A System for Quantitative Determination of Cytotoxic Activity of Antisera to Ascites Tumor Cells*

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SUMMARY

Criteria are described for recognizing early degenerative changes that occur when mouse ascites tumor cells are exposed to homologous or heterologous antisera. These permit determination of the same percentage of deformed cells, irrespective of whether or not vital staining dyes are used.

When 10 million cells of the L4946 or E9514-A ascites tumors were incubated at 37°C in 1 ml modified Locke's buffer with excess complement, the percentage of deformed cells leveled out between 1 and 3 hours' incubation at a value determined by antibody concentration. A titration curve, of percentage cells deformed after 2 hours' incubation against antiserum concentration, gave the cytotoxic titer, i.e., the final concentration of antiserum giving 50 per cent plus one-half the control level of deformed cells. For a rabbit antiserum to L4946 cells, the cytotoxic titer was 1.24 parts per thousand, with a standard deviation of 21 per cent. A relationship between cytotoxic titer and cell concentration in the medium was demonstrated, and dependence on pH, osmolarity, and complement concentration were investigated.

To relate cell deformation to cell death, serial dilutions of L4946 cells containing known percentages of deformed cells were inoculated into AKR mice and survival noted. The results indicate that the microscopic count underestimates the damage done to cells by antiserum. A method for estimating cell viability following inoculation of mice with tumor cells is described, which takes into account the period of temporary survival of mice dying from tumor, in addition to mere death or long-term survival.

To gain further insight into the role of immunity in cancer, sensitive tools are urgently needed. The present paper describes an attempt to standardize an in vitro assay to measure quantitatively the potency of antisera for killing tumor cells. A test system of this type seems relevant, since death of tumor cells is a desirable end-point.

Whereas the present work is most closely based on the work of Gorer and O'Gorman (15), Easty and Ambrose (7), Schrek and Preston (33), and Defendi and Colter (5), most investigators concerned with the effect of antisera on tumor cells have used some form of this system (1-3, 5, 7, 9-17, 19, 20, 27, 28, 32, 33). The system consists of a single cell suspension of living tumor cells in a suitable buffer, to which is added antiserum and complement. Dead or dying cells are recognized under the microscope with greater ease because, in contrast to normal cells, they are stained by a vital staining dye if injured by antiserum (1, 3, 5, 7, 15, 33) or by other agents (6, 9, 18, 24, 29), although negative results have also been reported (34, 35).

Several groups of workers have recently described in detail the morphologic changes that occur when ascites tumor cells are incubated with antiserum plus complement (2, 7, 11, 15, 19, 28, 32) or when such cells are injured by poisons (1, 9,
21), viruses (9), or x-rays (21). Remarkable agreement regarding both the sequence of degenerative changes and the consequent metabolic alterations has been achieved (2, 9, 10, 16, 17, 21–23, 26, 36). These studies, together with those on the relation between vital staining and cell death, have laid a solid foundation for the present work.

MATERIALS AND METHODS

Mouse breeding.—Mice of the strains AKR, C57BL/6, and C3Heb were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and were inbred in our laboratory. Development of our own sublines was prevented by obtaining three trios of nucleus breeding stock mice of each strain from the Jackson laboratory 3 times a year and using them for production stock breeding, but not beyond the third or fourth generation. Thus, our mice remained closely bound genetically to the Jackson stock, allowing us to supplement our production stock mice with theirs and to use these mice interchangeably.

Tumor transplantation.—Three types of mouse ascites tumors were used. The L4946 lymphocytic leukemia was transplanted intraperitoneally into 4–6-week-old AKR mice of either sex thrice weekly. In each transplantation, two mice received 0.2 ml. undiluted peritoneal fluid, and two mice 0.2 ml. of a 1:500 dilution with sterile saline. This schedule made available about 500 million L4946 cells for use on any day of the week. The E9514-A leukemia was obtained from the Jackson laboratory and transplanted similarly twice weekly in C3Heb mice, whereas Ehrlisch ascites tumor cells were transplanted once weekly in AKR mice.

