlates very well with the tumoricidal activity of the cells. The 23.5 kDa protein is strongly synthesized in *Propionibacterium acnes*-elicited macrophages, which have been activated in vivo. In human neutrophils a 23 kDa protein, which migrates within a pH range of 5.2–5.5 on two-dimensional gel, is synthesized and secreted following stimulation with granulocyte-macrophage colony stimulating factor and tumor necrosis factor [22]. These proteins which are probably associated with the activation of macrophages may be different from the 23 kDa protein shown here, because the 23 kDa protein is not induced by LPS and zymosan and has more basic isoelectric point than these proteins.

In the control cells, i.e., the freshly prepared macrophages without any further treatment, the 23 kDa protein band appeared in fluorography was very faint, suggesting that in vivo this protein is synthesized a low level and must be induced by various stresses. The mechanism(s) by which oxidative stress and sulfhydryl-reactive agents induce the synthesis of the 23 kDa protein and the function of this protein in macrophages remain to be investigated. Cloning of a cDNA for the 23 kDa protein is now being carried out. The cDNA clone will be obtained from a cDNA library of mouse peritoneal macrophages by differential hybridization using cDNA probes synthesized from poly(A)$^+$RNAs of the control macrophages and those exposed to stresses. The cDNA encoding the 23 kDa protein may be useful in understanding a possible function of the 23 kDa protein. It enables us to search the sequences of other proteins which have high homology to that of the 23 kDa protein. The cDNA will be of particular use to reveal the mechanism(s) by which the stress agents induce the synthesis of the 23 kDa protein.

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