Human Cell-mediated Cytotoxicity Estimated by Lymphocyte Titration

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SUMMARY

Human lymphocyte immunity to tumor-derived target cells was estimated by titrating lymphocyte concentration to achieve a 50% reduction of target cell survival. This lymphocyte titration assay gave estimates of cytotoxicity that were different from those obtained with the conventional cell-mediated cytotoxicity assays but were more proportional to lymphocyte activity. Estimates of cytotoxicity obtained using the lymphocyte titration assay were reproducible upon repeated testing over the course of several months and were relatively unaffected by two- to fourfold variations in target cell concentration. Target cell-specific cytotoxicity was reproducible but often did not appear to be tumor specific.

INTRODUCTION

Lymphocyte-mediated immunity is commonly estimated in the in vitro CMC3 assay. In the conventional assay, target cells are exposed to lymphocytes at a selected concentration, and target cell survival following exposure to lymphocytes is compared with survival following exposure either to medium alone or to lymphocytes from a control donor (8, 11). The results are commonly expressed in terms of %TCR caused by the test lymphocytes.

Although the purpose of the CMC assay is to estimate the cytotoxic activity of the lymphocytes, the %TCR caused by a particular concentration of lymphocytes is not proportional to their cytotoxic activity; doubling the activity of lymphocytes that reduced a target cell population by 80% cannot possibly give 160% reduction. As a result, changes in %TCR by lymphocytes obtained from the same donor but tested on different occasions do not directly reflect changes in the cytotoxicity activity.

A 2nd problem with the conventional CMC assay is that, because lymphocytes are tested at only 1 concentration, the relative activities of lymphocytes that are either very strongly or very weakly cytotoxic at the concentration tested cannot be reliably determined. As a result, the assay provides estimates of lymphocyte cytotoxic activity only within a narrow range, and changes in the activity falling outside the range estimated at a particular concentration cannot be detected.

The conventional CMC assay has been useful in 2 respects. It has been valuable in detecting the changes in cell-mediated tumor immunity produced by experimental manipulations of syngeneic animals. The genetically identical members of such populations respond in very similar ways, so the proper lymphocyte concentration can be determined by trial and error; tumors can be inoculated and their growth can be measured as often as necessary. However, we have found that the cytotoxic activity of lymphocytes from human donors varies greatly, and in many cases it lies outside the narrow range of the conventional CMC assay.

The assay has also been useful in comparing the activity of lymphocytes from populations with and without a given tumor, as it will determine whether the CMC is above or below some arbitrarily defined level. However, because the results are not proportional to lymphocyte activity, changes in %TCR during a patient’s clinical course do not reflect accurately the changes in cytotoxic activity. As a result, correlations of changes in CMC with changes in clinical course are difficult.

Because of these drawbacks in the CMC assay, we have modified it to produce results that are proportional to cytotoxic activity and to expand the range over which that activity can be estimated. In this report, we describe our LTA and the conditions that affect it.

MATERIALS AND METHODS

Lymphocyte Preparation. Venous blood (50 ml) was defibrinated by swirling it at 250 rpm with 2 paper clips in a 125-ml Erlenmeyer flask for 10 min at room temperature on a New Brunswick gyroratory shaker equipped with a device that tilted the Erlenmeyer flask at 45°. The defibrinated venous blood was mixed with 3% gelatin (P. Liener and Sons, America, Inc., St. Clair Shores, Mich.) in Roswell Park Memorial Institute Medium 1640 (Grand Island Biological Co., Grand Island, N.Y.) to produce a 3:1 blood:gelatin suspension and incubated at 37°. The majority of RBC sedimented within approximately 30 min (4). The leukocyte-containing supernatant fluid was collected and incubated...
for 30 min at 37° on approximately 1 g of nylon fiber (Fenwal Laboratories, Morton Grove, Ill.) which was loosely packed in a glass column. The nonadherent cells were gently washed out of the column with 50 ml of warm Roswell Park Memorial Institute Medium 1640 (6). Residual erythrocytes were removed from the eluate by incubating the pelleted cells in Tris-buffered ammonium chloride (2) at 4° for 10 min. The resulting lymphocyte preparation was washed 3 times in Waymouth medium containing 5% FCS (Armour Pharmaceutical Co., Kankakee, Ill.), and the concentration of cells was adjusted to 10 \times 10^6/ml in MNC medium. More than 95% of the cells had the morphological characteristics of small lymphocytes, and more than 95% of them were viable, as judged by their ability to exclude 0.1% trypan blue.

