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Protective Effect of Poly-2-Vinylpyridine-*N*-Oxide on Susceptibility of Silica-Treated Mice to Experimental Histoplasmosis

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We have studied the ability of poly-2-vinylpyridine-*N*-oxide (PVNO), a lysosomal stabilizing agent, to abrogate the cytotoxic effects of silica on macrophages. Male C3H/HeN mice were pretreated with PVNO and inoculated intravenously with silica particles. At 24 h after silica injection, silica-treated and -untreated mice were challenged intravenously with varying doses of live yeast cells of *Histoplasma capsulatum*. All mice receiving silica died when challenged with 5×10^5 yeast cells of *Histoplasma* sp. compared with no deaths in PVNO-pretreated animals and 10% mortality in controls not receiving PVNO or silica. When animals were given 2.5×10^5 yeast cells (a sublethal dose), the protective effect of PVNO was seen by a reduction in splenomegaly and viable *Histoplasma* sp. present in the spleen. Furthermore, PVNO alone showed a significant protective effect ($P < 0.05$) against a lethal challenge with *Histoplasma* sp. Prior treatment with PVNO also protected mouse peritoneal macrophages from the cytotoxic effects of silica particles in vitro. These results indicate that PVNO abrogates the cytotoxicity of silica particles on macrophages and also increases the resistance of mice to histoplasmosis.

Macrophages are involved in the expression of cell-mediated immunity to histoplasmosis. Activated macrophages have been shown to restrict the intracellular growth of *Histoplasma capsulatum* (7, 8) and even degrade the ingested organisms as shown by ultrastructural studies (A. Mathur and R. P. Tewari, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 20th, New Orleans, La., abstr. no. 43, 1980; 6). However, the role of macrophages in natural resistance to histoplasmosis has not been clearly defined. We have previously shown that treatment with silica, an agent which is selectively cytotoxic for macrophages, enhanced the susceptibility of mice to experimental histoplasmosis (17). Silica particles were also cytotoxic for mouse peritoneal macrophages in vitro.

Poly-2-vinylpyridine-*N*-oxide (PVNO) is known to prevent or reduce the toxic, fibrogenic, and immunosuppressive effects of silica, presumably by stabilizing macrophages (2, 12, 16). In an effort to further delineate the role of macrophages, we have examined whether PVNO administration can protect mice against silica-induced enhanced susceptibility to histoplasmosis and also its direct protective effect on the experimental infection. Mice were treated with silica or PVNO or both, and their susceptibility to varying challenge doses of *H. capsulatum* was evaluated. To further document the protective effect of PVNO, the in vitro phagocytic activity of normal peritoneal macrophages for yeast cells of *Histoplasma* sp. was studied with and without prior treatment of macrophages with silica or PVNO or both.

(These findings were presented in part at the 81st Annual Meeting of the American Society for Microbiology, 10 to 14 March 1981, Dallas, Tex.)

MATERIALS AND METHODS

Animals. Male C3H/HeN mice, weighing 18 to 20 g, were obtained from Harlan Industries, Inc., Indianapolis, Ind. The mice were randomly divided into groups of five, housed in plastic cages, and given food and water ad libitum.

Organism. Strain G-217B of *H. capsulatum*, a human isolate, was maintained in yeast phase on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.) slants. Cultures were stored at 4°C and transferred every 6 to 8 weeks, and the yeast cell suspensions were prepared from early logarithm yeast phase cultures grown in BHI broth. Yeast cells were harvested after 36 h of growth at 37°C on a gyratory shaker

with a shaking speed of 150 rpm. The cells were washed twice with sterile saline at 4°C. After a final centrifugation at 30 × *g* for 1 min to remove larger yeast aggregates, the suspensions were standardized spectrophotometrically to contain the desired number of organisms. The viability of cells, as determined by plating on BHI agar plates supplemented with 0.1% bovine serum albumin and 1% *Histoplasma* growth factor (3), was always >80%. Such cell suspensions primarily consisted of one and two cell infectious units as determined by hemacytometer counts. The mean lethal dose of the strain for C3H/HeN mice was 8×10^5 yeast cells given intravenously (i.v.).

