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Spontaneous Human Lymphocyte-Mediated Cytotoxicity Against Tumor Target Cells. IX. The Quantitation of Natural Killer Cell Activity

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On analysis of *in vitro* assays of human natural killer (NK) cell function the inadequacy of commonly used methods of expressing lytic activity was apparent. A comparison was made of the data obtained using modifications of two equations—the simple exponential fit and the von Krogh equations. Both of these equations were found to satisfy the following essential criteria for use in these assays. First, the majority of the results obtained in the chromium-release assay could be used in data reduction; second, the resultant "dose-response" curve was reduced to linearity; and third, a single numerical expression was obtained which was directly proportional to the cytotoxic activity. Of the two methods the more conventional exponential fit was found to be the simpler to use. The closeness of fit of the experimentally derived data to the ideal curves did not support the possibility that normal lymphocyte preparations contain suppressor cells capable of inhibiting NK activity. Data have also been presented showing that NK-sensitive targets could be categorized with respect to their susceptibility by comparing the slopes of the target cell survival curves obtained using the exponential fit equation. These observations are relevant to the accurate assessment of NK activity in patient populations and to the determination of the effects of disease and its treatment on this activity.

KEY WORDS: Human natural killer cells; chromium release.

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INTRODUCTION

In recent years, the phenomenon of spontaneous lymphocyte-mediated cytotoxicity (SLMC) or natural killer (NK) cell activity has become an increasingly popular field of investigation (1, 2). Although the majority of publications has dealt with animal experiments, the role of the NK cell in clinical immunology has also been the subject of intense research. In addition to studies evaluating NK activity in normal individuals (3–6), a number of reports have dealt with various disease entities such as malignancy (6–10), infectious mononucleosis (11), immunodeficiency (12, 13), "autoimmune" diseases (14), and multiple sclerosis (15). Other studies have examined the effects of various drugs and therapeutic procedures such as corticosteroids (16), immunotherapy (17), interferon therapy (18), and bone marrow transplantation (19, 20). In spite of the considerable amount of research which is being done on human NK cytotoxicity, very few reports have utilized methods of data reduction and expression which are meaningful in terms of indicating lytic activity, in either absolute or relative terms. Although in some cases data have been presented in graphical form showing a complete range of lymphocyte to target cell (L/T) ratios, this is frequently impractical for large patient groups, and the majority of investigators uses tables in which cytotoxicity is shown as the percentage chromium release at a single L/T ratio. The second most common form of data expression is the lytic unit (LU). The lytic unit is defined as that number of cells required to cause a defined amount of target cell lysis, and is usually ex-

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pressed as LU/10^6 lymphocytes so that the value increases with increasing lytic activity. As discussed below, the documentation of NK activity in terms of isolated percentage chromium-release values is unacceptable if it is intended that a comparison be made between different lymphocyte donors or between different cell subpopulations. Similarly, the use of lytic units is also prone to error, and is only as accurate as the method which is used to calculate the line.

In the present paper, four different forms of data expression are discussed: simple percentage chromium release, lytic units defined by linear regression analysis of the linear part of the sigmoid "dose-response" curve, lytic units defined by a simple exponential fit equation, and lytic units defined by the von Krogh equation (21). In evaluating these different equations, the primary criteria for a satisfactory method have been, first, that the majority of the data obtained experimentally could be reduced to linearity and, second, that the entire data set could be expressed as a single numerical parameter directly proportional to the lytic activity. In doing this, we have limited our analysis to data obtained using conventional chromium-release assay techniques, in which varying concentrations of effector cells are added to a fixed number of labeled target cells (22). Although this is by far the most common method of performing these assays, a considerable volume of data has been accumulated in which the cytotoxic cell-target cell interaction is treated as if it were an enzyme-substrate reaction (23-25). As yet, however, this methodology has not been applied as a means of comparing NK cell activity in different patient populations, and a detailed analysis of it is not intended in this paper.

MATERIALS AND METHODS

Lymphocyte preparation. Phagocytic cells were removed from 10 ml heparinized whole blood by incubation at 37° C with 100 mg carbonyl iron (Type E, A. D. McKay, New York), with occasional shaking. The blood was then passed twice over a large, 2.5-Kg magnet and 5 ml was layered on 3 ml of Ficoll-Isopaque (specific gravity 1.077) (26, 27). After centrifugation at 800*g* for 12 min at room temperature, the interface cells were washed three times in complete medium [RPMI 1640 containing penicillin (100 IU/ml), streptomycin (100 μg/ml), 10% fetal calf serum, and 10 m*M* Hepes buffer] and suspended at the required concentration of viable cells per milliliter.

