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Gel electrophoretic analysis of cellular and secreted proteins from resting and activated rat alveolar macrophages treated with pentamidine isethionate

James K. Selkirk*, C. He, B.A. Merrick

Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, USA

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Abstract

Pneumocystic carinii pneumonia, which is a major cause of death among patients suffering from acquired immunodeficiency syndrome, has often been treated successfully with pentamidine isethionate. This study examines pentamidine effects on cellular and secreted proteins from rat alveolar macrophages by two-dimensional gel electrophoresis and computerized image analysis. Over 100 secreted proteins were detected by fluorography. Fluorography showed pentamidine diminished tumor necrosis factor and interleukin-1 release along with other proteins. Effects of combined bacterial lipopolysaccharide and pentamidine were more pronounced on secreted versus cellular proteins in protein amount and pattern difference. Thus pentamidine exhibited a general repressive effect on cellular and secreted protein expression in resting and activated macrophages.

1. Introduction

Pentamidine isethionate [1,5-di(4-amidinophenoxy)pentane], was first used against protozoan infection in 1938 [1]. Although many other structural analogues were screened as trypanocides, pentamidine was the compound adopted for clinical use, although with caution due to its high toxicity. However, despite pentamidines' numerous deleterious side effects including nephrotoxicity, hepatotoxicity and hypotension, it was used with great success against epidemics of African trypanosomiasis in the post World War II period [2]. The drug was shown to be effective in infantile pneumocyctis carinii pneumonia (PCP) outbreaks in Europe, reducing the mortality rate from 50 down to 3.5%. However, it did not gain wide acceptance for general clinical use because of its multifocal toxicity, and also because subsequent occurrence of PCP was quite rare until the advent of acquired immunodeficiency syndrome (AIDS) which has now reached worldwide significance and is threatening to become a pandemic phenomenon. Since PCP has been shown to be one of the most common life-threatening infections in AIDS patients, pentamidine has again become prominent as one of the major drugs of choice for PCP infection.

Until recently, the long-term risks associated with the mechanisms of pentamidine toxicity had not received great emphasis because of the high

* Corresponding author.

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lethality of AIDS. However, as the longevity and survival of AIDS patients increase and the use of pentamidine becomes more common, adverse actions and long-term side effects of this drug will become increasingly important. It is already known that pentamidine interferes with numerous metabolic pathways and therefore has the potential for broad-based cytotoxicity. Originally pentamidine was administered only parenterally, either intramuscularly or intravenously, which resulted in deposition of the drug in virtually all organs and tissues in the body. Also, pentamidine has a slow turnover rate since treatment is followed by a relatively slow release from the body with the chemical present in urine up to eight weeks after cessation of therapy [3]. Although the lung is the site of PCP infection, pulmonary distribution after parenteral administration of pentamidine is relatively low as compared to the rest of the body. This observation led to adoption of an aerosolized route for pentamidine where high concentrations could be reached by direct deposition into the lungs [4], and with the hope of reducing severe systemic side effects. Subsequently, clinical studies did show that a daily dosage of 600 mg by inhalation resulted in general clinical improvement and survival [5]. However, more recent studies comparing parenteral and aerosol administration suggest that, while the aerosol route works well with mild infection, moderate or heavy infections are less successfully treated with this modality [6,7]. Currently, there is no adequate biochemical explanation as to why heavier infection of PCP should be less sensitive to pentamidine via direct deposition into the lungs.

In addition to its antimicrobial effects, aerosol-borne pentamidine may produce adverse effects upon resident alveolar macrophages which are essential components of the immunologic response to pulmonary infection and disease. Phagocytosis, recruitment and cytokine production are primary functions of macrophages which are activated by infectious organisms as a major line of defense in the pulmonary system. Activation of macrophages in in vitro culture can also be experimentally elicited by exposure to bacterial lipopolysaccharide (LPS) as the inducing agent. Since macrophage response to pentamidine may alter normal cellular biochemistry and therefore cytokine defense mechanisms may be impacted, we have evaluated electrophoretic patterns from both cellular and secreted proteins, as well as determined the presence of tumor necrosis factor (TNFα) and interleukin-1 (IL-1α) from isolated rat alveolar macrophages under resting and activated conditions using Western blotting with polyoclonal antibodies, two-dimensional polyacrylamide gel electrophoresis and computerized image analysis.