Antisera.—Rabbit antisera to L4946 cells were prepared by injecting 1.5 ml. Freund-McDermott adjuvant subcutaneously into each of two rabbits. The adjuvant was prepared by grinding in a mortar, until a smooth suspension was obtained, 9 ml. of a 1:1 mixture of Falba oil (Pfaltz & Bauer, Inc., Empire State Bldg., New York, N.Y.) and mineral oil, 15 mg. inactivated B.C.G. vaccine, and 3 ml. of a 50 per cent suspension of washed L4946 cells. Next, 0.6 ml. of 50 per cent washed L4946 cells was injected intravenously thrice weekly for a total of seven injections. After a test bleeding, the entire series was repeated. Ten days after the second series of injections, rabbits were bled from the central artery of the ear, by a modification of Cohen’s technic (4). Sera were stored frozen in 3-ml. aliquots at −10°C.

Mouse antisera were made in C57BL/6 mice and C3Heb mice by injecting intraperitoneally into each mouse 300,000 washed viable L4946 cells, followed 5 weeks later by 5 million such cells, and by exsanguinating 10 days later (14). The sera were pooled and stored at −10°C.

Complement.—Normal adult guinea pig blood was obtained by external cardiac puncture under ether anesthesia. Sera from nine to twelve animals were pooled and stored at −10°C in 3-ml. portions. Adult guinea pigs survived repeated collections of 10 ml. blood.

Buffer.—The fortified Locke’s buffer could be stored for 10 days at 3°C. in concentrated form. It contained 40.2 gm. sodium chloride, 1.96 gm. potassium chloride, 4.12 gm. sodium citrate, 2 H2O; these were dissolved in approximately 800 ml. distilled water; 1.06 gm. calcium chloride (anhydrous) was dissolved separately in approximately 100 ml. water and added to the other solutions; finally, aqueous solutions containing 100,000 units penicillin G and 100,000 µg. streptomycin were added, and the volume made up to 1 liter in a volumetric flask; then a slight pH adjustment was made to pH 7.4–7.5. For each experiment, one volume of concentrated buffer was diluted with 4 volumes of distilled water to give a buffer of 285 milliosmoles per liter, as determined in the Fiske osmometer (Fiske Associates, Inc., 500 Maple Street, Danvers, Massachusetts). This 285 milliosmolar buffer is hereafter called “fortified buffer,” since it contained 20 units penicillin G and 20 µg. streptomycin per ml.

Staining medium.—50 mg. trypan blue was dissolved with heating in 100 ml. fortified buffer; after cooling, 150 mg. eosin was added with stirring. This dye mixture was stored at 3°C. and prepared fresh every 2 weeks; 0.1-ml. portions were pipetted into small (9 mm. I.D. X 7.5 cm.) test tubes before each experiment.

Tumor cell suspension.—To collect cells, the peritoneal cavity of two to four tumor-bearing mice was rinsed out 3 times by injecting and again withdrawing 1.5 ml. of fortified buffer. Cell suspensions even mildly pink from their content of erythrocytes were discarded. Cell suspensions were centrifuged for 12 minutes at 5°C. and 13 g (350 r.p.m.) in the International Centrifuge PR2. Under these mild conditions, about two-thirds of the cells were packed into a loose pellet. To obtain

1 Thanks are due to Dr. Russell T. Jordan, City of Hope Medical Center, Duarte, California, for supplying this tumor.
2 Thanks are due to Dr. Charlotte L. Maddock of the Children’s Hospital, Boston, Massachusetts, for supplying this tumor, and for keeping the L4946 leukemia available frozen in 6 per cent glucose at −10°C.
3 Thanks are due to the Research Foundation and the University of Chicago, for supplying the vaccine through the courtesy of Dr. S. R. Rosenthal, 70 West Hubbard Street, Chicago 10, Ill.
higher yields the supernatant could be removed and resuspended. When so stated, cells were washed once as follows: cells were resuspended in 10 ml. fortified buffer by gently rocking the stoppered tube back and forth, if necessary for several minutes, and recentrifuged.