NK cell assays. Natural killer cell activity was measured using the K562 chromium-release assay as previously described (7, 28). The reasons for K562 being the target of choice in these assays have also been outlined in detail (1, 29). Briefly, the technique consists of a 5-hr or overnight ^{51}Cr-release assay (22) using V-bottom microplates, in which a series of lymphocyte to target cell ratios is set up by mixing a constant number of ^{51}Cr-labeled target cells with a series of dilutions of effector cells. After incubation at 37° C in a humidified 5% CO_2 incubator, the plates are centrifuged and 100-μl aliquots are removed for counting in a well-type Ultrogamma II gamma counter (LKB-Wallac, Stockholm). The percentage cell-mediated lysis (% CML) was calculated as follows:

$$\% \text{ CML} = \frac{(\text{cpm test} - \text{cpm medium})}{(\text{cpm max} - \text{cpm medium})} \times 100\%.$$

The cpm for the total incorporated label was used for the cpm max, and was determined by counting a 100-μl aliquot of resuspended target cells. Parallel wells were set up in which 1% saponin detergent was used in place of medium. The cpm of a 100-μl aliquot of the supernatants from these wells was used to determine the maximum detergent-mediated chromium release, which was usually 80-90% of the total incorporated label.

Maintenance and labeling of cell lines. Cell lines were maintained in complete medium, without Hepes buffer, but with 5 m*M* extra L-glutamine and 60 μg/ml of Tylocine (30, 31). The following cell lines were used: K562, an erythroleukemic line derived from a patient with chronic myeloid leukemia (32), Molt 4 (human T cell), HL-60 (human promyelocytic leukemia) (35), 2774 (ovarian carcinoma), A549 (alveolar cell carcinoma), WI38 (human embryonic lung), Goodwin (adenocarcinoma of the lung), P815X2 (mouse mastocytoma), and EL-4 (mouse T-cell lymphoma). For the chromium-release assay, approximately 10^6 target cells in 0.1 ml of culture medium were incubated with 100 μCi of ^{51}Cr (sodium chromate, in isotonic saline, Amersham No. CJS-11, 50-400 mCi/mg Cr) for 1 hr at 37° C. The labeled cells were washed three times in medium and resuspended at the appropriate concentration (usually 2×10^4 cells/ml in this report).

Data processing. A nonlinear, least-squares fitting program (NOTLIN, obtained from the Queen's University Computing Centre and available on request) was used to fit the data to the proposed equations (see below). This program uses standard numerical derivative fitting techniques (34). In effect, this iterative procedure involves an initial estimation of the constants described in the equations, followed by a calculation of the line using these estimated constants. The distances from each experimentally obtained data point to the calculated line (the "residuals") are then calculated, squared, and summed. The program then attempts to find a minimum in the sum of the squares of the residuals by making new estimates of the constants such that the sum of squares of the residuals is reduced, and then continues to make new estimates of the constants until a minimum in this sum is found (convergence). Occasionally no minimum is found and the program stops without converging. However, even in these cases, the values of the parameters found are adequate for comparison purposes, and the fact that the data do not converge serves to alert the user that a particular lymphocyte preparation may be different in the kinetics of cell-mediated lysis from the norm. Thus, the use of this computer program, which assumes a certain type of interaction, permits the detection of variant data which may potentially occur. The actual equations used are described in the Results. The standard deviations for the parameters described by these equations were also determined (34).

For the data described in Fig. 3, a linear regression analysis and the calculation of the correlation coefficient, r, were done according to Sokal and Rohlf (35).

RESULTS

The basic requirements of data reduction methods—a comparison of different commonly used procedures. In evaluating different methods of quantitating NK activity, it is necessary to make a basic assumption in terms of what is expected of the method. The assumption is that if a preparation containing x lymphocytes/ml lyses the same number of target cells as another containing $2x$ (or $3x$ or $4x$) lymphocytes/ml, the cytotoxic activity of the former is twice (or three or four times) that of the latter. If this somewhat self-evident assumption is accepted, a series of curves can be drawn, each of which relates to the other in a defined way, simply by plotting the actual cytotoxicity values obtained experimentally against a series of proportionally related values of x. Figure 1 shows five curves, labeled a–e, of which curve c was drawn from real data and the other curves were drawn by shifting the abscissa to the right or left. Theoretically these curves could equally well represent data from five different donors, and the relationship of curves from different donors, in practice, should be similar to these theoretical curves if they are to be compared with each other (see below). In published studies of cell-mediated cytotoxicity against ^{51}Cr-labeled targets, the data have usually been expressed either by simply documenting the percentage chromium release at a single lymphocyte to target cell ratio or by drawing a straight line between the points believed to be on the linear portion of the sigmoid curve in order to determine the "lytic unit." The documentation of the NK function of different lymphocyte preparations by simply recording the percentage chromium release at one L/T ratio is a common procedure, but one which is virtually useless in terms of conveying information as to the comparative activities. This is self-evident from Fig. 1. At an L/T ratio of 1.25/1 the cytotoxicity of a vs c was 58 vs 23%, a factor of 2.5; while at 10/1 the values obtained were 78 vs 73%, a factor of 1.1. In actual fact, the lines were drawn as if there had been a fourfold difference in activity between the two. The use of percentage chromium-release data at one L/T ratio to compare different