2. Experimental

2.1. Cell culture and radiolabeling

Rat alveolar macrophage cultures obtained after pulmonary gavage of female F344 rats (12–16 weeks old), were washed and plated to remove non-adherent cells [8]. Cells were resuspended in RPMI 1640 medium supplemented with glutamine, penicillin and streptomycin. For electrophoresis and immunoblotting experiments, macrophages were plated at 10^6 cells/ml in 100-mm plates. After 1 h for attachment, cells were exposed to pentamidine solubilized in water for 0.5 h prior to bacterial LPS at 1 μg/ml. Immediately after exposure to LPS, [^{35}S]-methionine was added at 100 μCi/well. Cells were incubated for 24 h at 37°C in a plastic enclosure gassed with a mixture of oxygen–carbon dioxide (95:5). Log orders of pentamidine concentration were selected up to a limit of 10 μM in which cellular viability was maintained without cytotoxicity.

At harvest, culture medium was removed and cells were scraped, washed in phosphate-buffered saline (PBS), pelleted and stored at −80°C until electrophoretic analysis. Radiolabeled secreted proteins in the supernatant were dialyzed with PBS containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and concentrated with Amicon centrifugal membranes (M_r cut-off 10 000), and lyphophylized prior to storage at −80°C.

Incorporation of [^{35}S]-methionine into macrophage protein was evaluated in 12 well plates at

10^6 cells/well. Under the conditions described above, cells were incubated at 0, 0.1, 1.0 and 10.0 μM pentamidine with and without LPS for 24 h. Cold carrier bovine serum albumin was added to each cell sample which was washed with 10% trichloroacetic acid until only background ^{35}S counts appeared in the supernatant. Excess ^{35}S was removed from secreted proteins by centrifugal dialysis. Protein was dissolved in 0.1 M NaOH, neutralized with acetic acid and counted in Aquasol.

2.2. Two-dimensional gel electrophoresis, fluorography and immunoblotting

Immediately prior to analysis, whole cells or secreted proteins were dissolved in urea-based lysis buffer [8]. Cellular protein was electrophoresed at 250 000 dpm of acid-precipitable radioactivity or as 125 000 dpm of secreted protein. Protein samples were subjected to two-dimensional gel electrophoresis according to O'Farrell [9]. Isoelectric focusing was in a pH gradient ranging from pH 4 to 8. Protein separation according to M_r by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) used a 10–16% acrylamide gradient. Gels with ^{35}S-labeled proteins were impregnated with fluor (Amplify, Amersham, Arlington Heights, IL, USA), dried, then exposed to preflashed XAR-5 film (Kodak, Rochester, NY, USA) for one to two weeks at −80°C. Film was developed using a Konica processor.

Two-dimensional gels were electroblotted onto poly(vinylidene diflouride) membranes and blocked with gelatin (Norland) for 2 h. Blots were incubated overnight with murine polyclonal antibody for IL-1α and TNFα (Genzyme, Cambridge, MA, USA). Secondary antibody detection for IL-1 used alkaline phosphatase and peroxidase for TNF, respectively. Murine rTNF (Genzyme) was electrophoresed, separated, electroblotted and stained with colloidal gold (Bio-Rad).

2.3. Image analysis

Films were digitized with an Optronics Model P-1000 densitometry scanner and analyzed on an International Imaging Systems workstation. Digitized images were subjected to smoothing and background subtraction algorithms in order to reduce each 1536×1536 pixel image into numerical data [10]. Every protein on each image was numerically described by x,y coordinates, the center point, and peak and total integrated densities (0 to 256 gray value range), and also sigma x,y diameters for an elliptical spot shape. Initial computer generated images were edited for artifacts and then modeled with a bi-gaussian algorithm on a Trace Multiflow computer to apportion protein spot density. Data from 3–5 gels for each treatment were registered and compared, and subsequently compiled into one master image [10]. Similarly, master images from each treatment were constructed from replicate images and qualitatively compared in order to determine common and unique proteins.