The supernatant was discarded, and the packed cells were gently resuspended in 30 volumes of fortified buffer (for the larger Ehrlich ascites cells, in 15 volumes). Now a cell count was done on duplicate samples in a hemacytometer counting chamber, first resuspending to counteract rapid settling of cells. Then the cell suspension was diluted with buffer to the desired concentration, usually 20 million cells per ml., and stored at 3°C.

Experimental procedure.—Experiments were run in 14 mm. I.D. X 10 cm. test tubes. Each tube received fortified buffer, guinea pig serum, antiserum, and cell suspension. Unless noted otherwise, the final concentration of guinea pig serum was 20 per cent, the final concentration of cells 10 million per ml., and the total volume was brought to 1.00 ml. per tube with buffer. Cells were resuspended immediately before pipetting the suspension as the last addition; then the contents of each tube were mixed gently. Time zero was noted when the rack containing the open test tubes was set to rest in a water bath at 37.0 ± 0.3°C. This time was generally within 3 hours after start of cell collection from mice.

Up to twelve, 24, or 36 test tubes were run simultaneously, depending on whether one, two, or three operators were participating. One hour after start of the incubation, each operator began to stain and count the percentage of deformed cells in the first of his assigned tubes.

To stain cells, an incubating tube was mixed gently, 0.1 ml. removed with a 0.2-ml. blowout pipette and expelled into a small staining tube containing 0.1 ml. dye. After gentle mixing, a small drop was pipetted to fill a hemacytometer counting chamber of 0.1-mm. depth (Macalaster Bicknell No. 2261). Each operator used a standard binocular light-field microscope at high magnification (45X objective and 10X eyepieces). Three hand tally counters (Macalaster Bicknell No. 9671) were used simultaneously: one for every cell (nos. 1, 2, 3, and 4 cells), a second for deformed but unstained cells (no. 2 cells), a third for stained deformed cells (nos. 3 and 4 cells). Criteria for differentiating between these cell types are given later. Every cell (except those in large clumps) was counted in three or four fields, for a total of 200–250 cells per slide. For slides with deformed cell counts above 95 per cent, only normal cells (no. 1 cells) and unstained deformed cells (no. 2 cells) were counted in twenty fields, and the total cell count in two fields was used to estimate total count in twenty fields. Time of incubation at start of counting and the three cell counts were recorded. The entire procedure of sampling, staining, counting, and recording took less than 5 minutes per tube.

Each operator completed working through his tubes 2 hours after start of the incubation. Then operators exchanged tubes and began a second count of each tube. The proportion of deformed cells (nos. 2, 3, and 4 cells) was calculated as a percentage of all cells counted (nos. 1, 2, 3, and 4 cells). To compensate for the difference in time at which different tubes were counted, the two values for each tube were plotted against time of incubation on arithmetic graph paper and joined by a straight line. The percentage of deformed cells after 2 hours' incubation was read off this line and taken as the final result for the tube.

RESULTS

Initial experiments.—Locke's balanced salt solution proved superior to Gey's or Tyrode's in its capacity to conserve the integrity of L4946 cells incubated in it at 37°C for several hours. Integrity of cells was judged by exclusion of vital staining dyes, both in the presence and absence of rabbit antiserum to L4946 cells. Occasional clotting of cells was prevented by adding citrate to Locke's solution; further modifications are described below.

