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Thioglycollate-elicited murine macrophages are cytotoxic to *Mycoplasma arginini*-infected YAC-1 tumor cells

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Abstract

Macrophages are important components of natural immunity involved in inhibition of tumor growth and destruction of tumor cells. It is known that these cells can be activated for tumoricidal activity by lymphokines and bacterial products. We investigated whether YAC-1 tumor cells infected with *Mycoplasma arginini* stimulate nitric oxide (NO) release and macrophage cytotoxic activity. Thioglycollate-elicited macrophages from male BALB/c mice were co-cultured for 20 h with YAC-1 tumor cells infected or not with *Mycoplasma arginini*. The cytotoxic activity was evaluated by MTT assay and nitrite levels were determined with the Griess reagent. Thioglycollate-elicited macrophages co-cultured with noninfected YAC-1 cells showed low cytotoxic activity (34.7 ± 8.6%) and low production of NO (4.7 ± 3.1 µM NO_2^-). These macrophages co-cultured with mycoplasma-infected YAC-1 cells showed significantly higher cytotoxic activity (61.4 ± 9.1%; $P<0.05$) and higher NO production (48.5 ± 13 µM NO_2^-; $P<0.05$). Addition of L-NAME (10 mM), an inhibitor of NO synthesis, to these co-cultures reduced the cytotoxic activity to 37.4 ± 2% ($P<0.05$) and NO production to 3 ± 4 µM NO_2^- ($P<0.05$). The present data show that *Mycoplasma arginini* is able to induce macrophage cytotoxic activity and that this activity is partially mediated by NO.

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- Macrophages
- Cytotoxicity
- Mycoplasma
- Tumor cells
- Nitric oxide

Macrophages are usually found in tumor infiltrates where they exert cytostatic/cytotoxic activities against tumor cells. The tumoricidal activity is enhanced by activation of macrophages with bacterial products or cytokines (1,2). Recently nitric oxide (NO) has been indicated as a critical effector molecule for macrophage anti-tumor activity (3,4). Macrophages can be induced to release NO upon stimulation with a variety of stimuli such as bacterial products or cytokines (3,5). More recently it has been reported that mycoplasma-treated macrophages release large amounts of NO (6).

YAC-1 tumor cells have been classically used as targets for natural killer (NK) cells. Resident macrophages do not present anti-YAC-1 activity, but lymphokine-activated macrophages are able to kill YAC-1 cells (7). The mechanism by which lymphokine-

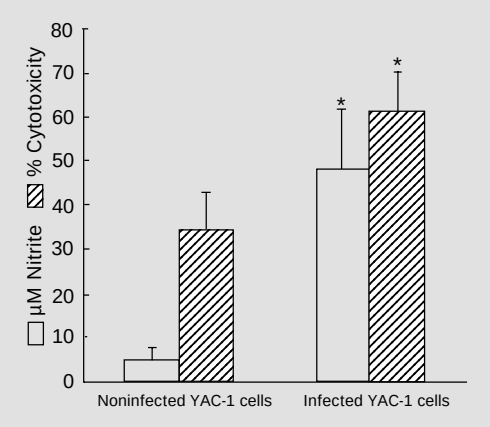

Figure 1 - *Mycoplasma arginini*-infected YAC-1 tumor cells induce macrophage cytotoxic activity and nitric oxide production. Thioglycollate-elicited peritoneal macrophages (2 x 10^5) were co-cultured with YAC-1 tumor cells infected or not with *Mycoplasma arginini* (5 x 10^4) for 20 h. Nitrite concentration was measured by the Griess reaction and macrophage cytotoxicity by the MTT assay. Results are reported as means ± SD for N = 4. *P<0.05 (Student *t*-test).

activated macrophages kill YAC-1 cells remains unsettled.

Based on these observations, we asked whether mycoplasma-infected YAC-1 tumor cells could stimulate macrophages to release NO and if this molecule, in turn, will lyse the infected YAC-1 cells.

Six- to eight-week-old male BALB/c mice were used throughout the experiments. Macrophages were harvested by lavage of the peritoneal cavity with 5 ml of phosphate buffered saline (PBS) 5 days after intraperitoneal injection of 1 ml of 3% thioglycollate medium (Gibco, Gaithersburg, MD). The cells were centrifuged and adjusted to 2 x 10^6 cells/ml in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10 mM HEPES, 11 mM sodium bicarbonate, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 5% fetal bovine serum. The cell suspensions were plated (100 µl/well) onto 96-well flat-bottomed plates (Corning, New York, NY) and incubated for 2.5 h for adherence in a humidified atmosphere with 5% CO_2 at 37°C. The nonadherent cells were removed by washing the wells with PBS. The adherent cells were 80-90% macrophages as judged by neutral red uptake.

Mycoplasma-free YAC-1 tumor cells were cultured with supernatants from *Mycoplasma arginini*-infected cells (20% v/v) in an antibiotic-free medium. Infection was confirmed by culture of supernatants in SP4 medium (8). The infected cells were maintained in complete medium. For cytotoxicity assays and induction of NO release, aliquots of tumor cell cultures were centrifuged, washed, resuspended in complete medium and adjusted to 5 x 10^5 cells/ml. Tumor cell suspensions (used as target cells) were added to macrophage cultures (effector cells) at a 4:1 effector to target (E:T) ratio. Effector and target cells (at the same cell number/well) were separately incubated in complete medium (100 µl/well) to establish control absorbance at 540 nm. The plates were incubated for 20 h in 5% CO_2 in a humidified atmosphere for cytotoxicity and NO release assays.