Preparation of silica particles. Silica particles (1 to 5 μm) obtained from Sigma Chemical Co., St. Louis, Mo., were purified as previously described (17). Briefly, silica was boiled in 1 N HCl to remove contaminating $FeCl_3$, washed with distilled water, dried, and sterilized by autoclaving. The day before use, silica particles were suspended in medium 199 (M199) plus 10% heat-inactivated (56°C for 30 min) fetal calf serum (FCS), and the suspension was sonicated for 30 s immediately before use.

Inoculation of mice with PVNO, silica, and *Histoplasma* sp. Mice were injected subcutaneously with 4 mg of PVNO (Polysciences Inc., Warrington, Pa.) in 0.2 ml of physiological saline, or 3 mg of silica particles i.v. in 0.5 ml of M199-FCS or both, or the suspending medium alone. PVNO was injected 24 h before the administration of silica particles. After 24 h, a set of silica or PVNO-treated mice or both along with controls were challenged i.v. with different doses of live yeast cells of *H. capsulatum*. The susceptibility of these mice to *Histoplasma* sp. was determined by 30-day mortality and mean body weights.

Enumeration of *H. capsulatum* in spleens. From each group, 10 mice injected with silica, PVNO, or medium alone were sacrificed 30 days after a sublethal infection with 2.5×10^5 yeast cells of *H. capsulatum* (i.v.). The spleens were removed aseptically and weighed individually. Serial dilutions of the spleen homogenates were plated on BHI agar plates supplemented with 0.1% bovine serum albumin and 1% *Histoplasma* growth factor (3). The plates were incubated at 30°C for 7 to 10 days and the CFU of *Histoplasma* sp. was recorded.

In vitro effect of PVNO and silica on macrophages. Nonelicited peritoneal cells were harvested from normal mice as previously described (18). Macrophage monolayers were prepared on 15-mm^2 glass coverslips in Nunc's four-well tissue culture chambers (2.5×10^5 cells per chamber) in M199 plus 20% FCS. After overnight incubation (16 to 18 h), the medium was removed and replaced with fresh medium containing 0.02% PVNO for 5 h. The monolayers were then washed three times with M199, and graded amounts of silica particles (20 to 100 μg/ml) suspended in M199 plus 10% FCS were added. After an additional 16 h of incubation, some monolayers were stained with 5 μg of acridine orange per ml (Eastman Kodak Co., Rochester, N.Y.) for 15 min. The macrophage monolayers were incubated for an additional 10 min in fresh M199–10% FCS to remove unbound acridine orange, mounted in phosphate buffered saline, and examined by fluorescent microscopy. Separate sets of macrophage monolayers were exposed to yeast cells of *Histoplasma* sp. for 1 h, washed three times with fresh M199, stained with acridine orange, and examined by fluorescent microscopy. The phagocytic activity of the macrophages was assessed by counting at least 200 cells.

Statistical analysis. The data were analyzed for statistical significance between control and test groups by the Student *t* test. The mortality data were analyzed by survival analysis techniques, and the body weight data were analyzed by one way analysis of variance.

RESULTS

Effect of PVNO and silica on susceptibility of mice to *H. capsulatum*. We evaluated the effect of PVNO on the silica-induced increased susceptibility of C3H/HeN mice to experimental histoplasmosis. At 24 h after subcutaneous injection of 4 mg of PVNO, mice were given 3 mg of silica i.v. After an additional 24 h, separate sets of silica-treated and -untreated mice were inoculated i.v. with 1×10^6 or 5×10^5 live yeast cells of *H. capsulatum*, and their susceptibility was assessed by 30-day mortality and weekly body weights. Silica-treated mice were more susceptible to infection with *Histoplasma* sp. than untreated controls, and prior treatment of mice with PVNO protected them against the deleterious effects of silica (Fig. 1). All mice given silica and 10^6 yeast cells of *Histoplasma* sp. died within 13 days, whereas 80% mortality was observed in mice given PVNO before silica treatment ($P < 0.01$). Furthermore, PVNO alone showed a significant protective effect against lethal challenge with *H. capsulatum* ($P < 0.05$).