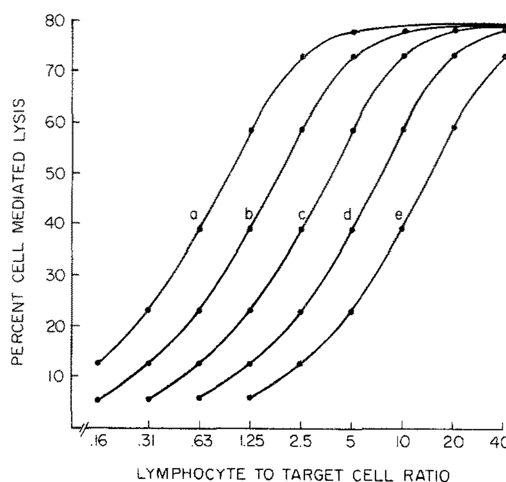

Fig. 1. Semilogarithmic plots of real data, curve c, compared with proportionally related theoretical curves. The relationship in NK activity between the curves is as follows: a = 4c, b = 2c, d = 0.5c, and e = 0.25c.

lymphocyte preparations is valid only if the plots of percentage chromium release vs lymphocyte numbers are linear and parallel, at the points at which the comparison is being made.

The other method commonly in use has been to estimate the number of effector cells required to produce a defined percentage lysis (the lytic unit). This is done by drawing a straight line using those points apparently on the linear part of the sigmoid curve and by extrapolating (if necessary) to the predefined levels of lysis. In previous publications (7, 12), we have used a similar method except that extrapolation was avoided in most cases by making the reference point the average cytotoxicity attained by control lymphocytes at an L/T ratio of 5/1, a level of cytotoxicity almost invariably on the linear part of the sigmoid curve. In order for comparisons between different lymphocyte preparations to be valid, however, the lines drawn must be parallel, as is evident from Fig. 1. In practice, however, the points selected frequently inadvertently include the curved part of the line, at either the top or the bottom. The result of this is to artifactually decrease the slope of the line at the extremes of high or low NK cell activity. Depending on the actual level of cytotoxicity observed, the error is further compounded if extrapolation is required to estimate the lytic unit. The result of this is to falsely increase the values obtained for high-activity lymphocyte preparations, while decreasing the values obtained for low-activity preparations. Figure 2 illustrates the results obtained on analysis of a number of normal donors, each tested from 5 to 150 times over a 5-year period (median 7 times) (4). It can be seen in Fig. 2 that the slopes of the dose-response curves varied to some extent, in spite of our efforts to include only those points on the linear part of the curve. Statistically the slopes of the majority of these curves were not significantly different from each other, making valid comparisons between the lines possible. On the other hand (Fig. 3), linear regression analysis of slope vs cytotoxicity showed a highly significant correlation between these two variables ($r = 0.9235$; $P < 0.001$), indicating that the greater the difference between the control cytotoxicity and that of the patient, the greater the probability that the slopes will not be parallel, and that the data will not be comparable if this methodology for deriving lytic units is used.

In short, the expression and comparison of data using lytic units require the use of an equation which incorporates all of the data, and not an arbitrarily selected portion of it, and which renders the data linear. Two methods have been used to do this (Figs. 4 and 5)—the exponential fit equation, $y = 1 - e^{-kx}$, used as $y = 1 - e^{-N\alpha t}$ primarily by Miller *et al.* (36, 37), and the von Krogh equation, $y = 1/[1 + (K/x)^n]$, which has been used extensively in evaluating complement-mediated lysis in the form $\log x = \log K + 1/n \log[y/(1 - y)]$ (21, 38). In these equations the meaning of the symbols is as follows: y = fractional cell-mediated ^{51}Cr release; x, N = number of effector cells per target cell; k = the slope of