To determine whether vital staining dyes were cytotoxic, L4946 cells were incubated at 37°C with an equal volume of 0.1 per cent solutions of buffered methylene blue or trypan blue. Methylene blue proved unsuitable by staining all cells after 30 minutes' incubation. Trypan blue stained only 10 per cent of cells, either when incubated with the cells for 3 hours or when added after incubation. If rabbit antiserum to L4946 cells was added, presence of trypan blue during incubation gave strong protection to the L4946 cells. This effect was not investigated further, but on this account trypan blue was added only at the end of incubation in succeeding experiments.

pH effect.—Aliquots of an L4946 cell suspension were spun gently in test tubes, the supernatant removed and cells resuspended to 10 million cells/ml in buffer of known initial pH, containing 20 per cent guinea pig serum but no antiserum. The tubes were incubated at 37°C, and cell counts were done at hourly intervals. A curve of per cent normal cells against time was drawn for each tube and per cent normal cells at precisely hourly intervals read off and plotted in Chart 1.
On prolonged incubation at low pH levels, cells stained with eosin, but did so gradually and indistinctly. Due to cell metabolism, the pH of media with initial pH of 7.5-9.5 dropped substantially during incubation. Although the final pH of the tube with initial pH 10.7 was not determined, results of two repeat experiments showed a drop of approximately 1 pH unit for tubes with similar pH. All three experiments with L4946 and one with E9514-A cells showed a sharp drop-off of normal cells following incubation for 4 hours at an initial pH below 6.5-7 or above 9-9.5.

Osmolarity.—Three experiments were done in which the osmolarity of the medium containing L4946 cells was varied from 50 to 1,000 milliosmoles per liter. At higher osmolarity levels, beginning around 350 milliosmoles, noticeably higher deformation of cells occurred; at 650 milliosmoles only 2 per cent of cells of normal morphologic appearance survived after 4 hours' incubation at 37° C. At the low osmolarities, beginning around 150 milliosmoles, cell surfaces became higher serrated but seemed to maintain structural integrity. Because it was difficult to classify such cells, quantitative curves are not presented.

Antibiotics.—Addition of penicillin and streptomycin at levels used by Eaton and Scala (8) reduced the possibility of bacterial contamination when cells were incubated at 37° C. and later injected into mice. Even at the low levels used, a slight adverse effect on cell integrity was observed. Nevertheless, these antibiotics were included in the buffer for the sake of uniformity in all experiments.

Classification of cells as normal or deformed.—The series of changes consequent to addition of antiserum and complement to single cell suspensions of L4946 cells agreed with those described in detail by a number of able investigators (2, 7, 11, 15, 19, 28, 32). Regarding one point of difference between these authors, with respect to late morphologic changes: with the present degree of manipulation given L4946 cells, the nuclear membrane usually appeared to rupture first; later, the cytoplasmic membrane also might rupture. Occasionally, the reverse sequence appeared to occur, as did prolonged survival of both membranes.

Investigation of early changes subsequent to addition of antiserum led to classification of cells into four classes. Cells of normal morphology were classed as no. 1 cells; typical specimens are illustrated as cells a, b, and c in Chart 2. These cells had a sharp cytoplasmic outline seen as a narrow, intensely dark ring of continuous and fairly regular outline, were generally nontransparent, and did not stain on exposure to trypan blue or eosin. No. 2 cells were classed as deformed, despite their failure to stain with vital staining dyes; several different types are shown in Chart 2, since a deficiency in any one of the characteristics of typical no. 1 cells was sufficient for classification as a no. 2 cell. Cells d, e, f, and g show the most usual se-
quence of increasing size, loss of contrast in cytoplasmic outline, and, most important, separation of a clear cytoplasmic area. Cell k shows an elongated pseudopod-like projection, with loss of contrast of the cytoplasmic outline along the projection; this loss is also seen in cell i, together with increase in size and development of vacuoles. Cell k appears to have cytoplasmic material protruding beyond its membrane. Cell l shows knoblike surface irregularities which may herald the more pronounced cytoplasmic contortions of cell m. The scheme shown in Chart 2 requires only minor modification for cells such as the Ehrlich ascites (7) and Krebs ascites (11), which show moderate cytoplasmic granulation and vacuolization when fresh; for these cells, types i and l cells count as no. 1 cells.

