A Radioisotopic Microassay for Lymphotoxin Using Technetium-99 Labeled Cells[1,2] (37636)

ROLF F. BARTH, G. YANCEY GILLESPIE, AND WERNER ROSENAU

The Department of Pathology and Oncology, University of Kansas Medical Center, Kansas City, Kansas 66103; and The Department of Pathology, University of California, San Francisco, California 94122

The *in vitro* stimulation of lymphocytes, either specifically by antigens to which the donor has been sensitized or nonspecifically by phytohemagglutinin (PHA), results in the production of lymphotoxin (1, 2). This substance has been shown to produce nonspecific cytolysis of a variety of cell types (3) using assays based on either the enumeration of surviving cells by an electronic counter (4–6) or the cellular release (7–9) or incorporation (2, 9) of radioisotopes. Although assays based on the monitoring of ^{51}Cr release have been successfully employed (7–9), a high level of spontaneous leakage is a potentially serious problem in long term cultures (7, 8). The incorporation of ^{14}C-amino acids by surviving target cells has been successfully used to assess the cytocidal effect of lymphotoxin. This procedure, however, is time-consuming and requires disruption of surviving cells, followed by protein precipitation with trichloracetic acid, centrifugation, and finally solubilization of the pellet. Recently a microassay based on visual enumeration of surviving target cells has been reported (10) and found to be superior to the assay employing ^{14}C-amino acid incorporation (2), although difficulty in obtaining consistency in target cell number from test well to test well proved a troublesome but not insurmountable problem (10).

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Limitations of previously described methods for assessing lymphotoxin activity together with our recent success in employing technetium-99m (^{99m}Tc) as a radioisotopic label (11–14) prompted us to develop a new microassay employing murine target cells prelabeled with ^{99m}Tc. This is a metastable radioisotope ($T_{½} = 6.0$) emitting a gamma photon with an energy of 140 keV. The short labeling time, high specific activity, and low spontaneous release have proved to be significant advantages of ^{99m}Tc and the assay which we have developed appears to be a simple and sensitive means of assessing lymphotoxin activity.

Materials and Methods. Preparation of lymphotoxin. Lymphotoxin was prepared from supernatants of human lymphocyte cultures which had been stimulated with Phytohemagglutinin-*P* (Difco Laboratories, Detroit, Michigan) by sequential use of Sephadex and DEAE cellulose column chromatography and preparative acrylamide gel electrophoresis (6). This procedure resulted in greater than 2,000-fold purification. The lymphotoxin was dissolved in buffer (0.1 *M* glycine, 0.15 *M* NaCl, pH 9.5) and stored at —75° without loss of activity. Buffer eluates of blank acrylamide gels were used as control material. This control eluate was noncytotoxic and yielded the same results as control material derived from lymphocyte cultures to which PHA had been added only at the moment of terminating the incubation and then fractionated as described for lymphotoxin. A cytotoxic unit (CU) of lymphotoxin was defined as that amount producing a difference of 1,000 mouse L-cells (Clone 929) between control and lymphotoxin-treated cultures under specific

conditions (6).

Preparation of radiolabeled target cells. L-cells (Clone 929) which had been grown in medium RPMI 1640 supplemented with 20% newborn calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin were used as target cells. A single cell suspension was prepared from monolayer cultures by treating them with 0.25% trypsin in calcium and magnesium free phosphate buffered saline (pH 7.4). The cells were washed once in Hanks' Balanced Salt Solution (HBSS), resuspended in HBSS, and their final concentration was adjusted to 10^7 viable cells in 10 ml HBSS containing 100 μg of sodium chromate. Sodium pertechnetate (Na $^{99m}TcO_4$) was eluted with 0.9% saline from a New England Nuclear molybdenum–technetium generator. The procedure used to label cells was the same as that which we previously described (13, 14). Ten mCi of $^{99m}TcO_4^-$ were added to the cell suspension which was then incubated for 15 min at 37° with occasional shaking. Following this, the valence of ^{99m}Tc was reduced (15, 16) by the dropwise addition of 0.2–0.3 ml of a sterile solution of 0.2% $SnCl_2 \cdot 2H_2O$. The stannous chloride was dissolved in freshly prepared acid citrate dextrose (30 g $Na_3C_6H_5O_7$, 0.15 g NaH_2PO_4, and 2.0 g dextrose/liter of distilled H_2O, pH adjusted to 7.4) and sterilized by passage through a 0.22 μm Millipore filter. After an additional incubation for 15 min at 37°, the cells were sedimented by centrifugation at 400*g* for 10 min and the unbound radioisotope was removed by washing them three times in 35 cc vol of HBSS.