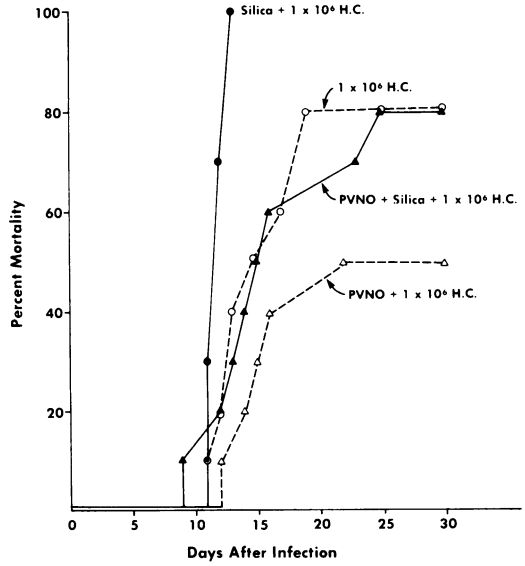

FIG. 1. Percent mortality of silica- or PVNO-treated mice or both after i.v. infection with 10^6 yeast cells of *H. capsulatum*. Each group consisted of 10 mice. Mean values are from two separate experiments.

The weekly body weights of mice supported the observations obtained by mortality rates.

The protective effect of PVNO was more dramatic when mice were challenged with 5×10^5 yeast cells of *H. capsulatum* (Fig. 2). All mice receiving silica and *Histoplasma* sp. died compared with no deaths in the mice given PVNO before silica treatment ($P < 0.001$). At 1 and 2 weeks, the mean body weights of mice receiving silica and 5×10^5 yeast cells of *Histoplasma* sp. were lower ($P < 0.01$) than mice given PVNO before silica or *Histoplasma* sp. or both (data not shown).

Effect of PVNO and silica on spleen weight and viable *Histoplasma* counts. The enhanced susceptibility of silica-treated mice is presumably the result of uncontrolled growth of the organisms within the susceptible host. We tested this assumption by determining the effect of PVNO pretreatment on spleen weights and the number of viable organisms in spleens of silica-treated mice 30 days after challenge with a sublethal dose of yeast cells of *Histoplasma* sp. (2.5×10^5 live yeast cells). Pretreatment with PVNO reduced the splenomegaly ($P < 0.001$) and decreased the viable counts of *Histoplasma* sp. in spleens of the infected mice ($P < 0.01$) that were associated with silica treatment (Table 1). Silica-treated mice had 23 times more viable *Histoplasma* sp. in their spleens than untreated controls ($P < 0.005$), and their mean spleen weight was approximately one and one-half times higher than those given *Histoplasma* sp. alone ($P < 0.005$).

In vitro effect of PVNO and silica on the viability and phagocytic activity of mouse peritoneal macrophages. To determine the effect of PVNO on the cytotoxicity of silica particles for macrophages in vitro, nonelicited mouse peritoneal macrophage cultures were exposed to silica with and without PVNO pretreatment, and the cytotoxicity was evaluated by fluorescent microscopy of acridine orange-stained cells. All control macrophages showed orange- to red-stained lysosomes with distinct greenish-yellow nuclei (Fig. 3a), and PVNO treatment did not alter their morphological characteristics. Silica was cytotoxic to macrophages as exhibited by a significant decrease in lysosomal staining and an alteration in cellular structure including the absence of distinct nuclear staining ($P < 0.001$), and the response was dose dependent (Table 2; Fig. 3b). Pretreatment of macrophages with PVNO (20 μg/ml) abrogated the cytotoxicity of silica particles ($P < 0.001$; Fig. 3c). Incorporation of PVNO (0.125 to 4 mg/ml) did not inhibit the growth of the yeast cells of *H. capsulatum* in vitro (data not shown).

