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An Improved *In Vitro* Assay for Lymphotoxin

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An *In Vitro* Microassay for Lymphotoxin (LT) is described. Target cell monolayers are established in the wells of a microtiter assay plate at a density of 100-500/well. Dilutions of Lymphotoxin containing medium are placed upon the target cell monolayers. The plates are sealed with a gas impervious film, and the cells are incubated for 24-48 hr. The cell numbers in each well are established initially, and after the incubation period. The percent destruction is based upon total cell counts of experimental wells compared to control wells. Comparison of the assay to the previously described assay, which is based upon ability of cells to incorporate ¹⁴C amino acids into protein, with several batches of LT, shows the microassay to be 25× more sensitive than the latter. Other advantages and disadvantages of the assay system are also described.

INTRODUCTION

Several investigators have reported that lymphoid cells from experimental animals and man can release soluble cell toxins into the culture medium when stimulated with mitogens, i.e., phytohemagglutinin (PHA) (1,2) or various antigens (3-5). The cell toxin released by PHA-stimulated human lymphocytes in culture has been termed lymphotoxin (LT) and is nonspecifically cytolytic for various cell types *in vitro* (6). We previously assayed the *in vitro* effects of LT on tube cultures of target L cells by measuring the uptake of ¹⁴C-amino acids (AA) into cellular protein (1). The present report describes a method which (a) does not require isotopes, (b) facilitates the convenient assay of many samples, (c) is more sensitive than our previous methods, and (d) can be performed with as little as 0.1 ml of medium.

METHODS AND RESULTS

Cells and media. Mouse L-929 cells (Flow Laboratories, Los Angeles, California) were used exclusively as the target cells in these studies. The culture

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medium employed was either RPMI 1640 (Grand Island Biological Company, Berkeley, Calif.) or Hanks Minimal Essential Medium (MEM) (Flow Labs, Los Angeles, Calif.), supplemented as previously described (1), and with 5% or 10% Fetal Calf Serum (FCS). Human LT was prepared from cultures of PHA-stimulated lymphoid cells obtained from surgically removed adenoid tissue, as previously described (6). Conditioned medium was obtained from the cell-free supernatants of 24–48 hr monolayer cultures of stock L cells. Labeling medium employed in the standard ^{14}C -AA viability assay was composed of MEM minus AA, supplemented with 3% dialyzed FCS and $0.5\ \mu\text{C}$ ^{14}C -AA hydrolysate/ml (Schwartz Bio Research, Orangeburg, N.J.) as described previously (1). Target L cells for use in the improved assay were established as monolayer cultures in 96 well microtiter plates (Falcon Plastics, Oxnard, Calif.) (Fig. 1).

Establishing target cell monolayers. A cell suspension was prepared by trypsinizing a stock L cell monolayer. The cell density was established by viable cell counts performed in a Neubauer chamber. Fresh, conditioned medium obtained from 24 hr stock L cell cultures was spun at $300g$ for 5 min to remove all cells and the pH adjusted to approximately 6.7 with 7% sterile acetic acid. Target L cells were then added to the conditioned medium from the stock cell suspension to give a final cell concentration of 500–2500/ml, and 0.2 ml of this suspension was added to each well. Care was taken to thoroughly mix the suspension before each aliquot of cells was removed to insure a homogeneous distribution of target cells in each well. The microplate was then sealed with a gas-impervious, pressure-sensitive film (Falcon Plastics) and incubated at 37° for 8–16 hr to allow the cells to establish monolayers. Tube cultures of target L cells (50,000 cells/ml/tube) were established at the same time, as previously described (1).

We found that L cells most reproducibly establish themselves as monolayers

FIG. 1. A top view of the microplate with pressure sensitive film in place.

in the microplate wells when cultured in conditioned medium. Under these conditions, as few as 50–100 cells could be employed in each well. We found that 250 cells/well was an optimal concentration which was easy to count, resulted in uniform wells and gave maximum sensitivity. Additional experiments revealed that the L cells could be maintained with normal division rates in the microplates for periods up to 5 days. Each well in the plate has a total capacity of 0.3 ml. One difficulty we encountered was that when 0.1 ml was used, monolayer cells established themselves around the edges of the wells. However, this was ablated when 0.2 ml vol was employed. The plates were used after the cells became attached to the plastic, usually within 8–16 hr.

Microassay. The film was removed from the plate and the medium decanted off the individual wells. Test and control media were then diluted with fresh 1640 and 0.1 or 0.2 ml aliquots of each dilution was added to duplicate test wells. The various control media were placed on cells in an identical fashion. We found that the results were most reproducible when target cells were maintained in the same basic medium as that used for the lymphocyte cultures. The top of the plate was then dried and resealed with fresh film and the number of cells in individual wells established by cell counts under 40X magnification on an Olympus Model FH microscope (Tokyo, Japan). The microscope was equipped with an eyepiece which contained a grid to facilitate counting a uniform area of the cell monolayer. The plate was incubated at 37° and examined periodically over 48–72 hr. Final cell counts were made in a similar fashion as primary cell counts. Microscopic examination revealed that in general the destructive reaction was faster in the microtiter plates than in the tube cultures. Complete cytolysis occurred in the microplates by 24–48 hr, depending on the LT batch, whereas the same LT exposed to tube L cell cultures required 48–72 hr. Figure 2A shows an individual microwell which contains a 48 hr HLT-treated L cell monolayer. Figure 2B shows a well containing a control L cell monolayer from the same plate. We counted all intact cells, rounded or extended, which remained on the monolayer. We did additional experiments employing fluorescein diacetate vital staining (7), which showed that all the rounded cells were nonviable. The number of rounded cells remaining in each well was determined by the time and the strength of the LT preparation. Vital stains and ¹⁴C-AA labeling studies were also performed, which demonstrated that no viable cells were present in the supernatants of these cultures.

