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Cytotoxic activity of BCG-activated macrophages against L929 tumor cells is nitric oxide-dependent

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Abstract

The tumoricidal activity of activated macrophages has been attributed largely to the release of tumor necrosis factor (TNF), or to the production of reactive oxygen or nitrogen intermediates. The L929 tumor cell line (a murine fibroblast-like cell) when treated with actinomycin D (ActD) has been used to measure TNFα cytotoxicity. In the present study, we determined the cytotoxic activity of BCG-activated peritoneal macrophages against ActD-untreated L929 tumor cells. Furthermore, we measured the production of hydrogen peroxide (H_2O_2), nitric oxide (NO) and TNF by macrophages cultured in the presence or absence of L929 cells. As expected, BCG-activated macrophages produced significant amounts of H_2O_2 (16.0 ± 3.0 µM), TNF (512 U/ml) and NO (71.5 ± 3.2 µM). TNF (256 U/ml) and NO (78.9 ± 9.7 µM) production was unchanged in co-cultures of L929 cells with BCG-activated macrophages but H_2O_2 production was totally inhibited. The cytotoxic activity was dependent on NO release since L-NAME (2.5, 5.0 and 10 mM), which blocks NO synthase, inhibited the killing of L929 cells. Addition of anti-TNF (20 µg/ml) antibodies to the cultures did not affect the tumoricidal activity of macrophages. Our results indicate that macrophage-mediated killing of L929 cells is largely dependent on NO production but independent of H_2O_2 or TNF release.

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Activated macrophages exhibit enhanced microbicidal and tumoricidal activities. Over the past 20 years, evidence has accumulated that these activities may be mediated by molecules such as tumor necrosis factor (TNF) (1), toxic products derived from the oxidative burst (2), and reactive nitrogen intermediates (3).

The L929 fibrosarcoma cell line when pretreated with actinomycin D (ActD) becomes extremely sensitive to TNF-mediated lysis (4). Consequently, it has been used extensively to measure the presence of TNF in body fluids and in culture supernatants. In the absence of ActD, L929 cells appear to be resistant to TNF-mediated cytotoxicity (5).

Although the role of hydrogen peroxide or reactive oxygen intermediates in tumor killing is well documented (2,6), the effect of oxygen reactive intermediates on L929 tumor cells has not been determined. However, Shoji et al. (5) reported that DNA damage induced by TNF in L929 cells is medi-

ated by formation of mitochondrial oxygen radicals.

Recently, the role of nitric oxide (NO) in tumor cell destruction by activated macrophages or by human monocytes has been described (7,8). The role of NO in L929 killing is still controversial. Cui and colleagues (9) showed that exposure to NO or to an NO donor does not result in DNA fragmentation of L929 cells. On the other hand, Fast et al. (10) found that exogenous NO is cytotoxic to L929 cells. Moreover, the latter investigators also showed that exposure of L929 cells to TNF results in NO formation. Nevertheless, inhibition of NO release by N-MMA did not block TNF-mediated cell killing (10). Controversy remains as to the involvement of NO in L929 killing by activated macrophages.

The purpose of the present study was to determine which of the effector molecules outlined above are released in co-cultures of activated macrophages with L929 cells and which exhibit tumoricidal activity.

The L929 cell line was originally obtained from the American Type Culture Collection (Rockville, MD, USA) and has been maintained in our laboratory for more than 8 years. L929 cells were harvested from culture by mild trypsin digestion after growing to confluence. In all experiments, 3.5 x 10^4 cells were seeded in 96-well flat-bottomed tissue culture plates and cultured overnight before the addition of macrophages. Activated macrophages were obtained from peritoneal cavities of C3H mice injected twice (days 0 and 14) with 2 mg of live or heat-killed BCG (ONCO BCG oral 500 mg, Instituto Butantan, São Paulo, Brazil) by washing the peritoneal cavity 4 days after the last intraperitoneal (*ip*) injection with 5 ml of ice-cold phosphate-buffered saline (PBS) and were diluted to 2 x 10^6 cells/ml.