In subsequent experiments, we studied the phagocytic activity of PVNO- or silica-treated macrophages or both for live yeast cells of *H. capsulatum*. Separate sets of cultures were exposed to live cells of *Histoplasma* sp. (macrophages to yeast cells, 1:5) for 1 h and processed as described in Materials and Methods. PVNO-treated and -untreated macrophages were phagocytic with 93 to 94% phagocytic cells (Fig. 3d), and the phagocytic activity was significantly decreased ($P < 0.001$) by silica treatment (Table 2; Fig. 3e). Pretreatment of macrophages with PVNO significantly reduced the cytotoxicity of silica particles ($P < 0.001$) as shown by partial restoration of the phagocytic activity of macrophages for *H. capsulatum* (Fig. 3f).

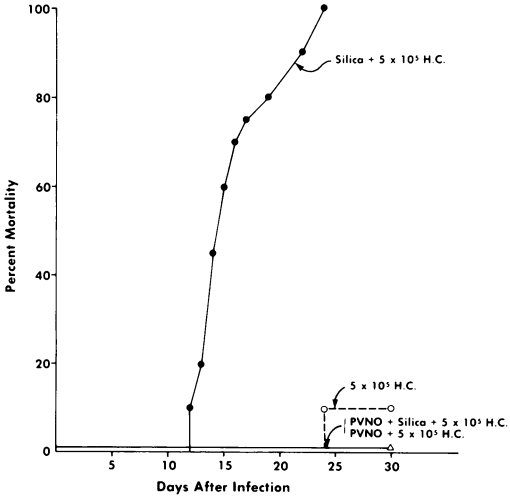

FIG. 2. Percent mortality of silica- or PVNO-treated mice or both after i.v. infection with 5×10^5 yeast cells of *H. capsulatum*. Each group consisted of 10 mice. Mean values are from two separate experiments.

TABLE 1. Spleen weights and recovery of viable organisms from mice 30 days after i.v. challenge with 2.5×10^5 yeast cells of *H. capsulatum*[a]

Treatment[b]	Spleen weight (mg ± SEM)	Viable *H. capsulatum*/ spleen (CFU ± SEM)
Controls	116 ± 5	
Histoplasma sp.	158 ± 10	100 ± 63
Histoplasma sp. + PVNO	156 ± 7	144 ± 47
Histoplasma sp. + silica	260 ± 7	2,300 ± 600
Histoplasma sp. + PVNO + silica	179 ± 10	160 ± 46

[a] Mean values of two separate experiments.
[b] Each group consisted of 10 mice.

DISCUSSION

The study has shown that PVNO, a lysosomal stabilizing agent, abrogates the deleterious ef-