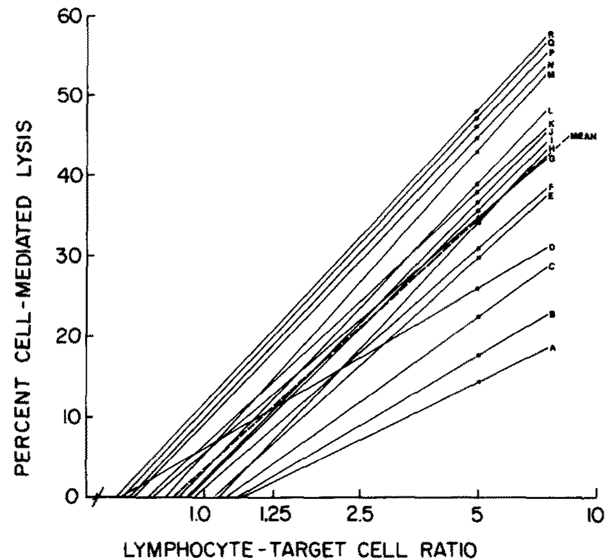

Fig. 2. The linear portions of the sigmoid semilogarithmic "dose-response" curves for 17 donors tested over 5 years, each tested 5-150 times.

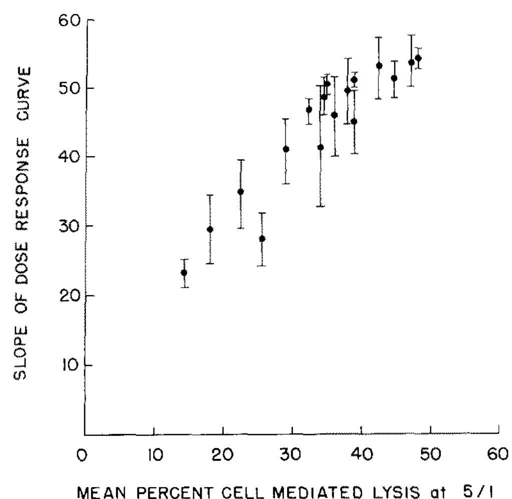

Fig. 3. The relationship of the slopes of the linear portion of the dose-response curves shown in Fig. 2 (±1 SD) to the calculated percentage cell-mediated cytotoxicity at an L/T ratio of 5/1.

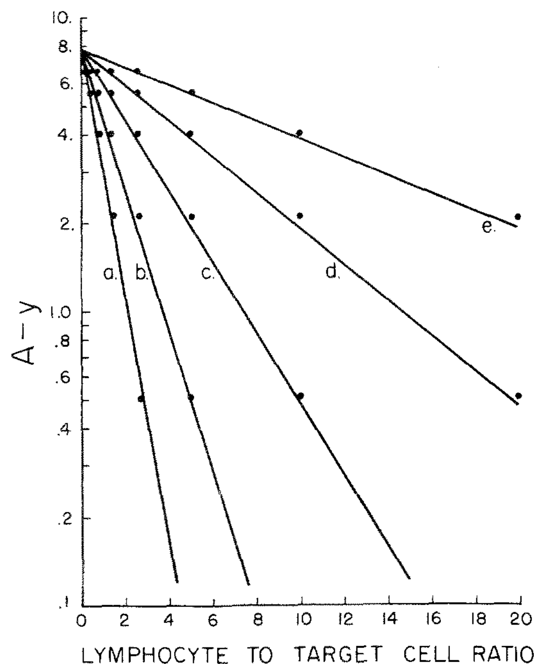

Fig. 4. A plot of the curves shown in Fig. 1 expressed as $\ln(A - y)$ vs x. The relationship between the slopes of these curves is as follows: $ka/kc = 4$, $kb/kc = 2$, $kd/kc = 0.5$, $ke/kc = 0.25$ ($A = 78$).

the line obtained by plotting logarithm $(A - y)$ vs x, and equal to αt; α = a constant; t = time of incubation at 37° C; K = a constant equal to the value of x when y = 50% of the asymptote of the dose–response curve, A; n = a constant, equal to the reciprocal of the slope of the von Krogh line ($1/n$) as it is conventionally expressed (see above).