3. Results and discussion

While the typical response of macrophages to treatment with LPS is an increase of approximately 35% over the control with regard to incorporation of [^{35}S]methionine into protein, Fig. 1 shows pentamidine slowed this effect when macrophages were activated with LPS. While pentamidine (10 μM) itself had little effect upon [^{35}S]methionine incorporation into macrophage protein, the expected LPS induction of ^{35}S labeling was depressed between 10 μM and 1 μM. When [^{35}S]methionine labeling was measured in secreted protein (see inset), the greatest effect of pentamidine was at 10 μM which depressed the LPS-induced increase in ^{35}S labeling of macrophage protein from 65 down to 17% above the control value. In contrast to cellular protein labeling, 10 μM pentamidine alone gave a decrease in secreted protein labeling of 15%.

Treatment with pentamidine did not greatly affect the relative distribution of cellular macrophage proteins as compared to the control pattern. The labeling time used in these experiments resulted in more than 700 proteins showing incorporation of radiolabel. The more prominent quantitative changes are noted in Fig. 2.

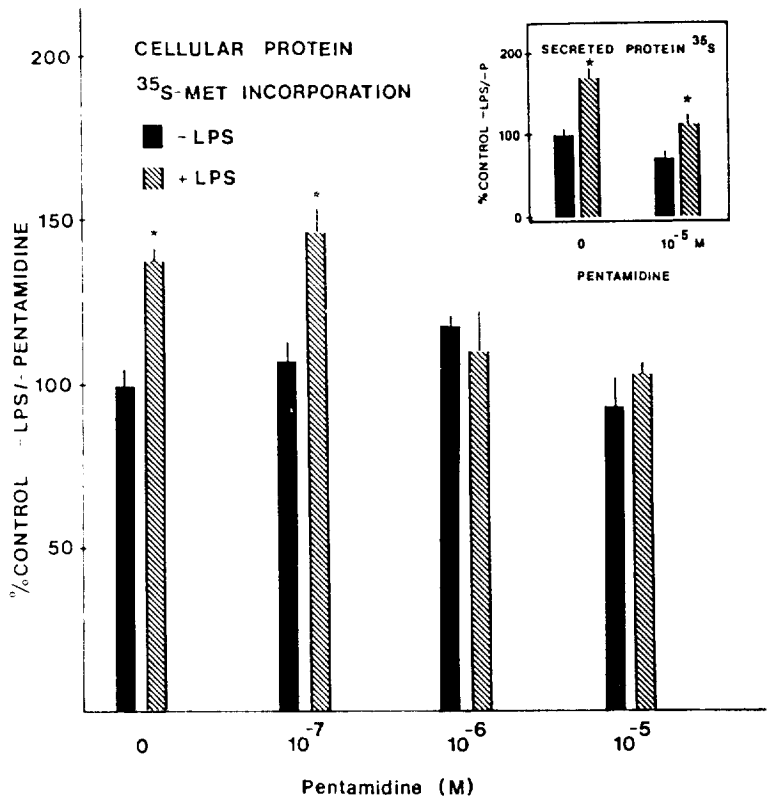

Fig. 1. Effect of bacterial lipopolysaccharide (LPS) and pentamidine on protein synthesis in rat alveolar macrophages. The histogram shows incorporation of [^{35}S]methionine for 24 h with increasing concentrations of pentamidine. Asterisks indicate significant differences at $p < 0.5\%$ from controls without LPS or pentamidine.