No. 3 and no. 4 cells are deformed cells that stain with trypan blue plus eosin, and represent late degenerative stages (2, 7, 11, 15, 28, 32). They are not shown in Chart 2, because no difficulty was experienced in distinguishing them from unstained cells (Figure 1). No. 3 cells are stained only in the nucleus, whereas no. 4 cells are stained in both cytoplasm and nucleus. Cell ghosts, often only faintly stained and faintly visible, were classed as no. 4 cells.

Effect of staining on cell classification.—The staining properties of comparable concentrations of eosin and trypan blue were distinctly different. Whereas eosin stained those cells which were less damaged than necessary for staining by trypan blue, trypan blue stained more vividly than eosin. For both vital staining dyes, a higher proportion of slightly damaged cells were stained at higher dye concentrations.

Chart 3 illustrates representative results obtained when different staining procedures were used simultaneously on a single cell suspension, in which between 36 and 64 per cent of the cells were deformed. Similar results were obtained in suspensions containing lower or higher percentages of deformed cells. Chart 3 shows that essentially the same total percentage of deformed cells (nos. 2, 3, and 4 cells) was obtained, irrespective of whether different concentrations of trypan blue, of eosin, of a mixture of the two dyes, or no dyes were used. With unstained cell suspensions, assessment of cell deformation was easier with the use of the phase rather than the light microscope, but identical results were obtained with either.

Despite these results, use of the staining procedure was continued, since it greatly reduced the percentage of cells requiring a critical decision for classification (see Figure 1). The percentage of no.

and 3 hours after start of incubation, when a mixture of eosin (0.15 per cent) and trypan blue (0.05 per cent) was used as staining medium; exceptions were seen during unusual conditions, as in trial of media of high acidity or low osmolarity (see above), or of high cell concentrations (see below). Inclusion of no. 2 cells into the deformed class eliminated the need for a time interval between the staining and counting of cells under the microscope.

Cell deformation and cell death.—To relate in vitro data of cell deformation to in vitro viability, three experiments were done in which tumor cells were incubated with antibody, deformation as-
unstained cells (no. 2 cells) in the deformed cell count.

Calculation of tumor cell viability from in vivo data.—Three methods were used above to calculate in vivo viability of tumor cells from mouse survival data. In the first method, each group (composed of two mice which received injections) was assigned a percentage mortality of 0, 50, or 100, depending on whether 0/2, 1/2, or 2/2 mice died from tumor injection. The LD50 cell dose was obtained from a curve of percentage mortality against the logarithm of numbers of cells injected. The values of this dose, which are comparable to the values in column 3 of Table 1, were 12, 7, 380, 200,000, and 780,000, and compare with 12, 7, 200, 200,000 and 630,000, respectively, obtained by the method of Reed and Muench (31).

The above methods ignored the fact that mice dying from their injection of tumor cells lived for different time periods before succumbing. To attempt to remedy this deficiency, the scoring system described in Table 2 was devised. It was based on the probability that a mouse dying relatively early after tumor injection had received many more cells than the minimum necessary to kill it. On the assumption that ten viable L4946 cells killed a mouse by multiplying logarithmically (30) to 200 million cells (25) in 18 days (see Table 1), then the initial cell dose multiplying at the same rate but killing a mouse within 11 days would be 6,900 cells. This simple calculation serves to explain assignment of high mortality scores to mice dying relatively early after inoculation, the mortality score being an index of the number of viable tumor cells in the inoculum.

Table 2 shows that a lower limit of 100 was chosen as the total score for a group of mice all dying from their injection of tumor. This score equaled the percentage mortality figure as calcul-

![Chart](image)

**Table 1**

**Incubation of L4946 Cells with Rabbit Antiserum and Transplantation into AKR Mice**

Percentage normal (no. 1) cells determined before and after transplantation. Transplantation begun 1 hour after start of incubation at 37°C, completed 2 hours later. Two mice transplanted at each cell concentration.

