Measurement of Cytotoxic Activity in Experimental Cancer

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Several tumor cell systems have been developed for investigating the cytotoxic activity of different substances. The Ehrlich ascytic tumor (EAT) has been studied extensively and used to increase our comprehension of immunotherapy (Yamamoto and Naraparaju. *Cancer Res* 57:2187–2192, 1997). In this study, we evaluated the ability of an enzymatic method to assess cytotoxic tumor activity in vitro. To prepare cytotoxic cells, lymphoid cells isolated from the lymph nodes of C57BL/6 mice were activated with interleukin-2 (IL-2). The cytotoxic activity to EAT cells was assessed by an enzymatic test using lactate dehydrogenase (LDH), an enzyme widely distributed in mammalian tissues which, in

the presence of NAD/NADH, converts lactate to pyruvate. IL-2 treatment of lymph node cells induced a greater percentage of lysis (75.47%) than effector cells that were not treated with IL-2 (42.38%). This suggests that IL-2 directly activates effector cells and not tumor cells. The results demonstrate that the LDH release-measurement method can be utilized to assay cytotoxic cell-mediated activity in which murine tumor cells act as the target. Levels of IL-2 appear to have a direct correlation to cellular immunity and treatment with these substances may strengthen immune function and decrease the pace of disease development. *J. Clin. Lab. Anal.* 18:27–30, 2004. © 2004 Wiley-Liss, Inc.

Key words: LDH; IL-2; tumor cells; cytotoxicity

INTRODUCTION

Within the context of immunological surveillance, mononuclear cell-mediated cytotoxic activity plays a critical role, particularly with regard to the pathophysiology of malignant diseases. A better understanding of that role may facilitate the development of new immunotherapeutic strategies. Interleukin-2 (IL-2) belongs to a class of soluble, regulatory proteins known as cytokines. IL-2 is a 133 amino acid glycoprotein secreted by T(CD4) lymphocytes and other cells following activation by antigens, mitogens, and other cytokines. It stimulates the proliferation and cytotoxicity of T lymphocytes and enhances the microbicidal and cytotoxic activities of natural killer (NK) cells, B lymphocytes, macrophages, and monocytes (1). Many studies of immunotherapy in murine and human systems have employed experimental approaches that use cytokines and associations with cytotoxic and biological agents to combat tumor cells (2–5). Cancer is a complex disease process that involves associations between invasive tumor cells, its products, and effector cells from the host immune response. Recently, there have

been many reports using cytokines for immune restoration (6–8).

Thus, in this study, we evaluated the effect of IL-2 on the cytotoxic activity in vitro of lymph node cells from C57BL/6 mice on Ehrlich ascytic tumor (EAT) cells. This cellular interaction in vitro was appropriately demonstrated by measuring lactate dehydrogenase (LDH). Although there are differences in the amount of LDH present in different cell types, the total enzyme activity in a sample of cell lysate is directly proportional to the concentration of cells in the sample (9). Therefore, cytotoxicity based on the corresponding amount of enzyme LDH released into the culture supernatant is considered a nonradioactive method that is rapid, reliable, and cost-effective (10). In this report, we

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demonstrate the capability of the LDH enzymatic method to measure cytotoxic activity of IL-2 treated cells isolated from pelvic lymph nodes on EAT cells.

MATERIALS AND METHODS

In this study, we used female isogenic C57BL/6 mice, weighing 20 g each, from the Biothery of Basic Immunology at Londrina State University. The animals were cared for in accordance with Human and Animals Ethics Committee of Londrina State University.

Effector Cells

The mononuclear cells were obtained from pelvic lymph nodes of mice killed by cervical displacement. The isolated cells were washed with RPMI-1640 medium. After centrifugation ($50 \times g$), these cells were incubated in RPMI medium without phenol (supplemented with 10% of fetal bovine serum and 0.1% Gentamycin) to the concentration of 10^5 cells/well in a plate of 96 wells (flat-bottom), in the presence or absence of IL-2 for 24 hr at 37°C . In the experiments IL-2 was used at $50 \text{ IU}/10^5$ cells.

Target Cells

After 12 days, cells of EAT were obtained from the C57BL/6 mice, following intraperitoneal inoculation. Cells were washed by centrifugation ($50 \times g$) and incubated in RPMI medium at a final concentration of 10^4 cells/mL.

Cytotoxic Activity Assay

Effectors cells were plated (96 flat-bottom) to 10^5 cells/well and tumor cells were added to 10^3 cells. Triton was used as a positive control for cellular lysis. The cytotoxic activity of lymph node cells on EAT cells was evaluated after 4 hr by the enzymatic measurement of LDH on the supernatant of cultures at 340 nm (11). The LDH method measures the oxidation of L-lactate to pyruvate with simultaneous reduction of nicotinamide adenine dinucleotide (NAD). Due to the appearance of reduced NAD (NADH), the change in absorbance at 340 nm is directly proportional to the LDH activity because other reactants are present in nonrate limiting quantities. This change is measured using a bichromatic (340,383 nm) rate technique.

This test was performed on the Dimension[®] clinical chemistry system, using the LDH FLEX[®] reagent cartridge (Dade Behring, Inc., Newark, NJ).