Those proteins with more than five-fold density difference are noted by letters (A–K). There were also five proteins that pentamidine either induced or sufficiently down-regulated to absence (solid triangles) which were modulated in the presence and absence of LPS. This indicated a specific pentamidine effect. The more prominent quantitative changes were seen in proteins A (M_r 63 000), C, D (52 000), E (37 000), F (29 000), J (26 000) and K (11 000). Two proteins were induced by LPS since both the M_r 69 000 and 11 000 proteins were missing in the pentamidine/–LPS panel. However, treatment with LPS also down-regulated two basic M_r 75 000 proteins as well as an acidic M_r 88 000 protein. The occurrence of both induction and down-regulation of proteins suggest a complex pattern of protein modulation by treatment with pentamidine.

Since macrophages secrete TNFα as well as IL-1α, which are both prominent in the general immune response, it was important to determine if pentamidine treatment altered the usual formation of these proteins by the LPS antigen. Fig. 3 shows the effect of pentamidine in the presence and absence of LPS. The left panel shows a small constitutive amount of TNFα while the right panel shows a considerable induction of TNFα as well as a small quantity of IL-1α. It would appear therefore that pentamidine does not specifically inhibit the formation of these important protective proteins during macrophage activation by LPS. In addition there was a considerable LPS-induced increase of a low-mo-

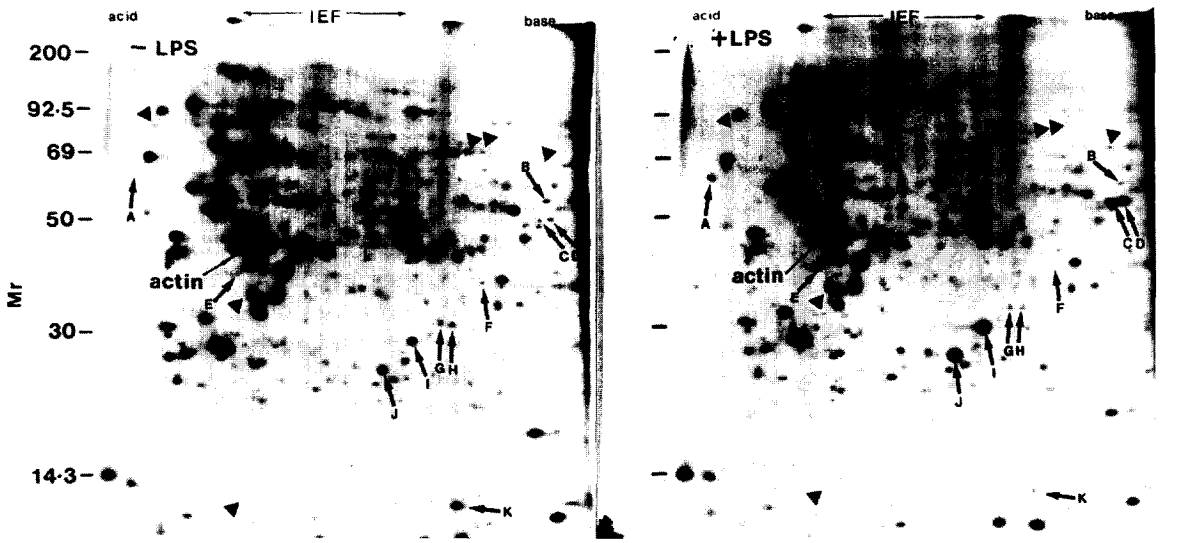

Fig. 2. Fluorographs from [^{35}S]methionine labeling of cellular proteins from pentamidine-treated rat alveolar macrophages after 24-h incubation with 10 μM pentamidine and with or without LPS. The presence (+) or absence (–) of LPS is indicated on the figure. Quantitative changes are indicated by arrows, while qualitative changes are marked triangles. M_r values $\times 10^{-3}$.

lecular-mass protein (M_r 13 500) which has not yet been identified. Identification of TNFα and IL-1α was previously confirmed by immunoblotting with specific antibodies [8].