Both of these equations assume that the asymptotic part of the curve, A, is 100%, which, in chromium-release assays using tumor target cells, is not the case (see Figs. 1 and 6). To overcome this problem, two methods have been used. The first involves calculating the percentage chromium release using detergent-lysed target cells to estimate the counts at 100% lysis. This results in a theoretical asymptote of 80–90% of the total incorporated label but, in our experience, almost always exceeds the asymptotic level achieved by cell-mediated lysis. As examples, in Fig. 1 detergent lysis resulted in 90% chromium release, while in Fig. 6 detergent lysis caused 85% chromium release. In each of these assays, the plateau achieved by maximum cell-mediated lysis was substantially lower, unusually so in Fig. 6. An alternative method has been to estimate the asymptote either experimentally (39) or by computer iteration (40) and to incorporate this value in the two equations as follows: $y = A(1 - e^{-kx})$ for the exponential fit and $y = A/[1 + (K/x)^n]$ for the von Krogh equation. Figures 4 and 5 show the results obtained when the curves in Fig. 1 were plotted using the modified exponential fit (Fig. 4) and the von Krogh equation (Fig. 5). It can be seen that the data becomes linear in both cases, and that the lines make use of the majority of the data points. The important parameters in these two equations

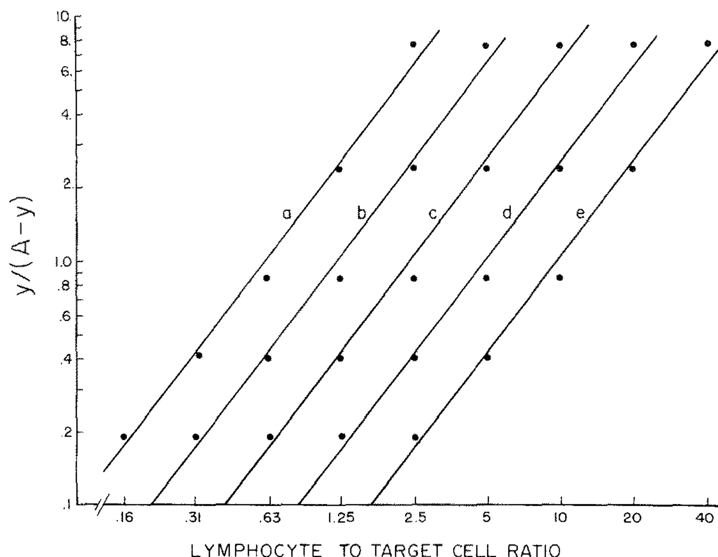

Fig. 5. A plot of the curves shown in Fig. 1 expressed as $y/(A - y)$ vs $\log x$. The relationship between the K values (and the x intercepts) of these lines is as follows: $Kc/Ka = 4$, $Kc/Kb = 2$, $Kc/Kd = 0.5$, $Kc/Ke = 0.25$ ($A = 78$).