**Target Cells.** The T24 target cells, which were derived from a transitional cell carcinoma of the bladder (TCC-B) (3), Malme-3M, derived from a melanoma, and CaKi-1, derived from a RCC, were provided by Dr. Jorgen Fogh, Sloan-Kettering Institute, New York, N. Y. Additional target cells obtained from surgical specimens of RCC and TCC of the bladder and renal pelvis were initiated in our laboratory by techniques previously described (5). Target cells were propagated in plastic tissue-culture flasks (Falcon Plastics, Los Angeles, Calif.) in Waymouth medium supplemented with 10% FCS, penicillin (100 units/ml), streptomycin (100 \mu g/ml), 2 mm glutamine, nonessential amino acids, and 10% tryptose phosphate broth. Target cells were maintained at a temperature of 37° in a humidified atmosphere of 5% carbon dioxide in air. All target cells used in these experiments were without evidence of *Mycoplasma* contamination by culture (Flow Laboratories, Bethesda, Md.) and electron microscopic examination. The karyotype of the frequently used target cell lines was routinely determined to minimize the chance of unrecognized cross-contamination. The karyotypes of the target cell lines reported here differed from each other and from that described for HeLa. (The chromosome analyses were performed by J. Cervenka, University of Minnesota.)

**LTA.** A single-cell suspension of the target cells was made using 0.125% trypsin in Versene, 1:5000. Target cells were adjusted to a suitable concentration in MNC medium supplemented with 10% FCS. Ten \mu l of target cell suspension were placed in each of the 72 wells of the Cooke Histoplate (Cooke Engineering, Alexandria, Va.) or in each of the 60 wells of the Falcon Micro Test plate and allowed to attach to the bottom of the well for at least 3 hr before the lymphocytes were added.

Suspensions of lymphocytes in concentrations of from 100 to 1.6 \times 10^4/10 \mu l were made by serial 2-fold dilutions. Ten \mu l of lymphocyte suspension of each concentration were placed in the wells of the columns, as indicated in Chart 1. MNC medium (10 \mu l) without lymphocytes was added to each of the wells in Columns 1, 4, 7, 10, and 12. The assay plates containing the lymphocytes and target cells were enclosed in self-sealing Zip-Lok plastic bags, together with a moistened sponge, and placed on a leveling platform in a humidified CO2 incubator at 37°.

The plates were inverted for approximately 10 min to allow most of lymphocytes to fall away from the target cells. Lymphocytes and nonadherent target cells were washed off with a device that delivered a stream of 0.05 M phosphate-buffered saline (pH 7.4) to each of the wells for a predetermined time ranging from 3 to 6 sec. The remaining adherent target cells were fixed in 95% ethanol. The ethanol was rinsed off by immersion in tap water, and the cells were stained with 0.1% crystal violet and counted with a Quantimet 720 Image/Analyzing Computer (Imanco, Monsey, N. Y.).

The mean number of target cells surviving exposure to lymphocytes at each concentration was compared with the mean number of cells surviving in the adjacent wells containing medium alone and was expressed as a percentage of target cell survival. The percentage of target cell survival was plotted against the lymphocyte concentration tested to generate a lymphocyte titration curve. From this curve, the number of lymphocytes required to cause 50% target cell destruction was determined and expressed as a LLD_{50}, calculated as follows:

\[
\text{LLD}_{50} = \frac{-\log_{2} L}{200 \times 10^{3}}
\]

where \(L\) equals the number of lymphocytes required to reduce target cell survival by 50% compared with survival in the presence of medium alone. Results were expressed as a negative logarithm to allow convenient expression of a wide range of cytotoxic activity and so that decreasing amounts of lymphocytes required to cause a 50% reduction in survival could be expressed as an increasing value. The number of lymphocytes required to kill 50% of the target cells was divided by 200,000 so that the subsequent LLD_{50} unit could be expressed as a small positive number.