Lymphotoxin assay. Ten thousand viable ^{99m}Tc-labeled L-cells (10 μliter) were dispensed into each of 96 wells of a Linbro tissue culture plate (IS-FB-96-TC) using a 500 μliter Hamilton syringe equipped with a repeating dispenser. Each well contained 0.2 ml RPMI 1640 supplemented with 10% newborn calf serum. The plate was incubated for 18 hr at 37° in a humidified atmosphere containing 5% CO_2 and 95% air prior to the addition of lymphotoxin or control material. A dose–response study was carried out by adding 0.1 ml of medium containing 100, 50, 25, or 12.5 CU of lymphotoxin or corresponding dilutions of control material and continuing incubation for an additional 28 hr. Identical sets of dilutions consisting of 6 replicates were set up in each half of the plate in order to permit comparison between gamma counting and visual enumeration of surviving cells. A time-response study was performed in a similar manner by adding 0.1 ml of medium containing 100 CU of lymphotoxin or corresponding dilutions of control material to the plate 12, 20, and 28 hr before termination of the experiment. At termination the medium was discarded and the wells were washed twice with HBSS to remove dead cells. In preliminary tests supravital staining with eosin-trypan blue revealed that the adherent cells excluded dye and the nonadherent cells did not. Following removal of dead cells the plate was air-dried and then half was spray-fixed with a surgical adhesive (Aeroplast), and the other half was fixed with absolute methanol for 10 min. The bottoms of those wells which had been spray-fixed were punched out, using a length of metal tubing 6 mm in diameter, placed in 10- × 75-mm tubes, and radioactivity determined with a Nuclear Chicago model 1185 gamma scintillation counter. The remaining half of the plate was stained with a 0.1% solution of crystal violet and the surviving adherent cells were enumerated by visual counting of two representative fields in each well using an inverted microscope equipped with an ocular reticle.

Results. Dose-response study. A maximum cytocidal effect on L-cells was observed after 28 hr of incubation with 100 units of lymphotoxin. This decreased somewhat with 50 units and was not evident with amounts less than that (Fig. 1). The number of surviving L-cells counted after 28 hr exposure to 100 CU of lymphotoxin was 10/0.11 mm^2 as enumerated by visual counting and 1100 cpm as determined by gamma scintillation counting for ^{99m}Tc compared to 85/0.11 mm^2 and 2250 cpm when a corresponding dilution of control material was used. Generally, there was good agreement between the number of surviving cells enumerated by visual counting and the cell number as determined by gamma scintillation counting for ^{99m}Tc. We had demonstrated previously the existence of a

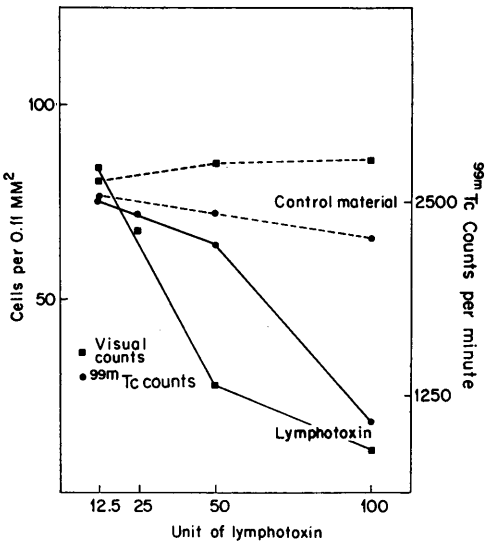

FIG. 1. Dose response of L-929 cells to lymphotoxin. ^{99m}Tc-labeled target cells were incubated with lymphotoxin or control material for 28 hr following which surviving adherent cells were enumerated by either visual or gamma scintillation counting. Each point represents the mean of six replicate determinations. Standard error was approximately ± 10%.

linear relationship between radioactivity expressed as cpm and graded numbers of cells (12) and this was confirmed by our present data.

The percent reduction of L-cells was calculated by subtracting the cpm (or number) of surviving cells in the presence of lymphotoxin (LT) from the cpm (or number) of surviving cells in the presence of control material (CM), dividing this by the cpm (or number) of surviving cells in the presence of control material, and multiplying the quotient by 100: percent reduction = $[cpm_{CM} - cpm_{LT}]/cpm_{CM} \times 100$.

Percent reduction was a linear function of the log units of lymphotoxin added to the culture (Fig. 2). The ^{99m}Tc microassay gave values approximately 10–15% greater than those determined by visual counting which suggests that the radioisotopic assay is a more sensitive test.

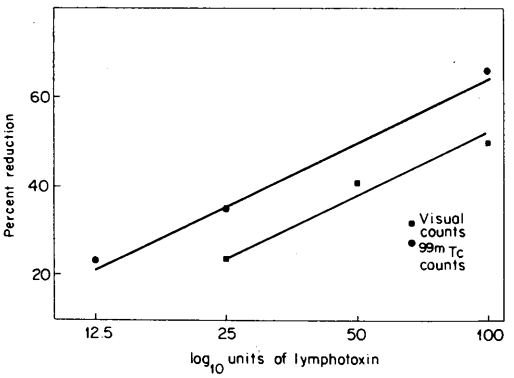

FIG. 2. Dose response of L-929 cells to lymphotoxin. The data are the same as those presented in Fig. 1. The percent reduction was calculated from the formula

$$\frac{[cpm(\text{or number})_{control} - cpm(\text{or number})_{LT}]}{cpm(\text{or number})_{control}} \times 100.$$