Statistical Analysis

The results were analyzed using the Student's *t*-test to calculate their statistical significance. *P*-values higher than 0.05 were not considered significant.

RESULTS

The cytotoxic activity of T cells is very important in an immunological response against the spread of malignant disease.

Effector cells were dispensed into microtitration plates of 96 wells (flat-bottom), followed by 10^3 target cells per well, giving effector-to-target (E:T) ratios of 100:1. For LDH assay, 80 μl from the culture supernatant was used. Figure 1 shows values (concentration of LDH = U/L) of specific killing measured. The culture of IL-2 treated cells + tumor cells presented LDH values significantly different in relation to other cultures.

In the LDH release assay, values were previously normalized by subtraction of the LDH backgrounds, originating from the spontaneous release of effector cells as demonstrated in Table 1. The effector cells treated

Fig. 1. Cytotoxicity assay by lactate dehydrogenase release (U/L). A, tumor cells + triton (positive control); B, effector cells; C, effectors cells + IL-2; D, tumor cells; E, effector cells + tumor cells; and F, IL-2 treated cells + tumor cells ($P < 0.05$). Values are mean $\pm$ SD. Each experiment was carried out in triplicate.

TABLE 1. Killing (percentage) against Ehrlich ascytic tumor cells as determined by lactate dehydrogenase (LDH) release

Group	Cellular lyse (%)
Target cells + IL-2	18,48
Effector cells + Target cells	42,38
Effector cells + Target cells + IL-2	75,47
Target cells + Triton	100

with IL-2 induced a greater percentage of lysis (75.47%) than effector cells not treated with IL-2 (42.38%). We also performed the culture cells with RPMI medium with phenol, and we verified the alteration of medium color as in normal cellular culture.

DISCUSSION

Cell-mediated immune responses constitute a major defense against the spread of cancer. Immunologic manipulation of the host with various systemic agents, in particular IL-2, frequently affects this natural phenomenon and can lead to the complete rejection of cancer (12). Therefore, the evaluation of cytotoxic reactions is of clinical importance in the understanding of tumor regression. Cytotoxicity is generally detected by the release of intracellular radioactive label from lysed cells, e.g., chromium 51 (^{51}Cr) release assay (13). However, the ^{51}Cr release assay requires special laboratory facilities and techniques for the storage of radioactive materials and for protection from exposure to radioactivity. Furthermore, the major disadvantage of ^{51}Cr labeling is the high spontaneous release of target cells combined with the need for an incubation step of 24 hr, and several cell washes.

To circumvent these limitations, we measured lymph node cell activity from the C57/BL6 mice using a nonradioactive assay that measured the release of LDH enzymatically.

As shown in Table 1 and Fig. 1, IL-2 administered directly to tumor cells induced a low percentage of cell lysis, most likely arising from the activation of immune cells present in low quantities in the locality of the tumor. The rationale for this approach can be that tumor infiltrating lymphocytes are enriched for tumor specific cytotoxic T lymphocytes (CTL) that were activated by IL-2. In contrast, lymph node cells treated with IL-2 induced a greater percentage of lysis (75.47%) than effector cells not treated with IL-2 (42.38%). This suggests that IL-2 directly activates effector cells and not tumor cells. This *in vitro* assay, based on the LDH measurement, showed itself to be an effective alternative to the ^{51}Cr release assay for the determination of cytotoxicity that is mediated by T-cells when tumor cells are used as the target.

It has been demonstrated that the LDH release assay was as sensitive as the Cr release assay in assessing NK activity in HIV-negative and HIV-positive subjects, and this may be an advantageous alternative to the ^{51}Cr release assay (11). Thus, cytotoxicity against many isolated tumor cells can be analyzed reliably, reproducibly, and without the use of isotopes by LDH release assay. Although some researchers have only investigated the use of this assay for NK activity, we demonstrated

that it can also be readily applied to measure other forms of cell-mediated activity, such as cellular cytotoxicity in tumor cell systems.

LDH has been used extensively as a marker for cell death, both *in vitro* and *in vivo*. The release of LDH into tissue culture medium accurately reflects cell viability *in vitro*. We have investigated the relationship between cell concentration and LDH activity in samples from cultures, taking into consideration the medium and the spontaneous release of effector and target cells when appropriate. In our protocols, we always used positive and negative controls. The associated immunological research has been complicated to some extent because, in conventional ^{51}Cr release assays, target cells frequently fail to incorporate sufficient amounts of ^{51}Cr and/or spontaneously release high amounts of ^{51}Cr .

The measurement of LDH activity *in vitro* provides a sensitive, accurate, and cost-effective alternative to the use of either radioisotopic or dye-based assays for determination of cytotoxicity. Using this assay, we were able to detect the cytotoxicity of IL-2 activated lymph node cells in mice.

Treatment of cancer patients in remission using immunotherapy with IL-2 is an approach that can be used to prolong the duration of remission. Activation of cytotoxic effector lymphocytes has been frequently suggested as an important mechanism for the pathophysiology of eradication of residual tumor populations.

Immune-based therapy is a new field of study. *In vitro* cytotoxic assays using LDH as the reporter of effector cell and tumor cell interaction may be a helpful addition to the study of cancer immunotherapy.

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