Macrophage anti-YAC-1 activity was evaluated by the MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide; Sigma) assay as described by Ferrari et al. (9). Briefly, 10 µl of MTT (5 mg/ml in PBS) was added to wells and the plates were incubated for 3 h. After this, 100 µl of 10% SDS in 0.01 N HCl was added in order to dissolve the formazan crystals generated by MTT reduction by living cells. The plates were incubated overnight and the absorbance was read at 540 nm in a microplate Labsystem Multiskan MS reader. Percent cytotoxicity was determined by the formula: % cytotoxicity = 1 - [(absorbance at 540 nm of effector + target cells) - (absorbance at 540 nm of effector cells)]/[absorbance at 540 nm of target cells] x 100.

NO release was determined by the standard Griess reaction as follows: 50 µl of test solutions was added to 96-well flat-bottomed plates containing 50 µl/well of Griess reagent (1% sulfanilamide/0.1% N-1-naphthylethylene diamine dihydrochloride/2.5% H_3PO_4). After 10 min at room temperature the absorbance of each well was measured in a Dynatech MR5000 microplate reader at 540 nm with a 620-nm reference filter and the nitrite concentration was determined from a sodium nitrite standard curve.

To prevent NO release we added 10 mM

L-NAME (N^{ω}-L-arginine methyl ester; Sigma), an inhibitor of NO synthesis to the co-cultures. This concentration of L-NAME did not affect the viability of macrophages or tumor cells as evaluated by the MTT assay.

Initially we compared NO production in co-cultures of thioglycollate-elicited macrophages with YAC-1 cells infected or not with mycoplasma. As shown in Figure 1, macrophages co-cultured with mycoplasma-free YAC-1 cells produced very low levels of NO (4.7 ± 3.1 µM NO_2^-). Conversely, high levels of NO (48.5 ± 13 µM of NO_2^-) were detected in co-cultures with mycoplasma-infected YAC-1 cells. Figure 1 also shows that thioglycollate-elicited macrophages co-cultured with mycoplasma-free YAC-1 cells exhibited low cytotoxic activity (34.7 ± 8.6%). In contrast, macrophage cytotoxic activity increased significantly when tested against *Mycoplasma arginini*-infected YAC-1 cells (61.4 ± 9.1%, $P<0.05$). Next, we asked whether inhibition of NO production by L-NAME would affect macrophage cytotoxicity. As shown in Figure 2, addition of 10 mM L-NAME to macrophage co-cultures with mycoplasma-infected YAC-1 cells almost completely inhibited NO release when compared with untreated co-cultures (51.3 ± 3 *vs* 3 ± 4 µM NO_2^-). Most importantly, inhibition of NO production was paralleled by a significant decrease in macrophage cytotoxicity (64.3 ± 4 *vs* 37.4 ± 2%).

Our results suggest that YAC-1 cells infected with *Mycoplasma arginini* stimulate macrophage NO production. In turn, NO exerts a cytotoxic activity against these tumor cells. However, it appears that YAC-1 cell killing does not rely entirely on NO release. This is documented in experiments where NO production was totally inhibited but significant cytotoxicity was still present (50%). Thus, it remains to be determined whether macrophages are able to kill YAC-1 cells by an NO-independent mechanism or contaminating NK cells are operating in this situation. Mycoplasma-induced macrophage NO production has been previously documented. For example, Mühlradt & Schade (10) found that murine resident peritoneal macrophages primed with interferon γ (IFNγ) released significant amounts of NO, when stimulated with a product extracted from *Mycoplasma fermentans*. Similarly, Yang et al. (11) showed that *Mycoplasma arginini*-infected L5178Y, a T-cell lymphoma, produces a triggering factor for NO production by thioglycollate-elicited macrophages primed with IFNγ. Interestingly, in our experiments *Mycoplasma arginini*-infected tumor cells were able to induce NO production in thioglycollate-elicited macrophages in the absence of exogenous cytokines.

Since mycoplasmas are potentially associated with animal and human diseases including immunodeficiencies (12) it is possible that macrophage activation induced by mycoplasma may contribute to clinical manifestations of these disorders. Finally, mycoplasma frequently contaminates cell cultures (13), and thus may alter macrophage or other cell functions assayed *in vitro*.

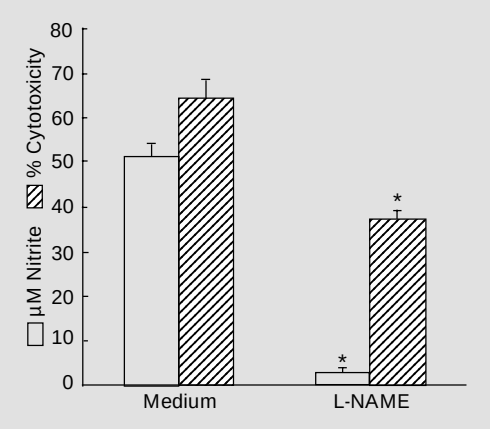

Figure 2 - Macrophage cytotoxic activity against *Mycoplasma arginini*-infected YAC-1 tumor cells is partially mediated by nitric oxide. Thioglycollate-elicited macrophages (2×10^5) were co-cultured with *Mycoplasma arginini*-infected YAC-1 tumor cells (5×10^4) in the presence or absence of 10 mM L-NAME for 20 h. Nitrite concentration was measured by the Griess reaction and macrophage cytotoxicity by the MTT assay. Results are reported as means ± SD for N = 4. *P<0.05 (Student *t*-test).

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