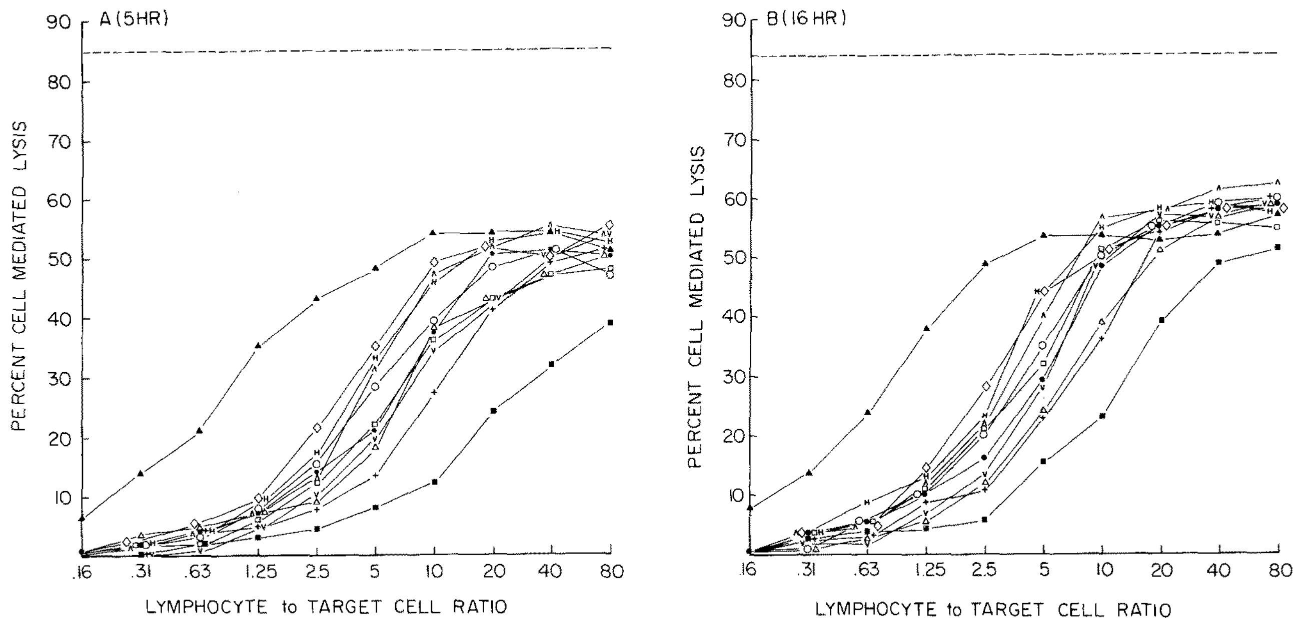

Fig. 6. (A) Cell-mediated cytotoxicity of lymphocytes from 11 normal donors vs K562, assayed at 5 hr. Total incorporated label = 100%. Dotted line, percentage maximum release by 1% saponin detergent. The symbols related to the donors listed in Tables I and II as follows: ●, 580; ○, 581; □, 582; ■, 583; △, 584; ▲, 585; +, 586; ◇, 587; H, 588; V, 589; /\, 590. (B) Cell-mediated cytotoxicity of lymphocytes from 11 normal donors vs K562, assayed at 16 hr. Symbols as for A.

are k and K, respectively. As can be seen from the figures and the data in the figure legends, these parameters are directly proportional to the relative cytotoxic activity for each of the five lines, thus satisfying the main requirement for a method of quantitating cytotoxic activity.

To assess the practical usefulness of the two equations, the methods were applied to the analysis of an actual experiment. Figures 6A and B show the results of a K562 chromium-release assay of NK activity, done using lymphocytes from 11 normal male and female donors. The supernatants were harvested at 5 and 16 hr and the data have been plotted on a semilogarithmic plot where 100% represents the maximum incorporated label. Several important points are evident from these figures. First, the linear parts of all of the curves are approximately parallel, as they should be if the lines are to be comparable. Second, the relationships between the different curves observed at 5 and 16 hr are similar, indicating that there is very little difference in terms of comparisons between donors at the two time points. Third, even with the lowest activity donor, the plateau of maximum lysis achieved, if enough effector cells are added, is the same as for the other donors. This observation is also a prerequisite if k or K values are to be compared between different donors, since the A values used in the calculations should be the same. Fourth, the plateau of maximum cell-mediated lysis is much lower than the percentage lysis caused by detergent (85% vs a mean A of 54% in Fig. 6A and 58.1% in Fig. 6B). These A values are low, the usual maximum cell-mediated lysis being between 70 and 80% in our experience. This is fortuitous because it serves to illustrate some of the problems which are met in data processing, since the greater the discrepancy between the detergent-mediated and the cell-mediated maximum lysis, the poorer the fit will be if an equation using an asymptote of 100% is used. The fit, and hence the estimation of lytic units, will be poorer still if the total incorporated label (either with or without subtraction of spontaneous release) is used as the "maximum" in the calculation of percentage chromium release, unless the appropriate value of A is used in the equation.

The analysis of the data in Fig. 6 is shown in Table I (calculated using the exponential fit equation) and Table II (calculated using the von Krogh equation). The rationale in preparing these tables was to compare the results obtained using both methods, as well as to assess the impact of fixing the A value, or of choosing an inappropriate A value, on the calculations of relative cytotoxicity and lytic units. In addition, the effect on the data of doing an overnight compared with a 5-hr assay was

Table I. The Effect of Varying A Values on the Calculation of Relative Lytic Activity and Lytic Units—Exponential Fit Equation

5-hr assay

Donor (Fig. 6A)	Slope $k(\times 10^{-3})^a$		k_i/geom mean k		20% Lytic units/10^6		
	$A = 85$	$A = 54$	$A = 85$	$A = 54$	Iter A	$A = 85$	$A = 54$
580	31.6	114	0.91	0.95	125	58.8	123
581	41.5	130	1.19	1.09	149	77.3	140
582	23.3	96.4	0.67	0.81	115	43.4	104
583	10.0	22.1	0.29	0.19	30.1	18.6	23.9
584	23.9	92.6	0.69	0.78	108	44.5	100
585	174	770	5.0	6.47	862	325	833
586	20.5	61.9	0.59	0.52	68.6	38.2	66.9
587	51.4	201	1.48	1.69	217	95.8	217
588	55.4	176	1.60	1.47	188	103	190
589	25.3	86.0	0.73	0.72	94.3	47.1	92.9
590	45.8	160	1.32	1.34	172	85.3	172

