Expression of Inducible Nitric Oxide Synthase by Stimulated Macrophages Correlates with Their Antihistoplasma Activity

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The antihistoplasma activity of recombinant murine gamma interferon (rMuIFN-γ)-treated macrophages of the RAW 264.7 cell line depends on the generation of nitric oxide (NO) from l-arginine. Macrophages of the P388D1 cell line treated with rMuIFN-γ do not produce NO or inhibit the intracellular growth of Histoplasma capsulatum. NO is generated by the inducible enzyme nitric oxide synthase (iNOS) formed by stimulated macrophages. Northern (RNA) blot analysis of RAW 264.7 cells revealed the expression of iNOS mRNA after exposure to rMuIFN-γ. In contrast, rMuIFN-γ-treated P388D1 cells did not produce detectable levels of iNOS. These data suggest that the failure of P388D1 cells to generate NO and to restrict the intracellular growth of H. capsulatum is due to a lack of expression of iNOS following treatment with rMuIFN-γ.

The dimorphic fungus Histoplasma capsulatum is a facultative intracellular pathogen of the mononuclear phagocyte system of susceptible animals. Gamma interferon (IFN-γ) plays a key role in host defense against histoplasmosis. Treatment of normal mouse peritoneal macrophages with recombinant murine IFN-γ (rMuIFN-γ) inhibits the intracellular growth of H. capsulatum (12). In contrast, resident red pulp splenic macrophages require a double signal (e.g., rMuIFN-γ and lipopolysaccharide [LPS]) in order to be fully activated to the antihistoplasma state (7). It has recently been shown that treatment of the mouse macrophage-like cell line RAW 264.7 with rMuIFN-γ inhibits the intracellular growth of H. capsulatum (10).

Macrophages contain the immunologically inducible enzyme nitric oxide synthase (iNOS) (8). Appropriately stimulated macrophages produce iNOS, which cleaves the guanidino nitrogen group from l-arginine to generate NO and l-citrulline (9). The production of NO by macrophages represents a key defense mechanism against some obligate and facultative intracellular pathogens (1, 4, 6). Treatment of RAW 264.7 cells with rMuIFN-γ results in the production of NO which correlates with the antihistoplasma activity of the cells (10). However, P388D1 macrophages do not restrict the intracellular growth of H. capsulatum following treatment with rMuIFN-γ alone or in combination with LPS (13). The goal of this study was to measure iNOS expression in rMuIFN-γ-treated RAW 264.7 and P388D1 cells in an attempt to (i) explain the differences between the cell lines in the release NO and (ii) correlate the expression of iNOS with antihistoplasma activity.

The RAW 264.7 and P388D1 macrophage cell lines were obtained from the American Type Culture Collection, Rockville, Md. (5). Both cell lines were cultured in modified Eagle’s medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% heat-inactivated fetal bovine serum and supplemented with 15 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 15 mM glucose, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (SMEM). The rMuIFN-γ was supplied by Genentech Inc., South San Francisco, Calif. RAW 264.7 and P388D1 cells were seeded separately at a concentration of 5 × 10⁶ cells onto 13-mm-diameter Thermonox coverslips (Nunc, Inc., Naperville, Ill.) in 24-well tissue culture plates (Corning Glass Works, Corning, N.Y.) and incubated at 37°C in a 5% CO₂ atmosphere for 2 h. The coverslips were washed vigorously with prewarmed Hanks’ balanced salt solution to remove nonadherent cells. Macrophage monolayers were then exposed to 1 ml of SMEM either with or without 5,000 U of rMuIFN-γ per ml for a 4-h preincubation. At the completion of the preincubation period, unopsonized yeast cells of H. capsulatum were washed with cold Hanks’ balanced salt solution and approximately 2 × 10⁵ to 3 × 10⁵ yeast cells were added to the macrophage monolayer. Two hours were allowed for phagocytosis, and then extracellular yeast cells were washed away. Two coverslips that were exposed to SMEM alone were washed and placed in 1 ml of methanol in order to fix the macrophage monolayer (7, 13). The methanol-fixed monolayers were then stained with periodic acid-Schiff stain (EM Diagnostic Systems, Inc., Gibbstown, N.J.; staining was done according to the manufacturer’s instructions) in order to determine the number of yeast cells per macrophage at the start of the experiment (7). Preexposure of both RAW 264.7 and P388D1 cells to rMuIFN-γ did not affect the percent phagocytosis compared with macrophages preexposed to SMEM alone (10, 12). The remaining monolayers, which were not fixed in methanol, were then incubated for an additional 18 h in the presence of the same factors as used in the preincubation protocol. At the completion of the 18-h incubation period, the monolayers were washed and fixed in methanol. Monolayers were stained with periodic acid-Schiff stain, and the mean number of yeast cells within infected macrophages was determined by counting the yeast cells in a minimum of 100 infected cells. The percent inhibition of growth was calculated as follows: [1 – (growth within macrophages exposed to rMuIFN-γ/growth within macrophages in SMEM)] × 100.

Plasmid CL-BS-Mac-NOS was kindly provided by Solomon Snyder, Johns Hopkins School of Medicine. The plasmid consisted of the full-length coding region of the mouse macrophage iNOS cDNA inserted into the Bluescript KS vector (Stratagene, La Jolla, Calif.) (8). Escherichia coli DH5α containing plasmid CL-BS-Mac-NOS was grown overnight in LB broth, and the plasmid was isolated by using the Magic Maxiprep DNA Purification System (Promega, Madison, Wis.). The purified plasmid was cut with the restriction enzyme

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NorI, and the 4,100-bp iNOS cDNA fragment was isolated by gel electrophoresis. Total RNA was isolated from RAW 264.7 and P388D, cells by using the Total RNA Isolation System kit (Promega, Madison, Wis.). Northern (RNA) blot analysis of total RNA was performed according to standard techniques by using the 32P-labeled iNOS cDNA as a probe (11).

Nakamura et al. (10) have shown that macrophages of the RAW 264.7 cell line produces NO2− which correlates with activation to an antihistoplasma state. The data in Table 1 show that treatment of P388D1 macrophages with rMuIFN-γ (5,000 U/ml) resulted in the production of only baseline levels of NO2− and no inhibition of growth of the fungus. In contrast, high levels of NO2− (24.4 ± 0.4 μM) were produced by activated RAW 264.7 cells, which correlated well with the induced fungistasis. Following an 18-h incubation period, total RNA was isolated from macrophages exposed to rMuIFN-γ or SMEM alone. Northern blot analysis of rMuIFN-γ-treated RAW 264.7 cells probed with iNOS cDNA showed that these cells produced a 4.1-kb band corresponding to the iNOS mRNA (Fig. 1) (8). A faint band corresponding to iNOS mRNA was detected from RAW 264.7 cells exposed to SMEM only (Fig. 1). iNOS mRNA was not detected from P388D1 cells treated with either rMuIFN-γ or medium alone, even though twice as much RNA was loaded into the lanes compared with the amount loaded for RAW 264.7 macrophages (Fig. 1). The inability of rMuIFN-γ-treated P388D1 to produce detectable levels of iNOS corresponds with their failure to release NO2− (Table 1). These data provide one possible explanation for the failure of P388D1 cells to be activated to an antihistoplasma state in response to rMuIFN-γ.

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