**RESULTS**

Lymphocyte titration curves generated by plotting the percentage of target cell survival against lymphocyte concentration for lymphocytes derived from 5 representative donors are shown in Chart 2. *Curve A* is that of a lymphocyte population without significant cytotoxic activity; it closely follows the 100% survival line and indicates that target cell survival even in the presence of high concentrations of lymphocytes is undiminished from survival in the presence of medium alone. We found that such complete absence of
target cell survival approached 100% as the lymphocyte would have been considered cytotoxic on not cytotoxic following exposure to lymphocytes from Donor C versus concentration of approximately 6 \times 10^3!weIl, lymphocytes from Donor B with target cell survival following exposure to the same conventional test been carried out with a lymphocyte concentration of lymphocytes from Donor B. Had the test been run at approximately 1.6 or 25 \times 10^3 lymphocytes per well, there would have been no significant difference in target cell survival following exposure to lymphocytes from Donor C versus Donor B. In other words, the lymphocytes from Donor C would have been considered cytotoxic or not cytotoxic simply as a function of the tested concentration of lymphocytes.

The paradoxical presence or absence of cytotoxicity as a function of the concentration of lymphocytes would not have been corrected by basing the estimate of cytotoxicity on target cell survival in the medium control. At a concentration of approximately 6 \times 10^3, lymphocytes from Donor B or C would not have been considered cytotoxic. At a concentration of approximately 12.5 \times 10^3, Donor C, but not Donor B, would have been considered to have had cytotoxic lymphocytes, while, at a concentration of 25 \times 10^3, lymphocytes from both donors would have been considered cytotoxic. When the estimate of lymphocyte cytotoxic activity is expressed in terms of the LLD_{50}, lymphocytes from both Donors B and C are considered approximately equally cytotoxic (LLD_{50}'s of 2.9 and 3.0, respectively).

If a conventional CMC assay had been carried out with lymphocytes from these donors and at a concentration of approximately 25 \times 10^3, lymphocytes from Donors D and E would have reduced target cell survival greatly in comparison with Donors A, B, or C, or the medium control. However, no estimate of the relative cytotoxic activities of lymphocytes from Donors D and E could have been obtained. Titration clearly shows that far fewer lymphocytes are required from Donor E than from Donor D to achieve 50% tumor cell reduction.

From the preceding examples, it is apparent that estimates of cell-mediated immunity expressed in terms of the LLD_{50} may be quite different from estimates expressed as the %TCR obtained from the conventional CMC assay.

Reproducibility of the Assay. The reproducibility of the LTA was estimated by performing duplicate assays on the same day. In a series of 87 such experiments, performed over the course of 6 months using lymphocytes obtained at the same bleeding and target cells plated at the same time, the mean difference between duplicate tests was 0.2 LLD_{50} unit, with a range of 0 to 0.5 LLD_{50} unit (S. E., 0.17). The results of tests in which lymphocytes were obtained from separate, separately processed blood specimens drawn from the same donor on the same day and tested against target cells plated at the same time are shown in Table 1. The difference between the highest and lowest LLD_{50} was 0.2 and 0.4 LLD_{50} unit against target cells T24 and Malme 3M, respectively.

The standard error of the 6 replicate wells in each column averaged about 10% of the mean number of target cells surviving in that column.

The reproducibility of the assay over time with lymphocytes from a control donor in whom no change in cell-mediated immunity was anticipated is shown in Chart 3. The activity against T24 target cell line, which had been propagated under uniform conditions for many months, showed that the repeated LLD_{50} determinations varied within a range of 0.5 LLD_{50} unit. In this example, the reproducibility of the LLD_{50} estimate of CMC during testing over several months suggests that the LTA is capable of giving a reproducible estimate of CMC activity and that target cell susceptibility to lymphocyte damage may be stable under optimal conditions.