16-hr assay

Donor (Fig. 6B)	Slope $k(\times 10^{-3})^a$		k_i/geom mean k		20% Lytic units/10^6		
	$A = 85$	$A = 62$	$A = 85$	$A = 62$	Iter A	$A = 85$	$A = 62$
580	56.3	132	0.87	0.87	174	105	169
581	65.1	156	1.00	1.02	209	121	200
582	65.3	155	1.00	1.02	211	122	199
583	20.4	46.9	0.31	0.31	66	38.1	60
584	39.8	92.2	0.61	0.61	124	74.1	118
585	256	715	3.93	4.71	1042	480	926
586	39.5	86.8	0.61	0.57	112	73.6	111
587	86.4	219	1.33	1.44	298	161	281
588	99.3	221	1.53	1.46	287	185	284
589	57.7	122	0.89	0.80	159	108	156
590	90.9	194	1.40	1.27	250	170	252

[a] The A values used were derived as follows: $A = 85$, detergent-mediated lysis: $A = 54$ and 62, maximum cell-mediated lysis (iterated from the dose-response curve showing the highest maximum values at 5 and 16 hr, respectively); Iter A, maximum cell-mediated lysis separately iterated for each individual dose-response curve. K562/well = 2×10^3.

also assessed. As stated above, in order for data from different lymphocyte preparations to be comparable, the A values used in data calculation must be the same, and a decision has to be made as to how it should be chosen. If complete curves have been obtained experimentally, the A values can be iterated accurately by the computer as described in Materials and Methods (or determined graphically) and the resultant set of A values should not differ significantly from each other. On the other hand, it is unusual that maximum lysis is attained by all of the lymphocyte preparations in any particular experiment, and the accuracy of the iteration diminishes the further the highest observed cytotoxicity is from the maximum. Two alternatives to the use of individually iterated A values are to use the maximum cell-mediated lysis shown by the highest donor in the group, determined either graphically or by iteration, and to use the percentage chromium release caused by detergent. The former is the more accurate in terms of producing the k or K values characteristic of the cells in question, but, as shown in Tables I and II, the choice of the higher, somewhat inappropriate A value actually has very little effect on the number obtained for the cytotoxic activity of a particular individual relative to that of other donors (k_i/mean k, Table I, and mean K/K_i, Table II). It can also be seen from these tables that the results obtained for relative cytotoxic activity are quite similar irrespective of whether they are determined at 5 hours or overnight. If anything, the lower donors "catch up" to some extent during the longer incubation time.

Because the lytic unit is a useful concept, it is obviously important that any equation used results in an accurate estimation of the number of lymphocytes required to lyse a predetermined percentage of target cells. That this is indeed the case for both the exponential fit equation and the von Krogh equation is shown in Fig. 7. In this figure, the solid and dotted lines represent the curves obtained using each of the two equations, while the filled circles

Table II. The Effect of Varying A Values on the Calculation of Relative Lytic Activity and Lytic Units—von Krogh Equation

5-hr assay

Donor (Fig. 6A)	K values[a]		Geom mean K/K_i		20% Lytic units/10^6		
	$A = 85$	$A = 56$	$A = 85$	$A = 56$	Iter A	$A = 85$	$A = 56$
580	21.2	6.1	0.88	0.96	156	133	127
581	22.1	5.3	0.84	1.10	154	151	152
582	28.6	7.3	0.65	0.80	120	110	117
583	91.3	31.8	0.20	0.18	31	27	29
584	27.8	7.5	0.67	0.78	110	110	110
585	3.1	0.9	6.00	6.48	956	1011	958
586	32.6	11.1	0.57	0.53	70	68	70
587	8.8	3.4	2.11	1.71	214	207	212
588	9.8	4.0	1.90	1.46	183	179	182
589	25.5	7.9	0.73	0.74	98	93	97
590	12.2	4.4	1.52	1.33	161	164	161

16-hr assay

Donor (Fig. 6B)	K values[a]		Geom mean K/K_i		20% Lytic units/10^6		
	$A = 85$	$A = 65$	$A = 85$	$A = 65$	Iter A	$A = 85$	$A = 65$
580	12.2	5.4	0.82	0.86	180	206	184
581	10.8	4.6	0.92	1.01	207	229	209
582	11.5	4.6	0.87	1.01	210	245	217
583	33.1	15.7	0.30	0.30	71	71	70
584	16.2	7.6	0.61	0.61	126	132	127
585	2.0	1.0	4.98	4.64	1082	1093	1077
586	16.4	8.2	0.61	0.57	111	122	111
587	7.6	3.3	1.31	1.41	292	309	296
588	5.2	3.2	1.92	1.45	273	272	272
589	12.8	5.9	0.78	0.79	142	165	146
590	7.1	3.6	1.41	1.29	238	267	239