<table>
<thead>
<tr>
<th>Normal cells (%)</th>
<th>Antiserum concentration*</th>
<th>Cell dose at mortality score of 50†</th>
<th>Relative viability of cells‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>0</td>
<td>11</td>
<td>91</td>
</tr>
<tr>
<td>40</td>
<td>1</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>1.5</td>
<td>3</td>
<td>200</td>
<td>3.8</td>
</tr>
<tr>
<td>0.25</td>
<td>10</td>
<td>130,000</td>
<td>0.008</td>
</tr>
<tr>
<td>0.10</td>
<td>30</td>
<td>580,000</td>
<td>0.002</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Approximate number of cells injected I.P. into AKR mice</th>
<th>Period of survival of injected mice (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>50,000</td>
<td>s, s b</td>
</tr>
<tr>
<td>200,000</td>
<td>s, s c</td>
</tr>
<tr>
<td>1,000,000</td>
<td>s, s d</td>
</tr>
</tbody>
</table>

* Parts per thousand, final concentration.
† Read off curves in Chart 4.
‡ Relative viability of cells = 100 X minimum cell dose at mortality score of 50 (minimum value in column 3) respective cell dose at mortality score of 50 (respective value in column 3)
* A number signifies the day after injection on which a mouse died with histologically confirmed tumor; e.g., 18 means death on day 18 after injection.
* d Signifies death without histological evidence of tumor; the mouse died on day 14 with questionable bacteremia.
lated by the first two methods. The upper limit of 150 was chosen empirically such that the cell dose at a mortality score of 50 (read from Chart 4 and recorded in column 3 of Table 1) was comparable to the LD₀₅₀ doses determined by the first two methods. This cell dose was a more representative index of the mortality data than the LD₅₀ since it included consideration of temporary survival of mice dying from tumor.

Time curves.—The change in deformed cell count with time is illustrated in Charts 5 and 6. Both control and experimental tubes containing antiserum showed a relatively small and approximately linear increase in percentage deformed cells between 1 and 3 hours after start of incubation. The percentage deformed cells at 2 hours was chosen as the final result for each tube, since it could be obtained from only two cell counts, one done before and another after 2 hours' incubation.

Cytotoxic titer.—Chart 7 shows a semi-logarithmic plot of percentage deformed cells at 2 hours' incubation against final concentration of antiserum. The resulting curve is a titration curve, showing increasing deformation (or damage) of tumor cells by increasing concentrations of antiserum. The cytotoxic titer was read off from this curve as the final concentration of antiserum giving 50 per cent deformation of normal cells present in the control tube. This definition implies a correction for the percentage of deformed cells in control tubes, which varied from 8 to 23 per cent in fourteen repeat experiments. Thus, the cytotoxic titer was read off at a value of 50 per cent plus one-half the control percentage of deformed cells (large arrow in Chart 7) rather than at 50 per cent deformed cells (small arrow in Chart 7). A high cytotoxic titer is defined as connoting a strong antiserum with high cytotoxic activity, requiring a low final concentration of antiserum for producing cell deformation.

In fourteen repeat experiments, the mean cytotoxic titer of a rabbit anti-L4946 serum was 1.24 parts per thousand with a standard deviation of 0.26, or 21 per cent. No significant difference was observed whether or not the cells were washed. Homologous antisera prepared against L4946 cells in C57BL/6 and C3Heb mice had 61- to 17-fold

![Chart 5](chart5.png)

**Chart 5.**—Percentage deformed cells of L4946 ascites tumor after incubation for various time periods at 37°C. with varying concentrations of rabbit anti-L4946 serum. Each tube contained 20 per cent guinea pig serum, antiserum (Ab) at stated final concentration (%o=parts per thousand), 10 million L4946 cells, and fortified buffer to 1.0 ml.