A method was developed to measure hydrogen peroxide (H_2O_2), TNF and NO production that allowed us to determine the release of these effector molecules sequentially by the same macrophage population (cultured alone or co-cultured with L929 cells). We first measured H_2O_2 release by the horseradish peroxidase-dependent phenol red oxidation method developed by Pick and Keisari (11) and adapted by Russo et al. (12). BCG-activated peritoneal cells (2 x 10^5/100 µl) were plated onto each empty well or overlaid on cultured L929 cells and incubated for 1 h in Dulbecco's PBS containing 5 mM dextrose, 0.28 mM phenol red and 5 µg horseradish peroxidase at 37°C in a humidified atmosphere of 5% CO_2. After incubation, the plates were centrifuged at 150 *g* for 3 min, 100 µl of the supernatants were transferred to microtiter plates containing 10 µl of NaOH to stop the reaction, and absorbance was read at 620 nm with a Dynatech microplate reader. Next, the cells were washed three times and cultured in RPMI 1640 with 5% fetal calf serum (FCS) (100 µl/well) for 4 h. After centrifugation, 50 µl were collected from each well and TNF was measured by the lytic assay of ActD-treated L929 cells (4). Fifty microliters of complete medium were added back to the wells and the plates were incubated for an additional 48 h. NO production was quantified by the accumulation of nitrite in the supernatants using the standard Griess method (13).

As shown in Table 1, BCG-activated macrophages released significant amounts of H_2O_2, TNF and NO when cultured in the absence of L929 cells. Similar results were obtained with heat-killed BCG (data not shown). However, in the presence of L929 cells, H_2O_2 production was completely inhibited while TNF and NO release were not affected. Since H_2O_2 release was totally suppressed we conclude that this molecule does not participate in L929 killing in our system. Next we tested whether the addition of anti-TNF antibodies (Endogen, Cambridge, MA, USA) or N^{ω}-L-arginine methyl esther (L-NAME) (Sigma Chemical Co., St. Louis, MO, USA), an inhibitor of NO synthase, would affect the L929 killing. The killing of

L929 cells was determined by staining the remaining cells with crystal violet (4) and cytolytic activity is reported as percent tumor cytotoxicity, where % cytotoxicity = (1 - A_{620} of L929 cells co-cultured with macrophages/A_{620} of control L929 cells) x 100.

As shown in Figure 1, addition of 20 µg/ml of anti-TNF antibodies that were capable of neutralizing more than 2,000 units of TNF did not affect NO production or macrophage cytotoxicity against L929 cells. In contrast, addition of L-NAME to the co-cultures inhibited NO release and macrophage cytotoxicity. The inhibition of NO production by L-NAME was dose dependent. However, cytotoxicity did not follow this pattern. Although, a clear inhibition of cytotoxicity (40%) was observed with L-NAME, increasing its concentration did not further increase L929 cell lysis.

The most important finding of the present study is that BCG-activated macrophages kill L929 tumor cells by a mechanism that is dependent on NO production but independent of H_2O_2 or TNF release.

Since the H_2O_2 release was totally inhibited upon macrophage contact with L929 cells we ruled out the participation of this metabolite in the killing process. Moreover, we have found that resident macrophages, which do not release oxygen intermediates, were able to kill L929 cells via an NO-dependent mechanism (Nascimento FRF, Ribeiro-Dias F and Russo M, unpublished results). The mechanism by which the oxidative burst is inhibited in co-cultures is unknown. We have previously shown that adherence and spreading of BCG-activated macrophages onto a polystyrene plastic surface is a sufficient signal to trigger H_2O_2 release (12). In our experiments we have observed by inverted phase microscopy that macrophages did not spread when co-cultured with L929 cells, whereas rapid macrophage spreading did occur in the absence of L929 cells. Thus, it appears that the contact between macrophages and L929 cells is insufficient to trigger the respiratory burst. In agreement with this assumption, it has been shown that phagocytes cultured on biological surfaces such as endothelial cells or extracellular matrix proteins are unable to release reactive oxygen intermediates (14; Rodriguez D, Nascimento FRF, Postól E and

Table 1 – Effect of L929 tumor cells on production of H_2O_2, NO and TNF by BCG-activated macrophages.