The overall modulation of secreted proteins was assessed using image analysis as seen in Fig. 4. Treatment with LPS showed the majority of secreted proteins (128, shaded spots) to be unchanged with 8 proteins (+) down-regulated and 53 proteins (○) induced by LPS. The addi-

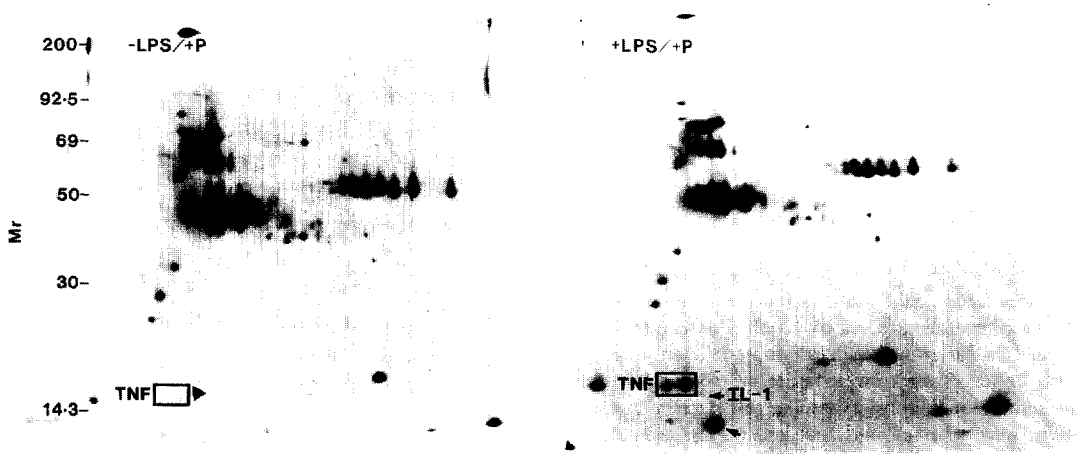

Fig. 3. Fluorographs from [^{35}S]methionine-labeled secreted proteins from rat alveolar macrophages after 24-h incubation with or without LPS. Immunoblotting confirmed the presence of TNFα and IL-1α. P – Pentamidine. M_r values $\times 10^{-3}$.