![Chart 6](chart6.png)

**Chart 6.**—Same experiment as in Chart 5, but with E9514A ascites tumor cells.

**TABLE 2**

<table>
<thead>
<tr>
<th>Day of death after injection</th>
<th>Mortality score for each mouse dying from tumor (two mice in each group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11 and under*</td>
<td>75</td>
</tr>
<tr>
<td>12</td>
<td>71</td>
</tr>
<tr>
<td>13</td>
<td>64</td>
</tr>
<tr>
<td>14</td>
<td>66</td>
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<tr>
<td>15</td>
<td>60</td>
</tr>
<tr>
<td>16</td>
<td>57</td>
</tr>
<tr>
<td>17</td>
<td>53</td>
</tr>
<tr>
<td>18 and over*</td>
<td>30</td>
</tr>
</tbody>
</table>

* In the present experiment, mice died at latest on day 18, at earliest on day 11 after injection.
weaker cytotoxic activity, with titers of, respectively, 7.6 and 2.1 per cent; mouse antiserum gave time curves and cell titration curves identical with those in Charts 5, 6, and 7.

**Complement addition.**—Chart 8 illustrates one of three experiments in which the concentrations of guinea pig serum in the system were varied. The results show that, at a concentration around 20 per cent, guinea pig serum was present in sufficient excess, so that its precise concentration had little effect on the cytotoxic action of antiserum.

In experiments with L4946 cells, pooled normal rabbit serum proved perceptibly cytotoxic at 5 per cent and almost completely cytotoxic at 20 per cent concentration. Pooled normal mouse serum was nontoxic.

**Inactivation of antiserum.**—The cytotoxic titers of active rabbit antiserum and of antiserum inactivated for 20 minutes at 56°C was determined on the same L4946 cell suspension in the presence of 20 per cent guinea pig serum. Two such experiments by chance gave identical results: a 3 per cent decrease in cytotoxicity of the inactivated antiserum. These experiments indicate that inactivation of rabbit antiserum has no significant effect on cytotoxic titer when excess complement is present.

**Cell concentration.**—Four experiments were done in which the cytotoxic titer against L4946 cells was determined simultaneously at final concentrations of 2.5, 5, 10, 20, and 40 million cells per ml.

![Chart 7](image-url) **Chart 7.**—Effect of rabbit anti-L4946 serum on the percentage deformed L4946 cells, following incubation for 2 hours at 37°C with 20 per cent guinea pig serum.

![Chart 8](image-url) **Chart 8.**—Effect of guinea pig serum on the percentage L4946 cells deformed by rabbit anti-L4946 serum following incubation for 2 hours at 37°C.

In two experiments, guinea pig complement was present at 20 per cent final concentration. Chart 9 shows that direct proportionality was not obtained. However, the relationship was approximately linear between 5 and 20 million cells per ml., and the cytotoxic titer approximately proportional to cell concentration. At the highest cell concentration an unusually high proportion of no. 2 cells was observed, suggesting that an excess of metabolic products was damaging the cells. In two further experiments, complement was added in proportion to cell concentration. Results were similar to those in Chart 9, except the corresponding curves were slightly concave rather than convex.

Chart 9 was used to correct the cytotoxic titer to the basis of 10 million cells per ml. for experiments run at different cell concentrations. Chart 9 also shows that the assay becomes more sensitive at low cell concentrations, since less antibody is needed to titrate the cells.
Volume of medium.—The cytotoxic titer of rabbit anti-L4946 serum was determined simultaneously in total volumes of 1.00 ml. and 0.25 ml. At the lower total volume, the incubating tubes were 9 mm. I.D. X 7.5 cm., and 0.05-ml. aliquots of cell suspension were stained with 0.06-ml. aliquots of dye. In two identical experiments, the titer in 0.25-ml. volume was, respectively, 15 per cent higher and 5 per cent lower than in 1.00-ml. volume. This indicates no significant change in titer with fourfold reduction in volume of medium. One advantage of using the lower volume is that relatively minute quantities of antiserum suffice for the assay; one disadvantage, that accuracy is reduced unless great care is taken in pipetting.