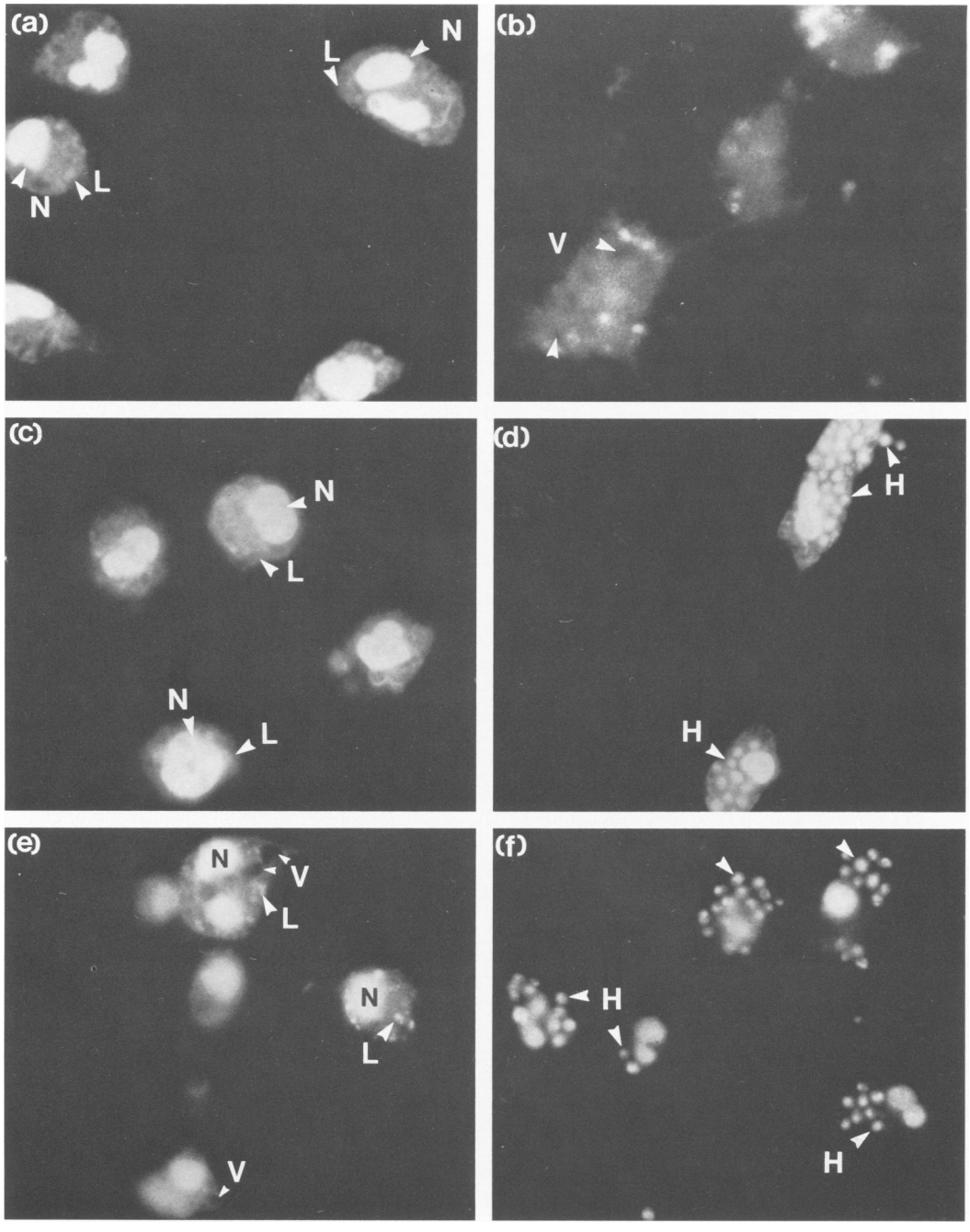

FIG. 3. Fluorescent microscopy of acridine orange-stained peritoneal macrophages (X1250). (a) Normal macrophages with diffuse lysosomal (L) staining in the cytoplasm and distinctly stained nuclei (N). (b) Macrophages after 16 h of incubation with 50 μg of silica particles per ml resulting in the loss of distinct lysosomal and nuclear staining characteristics. Black-appearing vacuoles (V) with silica particles are seen. (c) Macrophages after 5 h of incubation with 0.02% PVNO followed by a further 16 h of incubation with 50 μg of silica particles per ml. The protective effect of PVNO is shown by partial restoration of nuclear (N) and lysosomal (L) staining characteristics. (d) Normal macrophages after 1 h of exposure to live yeast cells of *H. capsulatum* showing intracellular-stained organisms (H). (e) Macrophages incubated with 20 μg of silica particles per ml for 16 h before exposure to live yeast cells of *H. capsulatum*. Macrophages showed distinct nuclei (N), some large faintly stained lysosomes (L), and black-appearing vacuoles (V) with silica particles. (f) Macrophages after 5 h of incubation with 0.02% PVNO followed by a further incubation with 20 μg of silica particles per ml for 16 h before exposure to live *H. capsulatum* for 1 h. Protective effect of PVNO is shown by partial restoration of phagocytosis of yeast cells (H).

TABLE 2. Effect of PVNO and silica on the viability and phagocytic activity of macrophages[a]

Treatment (concn/ml)	% Cells with lysosomal staining (±SEM)	% Cells with distinct nuclei (± SEM)	% Phagocytic cells (±SEM)
Controls (medium)	100 ± 0	100 ± 0	93 ± 2.8
20 μg of PVNO[b]	100 ± 0	100 ± 0	94 ± 3.2
20 μg of silica	8 ± 3	66 ± 0.7	53 ± 2.8
20 μg of silica + 20 μg of PVNO	70 ± 14	100 ± 0	74 ± 8.5
50 μg of silica	1 ± 1	18 ± 0.7	2 ± 2.4
50 μg of silica + 20 μg of PVNO	60 ± 25	99 ± 0	76 ± 14
100 μg of silica	0 ± 0	0.5 ± 0	0 ± 0
100 μg of silica + 20 μg of PVNO	10 ± 7	82 ± 3	45 ± 14

[a] Mean values from two separate experiments obtained by counting at least 200 macrophages per group per experiment.