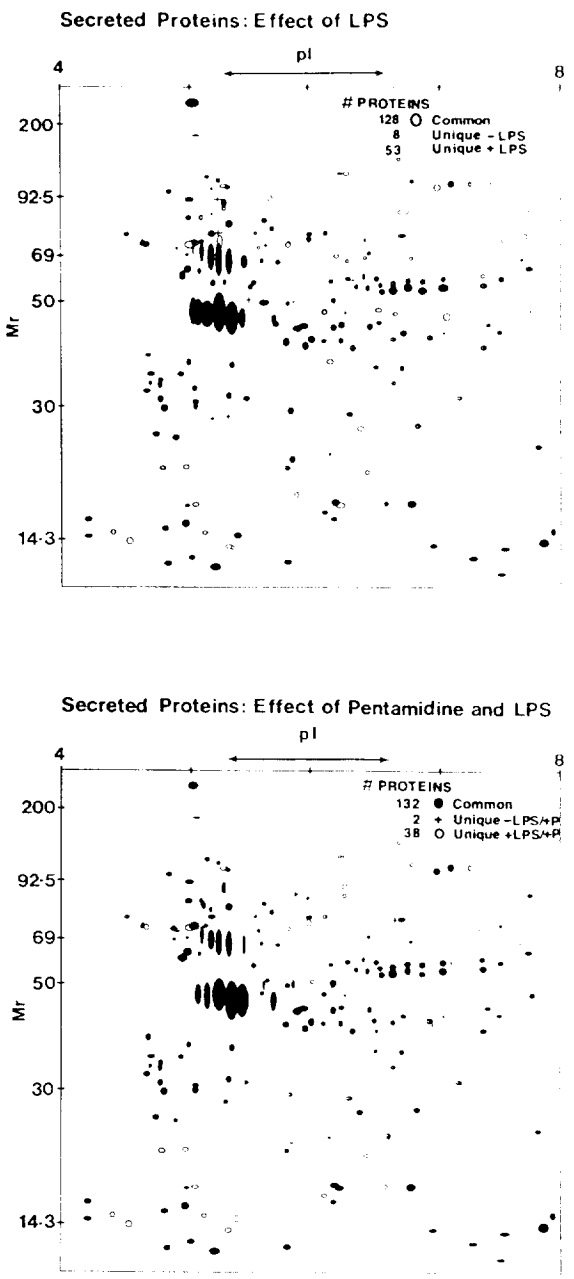

Fig. 4. Computerized comparison of secreted proteins from alveolar macrophages as affected by 10 μM pentamidine (P) and bacterial LPS for 24 h. [^{35}S]Methionine-labeled proteins secreted into culture medium were concentrated and subjected to two-dimensional polyacrylamide gel electrophoresis. Fluorographs were scanned and analyzed by image analysis algorithms. Master composite images as shown above represent shared (common) and different (unique) proteins with and without LPS in the absence or presence of pentamidine.

tion of pentamidine caused relatively little qualitative change in the protein profile under these conditions with 132 proteins common with and without LPS. Only 2 proteins were down-regulated below the resolution of analysis, although pentamidine did reduce the inductive effect of LPS with only 38 proteins up-regulated.

Pentamidine isethionate is structurally categorized in the aromatic diamidine family of compounds that are extensively used as trypanocides [11]. The mechanism of action of pentamidine is still uncertain, although it appears to inhibit dihydrofolate reductase and deplete folic acid [12]. It also binds to DNA in a non-intercalative way, and is known to preferentially attach to the kinetoplast in trypanosomes [13]. Pentamidine also inhibits proteases [14], oxygen consumption in cells [14], RNA polymerase [15], nucleic acid, protein and phosopholipid synthesis [16,17], all of which supports the concept of this drug being a multifocal cellular toxicant. This wide range of toxicity against normal cellular processes is probably the reason that pentamidine is lethal to non-dividing cells and also serves as evidence that interaction with DNA is not the only mode of action [18].

The striking similarity of control and pentamidine-treated protein patterns observed in this study also supports the concept that this compound is functioning as a general cell toxicant which attacks numerous metabolic pathways although pentamidine also stimulated the production of a number of specific proteins (Figs. 2 and 4), whose structure and function have yet to be determined. Furthermore, it was clear that LPS caused a significant induction of TNFα and a small induction of IL-1α, both of which were to some degree reduced by the presence of pentamidine. However, TNFα and IL-1α are still secreted (Fig. 1, inset), suggesting that these important cytokines are still produced and this part of the signaling pathway for pulmonary defense is still intact. These cytokine results also suggest pentamidine to be an overall cellular toxicant, rather than a specific inhibitor of these two particular cytokines. Although visible on the gel and confirmed by immunoblotting, IL-1α was too light for densitometric quantitation. Since

these cytokines are important to the immune response it is important that they are not inhibited, and that a patient's treatment regime of pentamidine be so designed that perturbation of normal cellular biochemistry is minimized while the efficacy of the drug is maximized.

Comparative image analysis of the secreted proteins showed a complex series of biochemical changes mostly through stimulation by LPS, although a few proteins were induced by the presence of pentamidine alone.

With the growth of the number of AIDS patients, the clinical literature on the health effects and efficacy of pentamidine for treatment of PCP has presented a broad spectrum of physiological effects. The results of this study are the first presentation of cellular protein patterns of alveolar cells treated with pentamidine. The combination of two-dimensional gel electrophoresis and computerized image analysis has shown specific protein perturbations against a broad background of shared proteins by both normal and pentamidine-treated cells. There appears to be a combination of organ toxicity, which probably accounts for the numerous side effects seen in vivo, coupled with a modulation of a separate subset of proteins some of which are induced and several of which are down-regulated. At the present time, the function of these proteins is unknown. It is, however, important that they be isolated and identified in order to determine their placement in their respective biochemical pathways and to formulate a generalized mechanism of pentamidine cytotoxicity.

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