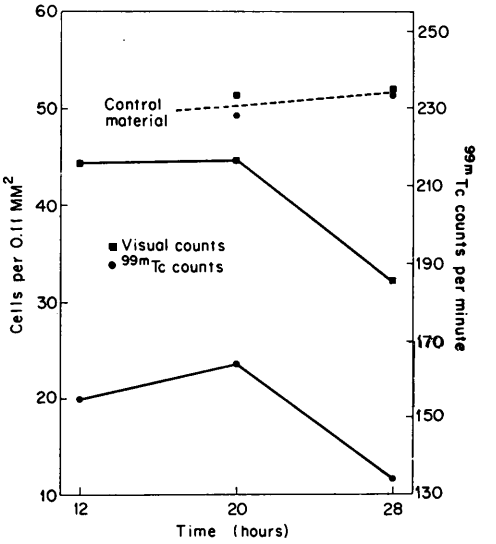

FIG. 3. Time response of L-929 cells to lymphotoxin. 100 cytotoxic units of lymphotoxin or a corresponding dilution of control material was added to the test plate 12, 20, and 28 hr prior to termination of the experiment. Surviving adherent cells were enumerated by visual or gamma scintillation counting at 28 hr. Each point represents the mean of six replicate determinations.

Time-dependency study. The cytolytic effect of 100 CU of lymphotoxin was first evident after 20 hr of incubation and was maximal after 28 hr (Fig. 3). The number of surviving adherent cells enumerated both visually and by gamma scintillation counting paralleled one another although higher cell numbers were recorded by the visual counting assay. This may have been due to the

proliferation of surviving cells following lysis. The radioisotopic pool of ^{99m}Tc on the other hand remained constant irrespective of the amount of cell proliferation and therefore was a more accurate indicator of cell lysis.

Discussion. The technetium-99m microassay is a sensitive, easily performed, and rapid test for lymphotoxin activity which appears to have a number of advantages over previously reported *in vitro* methods. Tube assays based on either electronic counting (1, 5) or incorporation of ^{14}C-amino acids by surviving cells (2, 9) require an inoculation density in the range of $1–2 \times 10^5$ cells per tube. Comparison of the assay using ^{14}C-amino acid incorporation (2) with a microassay based on visual enumeration of surviving cells (10) revealed the latter method to be significantly more sensitive. This enhanced sensitivity was attributed to the smaller number of cells per volume of toxic medium in the microassay (1,000–5,000 cell/well), thus increasing the number of lymphotoxin molecules available per cell (10). These observations would also appear to apply to the ^{99m}Tc microassay which we have developed since only 5,000–10,000 cells are required per well.

The ^{14}C-amino acid radioisotopic tube assay requires laborious, repetitive precipitation of labeled proteins from surviving cells (2, 9). In the ^{99m}Tc microassay surviving adherent cells can be enumerated immediately by gamma scintillation counting of the punched-out well bottoms. Visual counting of surviving cells is a tedious procedure which may be influenced by human subjectivity. In contrast, ^{99m}Tc-labeled cells are machine counted and therefore not influenced by this factor. Although a skilled technician may be able to count surviving cells accurately it requires hours instead of minutes to cover an entire 96-well plate. The ^{99m}Tc microassay is based on the detachment of dead and injured target cells and this appears to correlate with ^{51}Cr release (17) suggesting that it represents specific cellular injury or damage and not merely random detachment of undamaged viable cells. The spontaneous release of ^{51}Cr can be as much as 50% of the specific release after 40–48-hr incubation (18) and this may be a serious limitation. This problem is not encountered with ^{99m}Tc, which apparently is not released from monolayer cells in significant amounts in a reutilizable form (13). Furthermore, since the pool of radioisotope remains constant the enumeration of cells by gamma counting is not affected by their proliferation and is a true measure of cell lysis.

The high specific activity of the radioisotope adequately compensates for the 6-hr half life of ^{99m}Tc. Advantages of ^{99m}Tc include rapid labeling time without dependence upon mitosis for incorporation, low spontaneous release, nonreutilizability, and ready availability of the radioisotope in institutions where diagnostic clinical scanning is performed (13).

Summary. We have developed a new microassay for lymphotoxin utilizing murine L-929 target cells prelabeled with technetium-99m, a high specific activity, metastable gamma emitter which is bound to living cells and not released in appreciable quantities by dead or injured cells. Target L-cells prelabeled with ^{99m}Tc were added to micro tissue culture plates at a concentration of 10,000 cells/well. Eighteen hr later lymphotoxin or control material was added and the cells were incubated for an additional 12–28 hr. A maximum cytocidal effect was observed after incubation of target cells for 28 hr with 100 CU of lymphotoxin. The number of surviving adherent cells as determined by gamma counting for ^{99m}Tc was in accord with that recorded by visual enumeration. The ^{99m}Tc microassay is a sensitive, easily performed, rapid test for lymphotoxin activity which appears to have a number of advantages over previously reported *in vitro* methods.

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