[b] PVNO alone did not inhibit the growth of *H. capsulatum* in vitro.

fects of silica on the natural resistance of mice to experimental histoplasmosis. These findings are analgous to that of O'Brien et al. (14) who have shown that PVNO pretreatment abrogated the silica-induced susceptibility of mice to *Salmonella typhimurium*.

Silica, a selective toxic agent for macrophages, has been used to deplete macrophages from lymphoid cell suspensions in vitro (2, 11, 15) and, in experimental animals, to define the role of macrophages in resistance to *Salmonella typhimurium* (14), *Crytococcus neoformans* (13), *Trypanosoma cruzi* (10), and viruses (5, 19). Allison (1) has shown that when macrophages engulf protein-coated silica particles, the particles are enclosed within phagosomes which fuse with primary lysosomes. The proteolytic enzymes strip off the protein coat, thus making silicic acid available to damage lysosomal membranes with the liberation of lysosomal enzymes into the cytoplasm, resulting in cell death.

PVNO is a negatively charged polymer which forms hydrogen bonds with silica and is known to prevent or reduce the toxic, fibrogenic, and immunosuppressive effects of silica, presumably by stabilizing lysosomes in macrophages (2, 9, 12, 16). The stabilizing effect of PVNO on macrophages appears to depend on its incorporation into lysosomes which then fuse with phagosomes containing toxic materials such as silica. Thus PVNO stabilizes macrophage phagolysosomes preventing their disruption that otherwise would result in cellular death (4, 16).

The protective effect of PVNO on silica-induced susceptibility was most pronounced when animals were challenged with 5×10^5 yeast cells of *Histoplasma* sp. which was below the mean lethal dose of the organism. When mice were challenged with a sublethal dose of 2.5×10^5 yeast cells of *Histoplasma* sp., silica-treated animals had 23 times more viable organisms in their spleens than untreated controls. At the same challenge dose, pretreatment of mice with PVNO reduced the viable counts of *Histoplasma* sp. in spleens to levels that were similar to those of controls, further illustrating its protective effect.

In the present study, we have also shown that pretreatment with PVNO abrogates the cytotoxicity of silica particles to macrophages in vitro. Normal and PVNO-treated macrophages showed orange- to red-stained lysosomes with distinct greenish-yellow nuclei when stained with acridine orange. Silica treatment resulted in decreased lysosomal staining with alteration in cellular structure, including loss of distinct nuclear staining. It is noteworthy that silica treatment decreased or abolished the phagocytic activity and PVNO pretreatment not only reduced the cytotoxicity of silica particles but also partially restored the phagocytic activity of macrophages for *H. capsulatum*.

It is of interest that mice pretreated with PVNO before receiving 10^6 yeast cells of *Histoplasma* sp. had a lower mortality than control animals given *Histoplasma* sp. alone. At a challenge dose of 5×10^5 *Histoplasma* sp., PVNO-treated mice had higher body weights at 3 and 4 weeks than the animals that received *Histoplasma* sp. alone. PVNO has been shown to protect macrophages against the toxic effect of silica particles (1 to 5 μm) by the stabilization of lysosomal membranes. It is possible that PVNO may have a similar protective effect on phagolysosomes containing yeast cells of *Histoplasma* sp. which vary in size from 2 to 5 μm. This stabilization of phagolysosomes may prevent the rupture of macrophages containing viable multiplying yeast cells of *Histoplasma* sp., thus limiting the early spread of the organism to other macrophages.

In summary, these findings suggest that PVNO abrogates the detrimental effect of silica on natural resistance to experimental histoplas-

mosis and also indicate the importance of macrophage-dependent immune responses in resistance to the disease. Furthermore, PVNO also has a significant protective effect against histoplasmosis, and the mechanism of its action warrants further investigation.

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