DISCUSSION

The present assay system is identical in many respects to systems described previously. Thus, Gorer and O’Gorman (15) have shown that cell suspensions derived from ascites tumors, solid tumors, or spleen showed different degrees of staining by trypan blue or eosin, depending on the titer of isoantibody and the presence of complement. Easty and Ambrose (7) determined the potency of antiserum by measuring the time required for lysis of single cell suspensions of mouse tumor cells or of normal tissue cells. Schreck and Preston (33) titred single cell suspensions of the Bagg rat lymphosarcoma, using the proportion of vitally stained cells at the end of 4 hours’ incubation as their endpoint. Defendi and Colter (5) found that antiserum caused a progressive decrease with time in percentage of vital staining of trypsinized tissue culture cell suspensions, and used the rate of staining as a measure of antiserum potency.

At first sight, the kinetic data of Defendi and Colter (5) appeared to contradict the present work. However, when the results in Charts 5 and 6 were plotted by their method, reasonable facsimiles of their charts were obtained. The main points of difference are their use of higher concentrations of antiserum, interest in initial rates of staining, and use of trypsinized tissue culture cells.

The main finding of the present study is that the percentage of deformed ascites tumor cells increased only slightly between 1 and 3 hours when the cells were incubated with antiserum and complement at 37° C. This finding held in two experiments with Ehrlich ascites tumor, as well as with L4946 and E8514-A cells. As a result, the percentage of deformed cells in many experimental tubes could be determined in a single experiment.

Estimation of the percentage of deformed cells was raised beyond an empirical method dependent on dye concentration, by inclusion of cells deformed but unstained by vital staining dyes. However, it added complication to a method that was otherwise very simple. Time and titration curves identical in shape and only slightly displaced in position were obtained when cells were classified into only two types, unstained (nos. 1 and 2 cells) and stained (nos. 3 and 4 cells). Future investigators may wish to employ this simpler classification method without fear of loss in reproducibility.

In vivo results indicated that the in vitro count underestimated damage to cells, suggesting that the cell deformation seen under the microscope heralded the first irreversible stages of cell death. The scoring method used to estimate cell viability may be of interest. If its basic concept is valid, it should result in a smoothing out of experimental points in a plot such as Chart 4, and this in fact occurred. A good case could be made for future use of a much higher upper scoring limit than 150.

An interesting question left unanswered is why only a portion of the tumor cells became deformed at any given antiserum concentration. The very fact that the cell titration curve of Chart 7 is S-shaped indicates that a given tumor cell population displays a whole spectrum of resistance to the action of antiserum. Since this action is directed against the cytoplasmic membrane (2, 7, 11, 16, 17), the strength of this membrane must vary widely in a given cell population. Possible reasons for this variation may include age and mitotic state of the cell, with old cells perhaps being especially sensitive; damage of cells, either from roughness in manipulation or through use of trypsin (5) or deoxyribonuclease (3); and prior damage incurred in vivo from the normal immunological defense mechanisms of the host (33). The existence of a percentage of tumor cells especially resistant to the action of antiserum (3, 28, 33) is ascribed to the same factors responsible for the S-shape of the cell titration curve (Chart 7). Because of this shape, a relatively large amount of antiserum is required to destroy the last remaining viable cells.

Previous data (3, 7, 20, 33) suggest that the present assay system may be applicable to single cell suspensions of solid tumors or normal solid tissues.

REFERENCES

Fig. 1.—L4946 cells after 2 hours' incubation at 37° C. with 0.6 parts per thousand of rabbit antiserum and 32 percent guinea pig serum, then stained with eosin plus trypan blue. Seen in a hemocytometer chamber at 700X magnification are eleven normal (no. 1) cells, one unstained deformed (no. 2) cell (arrow), and six stained deformed (no. 4) cells.
A System for Quantitative Determination of Cytotoxic Activity of Antisera to Ascites Tumor Cells

Arnold E. Reif and Henry J. Norris

*Cancer Res* 1960;20:1235.

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