Factors Affecting the Results of LTA. Almost any change in the method of lymphocyte preparation or the conditions...
under which the target cells were propagated caused a shift in the LLD_{50}. To minimize such target cell variations, FCS was purchased in lots sufficient for more than a year when serial estimation of a patient's cell-mediated immunity was attempted.

We tried to determine the rate at which lymphocyte cytotoxic activity was manifested during an assay. Assay plates set up at the same time were terminated at various intervals after lymphocyte addition, and titration curves were determined. Results representative of such a series of experiments are shown in Chart 4. Cytotoxic activity increased for approximately 48 hr, after which it remained stable or declined. Similar experiments showed that the slope of the titration curve gradually increased with incubation periods greater than 48 hr as a result of the continued growth of target cells in the medium control wells, which raised the base line on which target cell survival was based. To detect maximum cytotoxicity, experiments were routinely incubated for 40 to 48 hr following the addition of MNC medium.

Cytotoxicity is influenced not only by the patient’s immunity but also by the sensitivity of the target cell to damage and by qualitative and quantitative variations in antigens expressed by that target cell. When early-passage cell lines were tested frequently with lymphocytes from the same donor, it was often found that the resulting LLD_{50} showed a gradual unidirectional change, either rising or falling. Under uniform cell-propagation conditions, the LLD_{50} usually stabilized within several months of the alteration of culture conditions or after the initiation of a target cell line. However, as shown in Chart 5, some cell lines showed a persistently rising LLD_{50} over a relatively long time when tested against lymphocytes from the same donor. It can be seen that against the Malme 3M cell line, lymphocytes from the same donor caused a progressive rise in the level of cytoxicity from approximately 1.7 to 5.5 LLD_{50} units. The donor of these lymphocytes was a healthy 42-year-old white male who neither worked in the laboratory nor had been transfused with whole blood. Over the same testing period, lymphocytes from other donors showed a similar rise in LLD_{50} against this target cell line.

The effector cell:target cell ratio has frequently been considered an important variable in estimating CMC. We attempted to estimate the effect of varying target cell concentration on the LLD_{50}. The results of 2 representative experiments are shown in Charts 6 and 7 and in Table 2. Target cells derived from TCC and RCC were plated at loading concentrations of 100, 200, and 400/well, and lymphocytes from a single donor—in this case, a patient with known RCC—were tested. When tested against the TCC cells, there was a fall in the LLD_{50} as the concentration of target cells increased, but when lymphocytes from this donor were tested at the same time against RCC-derived cells, there was a slight increase in LLD_{50} with increasing target cell concentration. In neither case, however, was the change in LLD_{50} proportional to the change in either the original target cell concentration or the number of target cells surviving in wells containing medium alone. For example, the same concentration of lymphocytes (approximately 6000) was capable of destroying 38 to 76 target cells, and 58 of 137 or 180 of 360 RCC-derived target cells.

The exact causes of the feeder effect are unknown, but we found that lymphocytes contributed to a feeder effect more frequently in early-passage cell strains, particularly...
Cytotoxicity of lymphocytes from RCC Patient 608 against different concentrations of target cells

<table>
<thead>
<tr>
<th>No. of target cells loaded/well</th>
<th>TCC</th>
<th>Mean no. of target cells surviving in medium control wells</th>
<th>LLD&lt;sub&gt;50&lt;/sub&gt;</th>
<th>RCC</th>
<th>Mean no. of target cells surviving in medium control wells</th>
<th>LLD&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
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<tr>
<td>100</td>
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<td>3.5</td>
<td>76</td>
<td>4.8</td>
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</tr>
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<td>200</td>
<td>92</td>
<td>2.7</td>
<td>137</td>
<td>4.9</td>
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</tr>
<tr>
<td>400</td>
<td>251</td>
<td>2.6</td>
<td>367</td>
<td>5.2</td>
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when target cells were plated at a low density. The effect of target cell density upon the feeder effect is shown in Chart 8. It can be seen that the augmented target cell survival in the presence of lymphocytes was most pronounced when target cells were plated at the lowest concentrations.