Peritoneal macrophages were cultured in the presence or in the absence of L929 cells. The H_2O_2 was measured after 1 h, TNFα after 4 h and NO after 48 h of culture. Values represent the mean ± SD for 5 individual mice. TNF U/ml were defined as the reciprocal of sample dilutions required to induce 50% lysis of L929 cells. Results are representative of one of three experiments run independently. *Significant differences between groups (P<0.05) (Student *t*-test).

	Macrophages (Mϕ)	Mϕ + L929 cells
H_2O_2 (µM)	16.0 ± 3.0	0.0*
TNF (U/ml)	512	256
NO (µM)	71.5 ± 3.2	78.9 ± 9.7

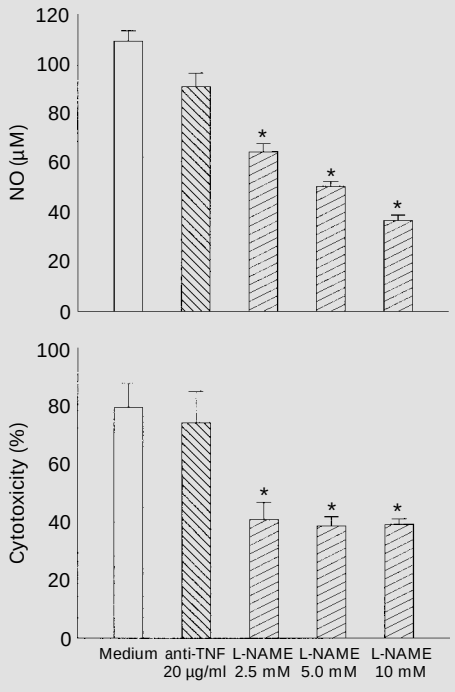

Figure 1 – Effect of anti-TNF antibodies or L-NAME (iNOS inhibitor) treatment on NO production and macrophage cytotoxicity. BCG-activated macrophages were co-cultured with L929 cells in the presence of anti-TNF antibodies (20 µg/ml) or L-NAME (2.5, 5.0 and 10 mM) and nitrite concentration and cytotoxicity were determined 48 h later. Values represent the mean ± SD for 5 individual mice. *Significant differences between groups (P<0.05) (Student *t*-test).

Russo M, unpublished results).

It is puzzling that TNF is also not involved in L929 cytotoxicity since it has been previously shown that L929 killing by BCG-activated macrophages was significantly inhibited by anti-TNF antibodies (15). Moreover, L929 cells have been widely used as a target for TNF-mediated cytotoxicity (4). The following possible explanations for these conflicting results may be proposed. First, L929 cells appear to be resistant to TNF lysis if pretreatment with ActD is withheld (5). In support of this observation, we have found that exposure of ActD-untreated L929 cells to high doses (>500 U/ml) of serum-rich murine TNF or recombinant human TNF (>2,000 U/ml) did not result in any L929 killing (Nascimento FRF, Ribeiro-Dias F and Russo M, unpublished results). Alternatively, L929 may be sensitive to the TNF-lytic pathway when TNF is added during the seeding process but not after L929 cells have reached confluence. These possibilities may reconcile our results with those published previously (15,16). In our experiments activated macrophages were added to monolayers of L929 cells cultured overnight, whereas in other studies trypsin-detached L929 cells were added to adherent macrophages (9,15,16). It follows that the cell cycle and sensitivity of L929 cells to TNF lysis may be quite different in these two experimental situations, a possibility which is currently being investigated in our laboratory.

Taken together, our results indicate that NO is a critical effector molecule for macrophage-mediated L929 cell cytotoxicity.

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