Comparison of the microassay technique with the ¹⁴C-AA incorporation technique was performed on various dilutions of the same batch of test and control media. After incubation at 37° for 48 hr, the tube culture monolayers were labeled with a ¹⁴C-AA hydrolysate (0.5 µc/ml) for 20 min. The cells were dissolved with 1.0 ml of 0.1 M KOH and precipitated with 10% trichloroacetic acid. The precipitate was solubilized and reprecipitated a total of three times. The final precipitate was collected on millipore filters and radioactivity was determined in a Beckman Model LS-233 Liquid Scintillation Spectrophotometer (Beckman Instruments, Fullerton, California). Counts per minute were converted to % destruction by the following algorithm:

$$\% \text{ destruction} = \frac{\text{CPM Control} - \text{CPM Experimental}}{\text{CPM Control}} \times 100$$

FIG. 2. (a) The appearance of the L cell monolayer after 48 hr of exposure to LT containing media. $\times 40$. (b) The appearance of the L cell monolayer after 48 hr of exposure to control media. $\times 40$.

The data obtained from the microassay were derived entirely from total cell counts which were converted to % destruction by the following formula:

$$\% \text{ destruction} = \frac{\text{Total cells control} - \text{Total cells experimental}}{\text{Total Cells Control}} \times 100$$

To avoid erroneous results, it was necessary to compare experimental wells to control wells, which contained a similar number of cells initially ($\pm 10\%$). A comparison of the two techniques for one batch of HLT is shown in Fig. 3. These experiments were repeated with three separate batches of LT medium. The standard errors for both tube and plate cultures were calculated and varied randomly from $\pm 2\%$ to $\pm 7\%$. This variation did not significantly affect the shape of the curves. Assuming an LD_{50} as the end point, it can be seen that the microassay is approximately $25\times$ more sensitive than the tube culture assay. Also of interest is the observation that no toxicity was detectable in the cell-free medium obtained from unstimulated lymphocyte cultures by the ^{14}C -AA incorporation technique in tubes, while it was detected in the undiluted medium by the microassay method. These results suggest there may be some low level of stimulation by foreign components in the tissue culture medium itself.

DISCUSSION

It is clear that the microassay technique described in this communication has distinct advantages over the previously described tube culture ^{14}C -AA incorporation technique for detecting *in vitro* LT-induced cytotoxicity. The first and most important is the increase in sensitivity. This phenomenon may be due to the reduced number of cells per volume of toxic medium; thus increasing the number of LT molecules available on a per cell basis. In the microassay, we use from 1,000–5,000 cells/ml toxic medium, whereas the tube culture assay uses 50–100,000 cells/ml

Fig. 3. A comparison of the sensitivity of L cells to LT induced destruction in micro plate and tube culture. The cells were assayed for viability as described in the text after 48 hr of exposure to test and control media.

TABLE 1

EXAMPLES OF TOTAL CELL COUNT FROM THE MICROPLATE ASSAY FOR LYMPHOTOXIN

LT dilution	Total Cell Counts		% destruction ^a
	Initial	48 hr	
Undiluted	30 ^a , 36 ^b	3 ^a , 3 ^b	97.6, 98.1
1:10	16 ^c , 44 ^d	16 ^c , 58 ^d	74.6, 71.6
Control	32 ^a , 39 ^b , 16 ^c , 48 ^d	124 ^a , 156 ^b , 63 ^c , 204 ^d	

^a Experimental counts a, b, c, d were compared to control counts a, b, c, d respectively, for calculation of % destruction. The algorithm for this calculation is given in the text.

toxic medium. Another important advantage is the relatively simple experiment required. There is no need for a CO₂ incubator and stock cells can be maintained in small cultures, thus decreasing the amounts of medium and other tissue culture reagents used. In addition, there is no need of radioisotopes or of a liquid scintillation spectrophotometer. Although the microplates are "disposable", they may be reused if care is taken to insure proper cleaning. They may be sterilized chemically, by UV irradiation or gas.

There are several disadvantages which should also be discussed. One of the most troublesome is achieving consistency in target cell numbers from well to well. This variability is more obvious when cell densities of 300 cells/well or less are employed. This variance can be overcome with experience or by performing initial cell counts of each well and comparing the final cell counts to control wells which varied by less than 10% of their initial counts. Table 1 gives examples of data which have been processed in this manner. In some of our studies, it was necessary to wash monolayers before addition of test media. We found that the Monolayers should be washed gently to avoid loss of cells. Rough Washing procedures can result in loss of up to 20% of the cells. It is of utmost importance that test medium be the same as the medium used to establish the monolayers. The monolayers at this low density do not apparently tolerate the shock of exposure to entirely different medium conditions and inconsistent results are obtained. The condition of the target cells also has its effects upon the assay. If for any reason the cells to be used are unhealthy, inconsistent results will be obtained. It can be argued that unhealthy target cells would give erroneous results in any system. This is undoubtedly true; however, the microassay system is especially sensitive to these conditions since the cells are maintained at such a low density.

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