Target-Cell Specificity. The target cell specificity of the cytotoxicity estimated by the LTA is illustrated by the results shown in Charts 9 and 10. Lymphocytes obtained from a normal donor and from donors with TCC and RCC were tested on the same day against target cells derived from a TCC (Chart 9) and RCC (Chart 10). Against the TCC target cell line, the lymphocytes from the TCC donor were significantly more cytotoxic than lymphocytes obtained from the
RCC donor. This relative cytotoxicity was reversed when lymphocytes from the 2 donors were tested against the RCC-derived target cell line. In each case, the normal donor had the lowest cytotoxic activity. Such target cell specificity is similar to the activity shown in "criss-cross" experiments using conventional CMC tests (8).

While we have found consistent, reproducible target cell specificity, such specificity is not necessarily associated with the tumor type of the lymphocyte donor. In other words, lymphocytes from a patient with RCC may be more cytotoxic to target cells derived from melanoma than to target cells derived from an RCC. The results of tests illustrating such a target cell-specific, but apparently tumor-unrelated, cytotoxicity are shown in Chart 11. The activity of lymphocytes from a control and a RCC donor was determined against target cells derived from RCC, TCC, and melanoma. Significant but quantitatively similar cytotoxicity was consistently shown by the lymphocytes from the normal donor tested over a period of several months. Lymphocytes from the RCC donor showed significantly higher cytotoxicity against the RCC target cells than did lymphocytes from the control donor, while the lymphocytes from the RCC donor had the same level of activity against the TCC-derived target cells as did lymphocytes from the control donor. Had the comparison been limited to these 2 target cells (TCC and RCC), it might have been concluded that tumor-specific immunity was being displayed. However, it can be seen that lymphocytes from the RCC donor had even greater cytotoxicity against the melanoma-derived target cell. The high LLD50 caused by the RCC donor lymphocytes against the melanoma-derived target cell cannot be explained totally as a result of an increased sensitivity of the melanoma-derived target cell to cytotoxic damage, because the LLD50 caused by lymphocytes from the healthy, nonlaboratory control donor was similar against all 3 kinds of target cells.

In the absence of demonstrated tumor-associated antigenic similarity between the RCC and melanoma, it might be concluded that antigens other than tumor-specific or associated antigens are being detected by lymphocytes from the RCC donor. Because of the multiple antigenic differences between a lymphocyte donor and the target cell donor, target cell specificity that is apparently unrelated to tumor specificity perhaps should not be surprising. However, this background of apparently nontumor cell-surface antigens seriously hampers the detection of possible tumor-associated immunity. To detect tumor-specific immunity against this background of multiple, presumably tumor-unassociated, antigenic differences and variable sensitivity to lymphocyte-mediated damage, we have carried out serial estimates of lymphocyte cytotoxicity during the clinical course of the patient.

Clinical Studies. It was postulated that perhaps changes in cytotoxicity, if confined to 1 kind of target cell and associated with the removal or recurrence of a tumor mass, might be a more dependable indicator of immunity directed against tumor-associated antigens. In Chart 12, the cell-mediated immunity of lymphocytes obtained from a patient with RCC was measured at frequent intervals against target cells derived from a TCC of the bladder, RCC, and malig-
Our results suggest that when the lymphocytes are greatly diluted, the activity of even strongly cytotoxic lymphocytes may be limited because of lack of sufficient contact with the target cells (contact limited). On the other hand, lymphocytes in the presence of target cells against which they have little activity may be limited in the percentage of target cells damaged, because not enough of the lymphocytes possess cytotoxic activity (immune cell limited).

The feeder effect was more pronounced at lower target cell concentrations. When target cell survival must be estimated by manual optical counting, lower target cell concentrations are generally selected, and it is thus possible that estimates of cytotoxic activity with the conventional CMC assay may be more affected by the feeder effect phenomenon than are the cytotoxic assays carried out with higher target cell concentrations. The speed of counting by electrooptical devices is not related to the number of cells counted, so target cell concentration can be adjusted to diminish feeder effects without prolonging the analysis.

We have found that, with some target cell lines under uniform conditions, the degree of cytotoxic damage caused by lymphocytes from a given control donor may be relatively stable for many months. However, under similar conditions and with lymphocytes from the same donor, we have also observed unidirectional shifts in LLD50’s (either rising or falling uniformly). These changes in cytotoxic damage may be of short duration (several weeks) or may occur over many months of testing. We will present elsewhere the evidence suggesting that these changes result both from changes in sensitivity to cytotoxic damage and from qualitative or quantitative differences in antigen expression. However, in order to differentiate the changes in LLD50, which result from alterations in the donor’s lymphocytes from those in the target cells, we have had to retest at short intervals lymphocytes from control donors in whom no change in target-cell immunity is anticipated.

The presence of target cell-specific cytotoxicity apparently unrelated to immunity against tumor-associated antigens has been reported previously. We have found that such cytotoxicity may persist over relatively long periods of time. Because most reactions test the activity of lymphocytes from one donor against target cells from other donors, one must expect many antigenic differences between the target and effector cells which are unrelated to malignancy. We have attempted to overcome the confounding effect of these multiple antigenic differences by using the patient’s cytotoxic activity before tumor removal or recurrence as the base line with which to compare activity after removal or recurrence. We postulate that changes in the activity directed against the specific target cell, when associated with changes in the tumor burden, may be a better indicator of tumor-associated immunity than are static, one-time estimates of activity against target cells from a variety of donors. The initial results of serial estimates of patient immunity during the course of the illness have been reported elsewhere (7). Although we have occasionally found a decline in lymphocyte cytotoxicity against the tumor-associated target cell line as reported by others (9), we have also found both short- and long-term rises in antitumor target cell cytotoxicity following reduction of the tumor burden.

Human Lymphocyte Titration

Chart 13. CMC displayed by lymphocytes obtained from a patient with benign prostatic hyperplasia (BPH) against target cells derived from a RCC, TCC, and melanoma before and after transurethral resection of prostate (TURP). Dx, diagnosis.

Our results suggest that when the lymphocytes are greatly diluted, the activity of even strongly cytotoxic lymphocytes may be limited because of lack of sufficient contact with the target cells (contact limited). On the other hand, lymphocytes in the presence of target cells against which they have little activity may be limited in the percentage of target cells damaged, because not enough of the lymphocytes possess cytotoxic activity (immune cell limited).

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Many patients fail to show significant changes in CMC when the tumor burden changes. The CMC assay estimates changes in only 1 portion of the immune response; it is possible that antibody changes also occur when the tumor burden changes so that LTA’s carried out in the presence and absence of serum from the lymphocyte donor may also demonstrate changes in immunity associated with such changes in clinical course.

The facility with which target cell survival can be determined by electrooptical image analysis has permitted us to examine the activity of lymphocytes from control donors against target cells from a large number of donors with various tumors. Contrary to what we expected, lymphocytes from control donors frequently showed significant, reproducible cytotoxic activity against the target cells. Even for lymphocytes that usually had relatively low levels of cytotoxic activity, target cells often could be found against which the lymphocytes had quite high and reproducible cytotoxic activity. In other words, the likelihood of detecting high levels of reproducible target cell-specific cytotoxic activity, even with control donors, increased as the variety of target cells tested increased. This cytotoxic activity occurred even in control donors with no history of a source of possible sensitization against HL-A antigens (e.g., pregnancy or whole-blood transfusion).

We do not know whether this intrinsic target cell-specific cytotoxicity is the result of a clinically undetected encounter with tumor-associated antigens, of activity against fetal-type antigens expressed by the target cells in response to tissue culture propagation, or of a response to other antigens or effects that are unrecognized. However, the fact that lymphocyte titration expands the range over which lymphocyte activity may be measured and the fact that results obtained from lymphocyte titration assays are proportional to cytotoxic activity gives an assay that is capable of asking more useful questions in the analysis of immune response of humans to their tumors.

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REFERENCES

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