[a]The A values used were derived as follows: $A = 85$, detergent-mediated lysis; $A = 56$ and 65, maximum cell-mediated lysis (iterated from the dose-response curve showing the highest maximum values at 5 and 16 hr, respectively); Iter A, maximum cell-mediated lysis separately iterated for each individual dose-response curve. K562/well = 2×10^3.

represent the actual data. Although the two equations do not produce exactly the same sigmoid curve from the same data, the lines are very close to each other and to the experimentally derived points, and both equations are obviously equally useful for the determination of lytic unit values. Based on a comparison of the residual (or unexplained) variance derived from each of the two models, using iterated A values and all of the 11 donors at both time points, it was found that both equations resulted in excellent fits of the computed curves to the data.

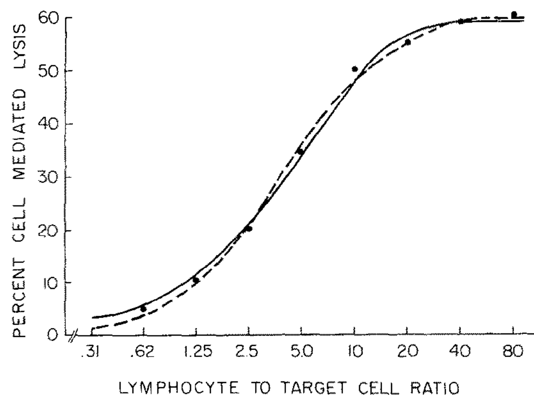

Fig. 7. Calculated curves given by the exponential fit equation (solid line) and the von Krogh equation (dotted line) compared with actual data points. Donor 581.

Potential sources of error. Although both equations result in a good correlation between the observed and the calculated points, the exponential fit equation is more sensitive to changes in the A value as far as determining the lytic unit is concerned (compare Table I, $A = 85$ vs the iterated A and $A = 54$, with the corresponding columns in Table II). This error is due to the investigator, however, and is not a defect in the equation, since it is obviously incorrect to use the higher A value when it is known that the maximum cell-mediated cytotoxicity plateaus at a point considerably lower than that indicated by detergent lysis or total incorporated la-

Table III. The Relationship Between Relative Cytotoxicity and the Definition of the Lytic Unit

Donor	Exponential fit equation $A = 54, k_1/k_2 = 0.029$		von Krogh equation $A = 56, K_2/K_1 = 0.028$		
	LU (20%)	LU (50%)	Slope ± SD	LU (20%)	LU (50%)
1. 583	23.9	4.3	0.94 ± 0.05	29	1.65
2. 585	832.2	148.0	1.13 ± 0.05	958	87
LU_1/LU_2	0.029	0.029		0.030	0.019

bel. The von Krogh equation yields a better fit under these conditions, because it contains a third variable, the slope of the line $1/n$. Unfortunately, this variable introduces a more serious error into the lytic unit calculation. Table III shows the data obtained from the two donors from Fig. 6A who were at the extremes of high and low cytotoxicity, the type of data which gives the most problems with analysis. It can be seen in this table that there is good agreement between the two equations as far as relative cytotoxicity is concerned (k_1/k_2 and K_2/K_1). Furthermore, the slopes of the lines obtained using the von Krogh equation are not significantly different, a requirement if the data are to be comparable. However, the divergence of the two lines is still sufficient to influence the relative values of the lytic units. Thus, selection of a lytic unit value of 50% instead of 20% results in a different conclusion as far as relative activity is concerned. As long as the appropriate A value is used, this error does not occur with the exponential fit equation (Table III).

The use of the exponential fit equation parameters to categorize NK-susceptible targets. As shown in Fig. 4, the straight line described by the exponential fit equation is simply a target cell survival curve with a slope of k which increases with increasing susceptibility of the target to lysis. Since the majority of "routine" assays of human NK function uses K562 as the target cell, it is reasonable to use this line as the standard for comparison with other lines. Table IV itemizes the relative susceptibility of a variety of cell lines in short-term (5-hr) and long-term (18-hr) assays, and expressed as the ratio of individual k values to the k value obtained for K562. All the lines were tested at the same time, and the k values used are the means of those of two donors. The rank order of target susceptibility was the same for both donors, and the data obtained in this one assay reflect our experience in general with these lines. First, Molt-4 is similar to K562 in NK susceptibility, as has been reported, and these two cell lines may be defined as "fast" targets (28). As a point of interest, long-term (1-year) culture of K562 in HSA caused only a 50% decrease in susceptibility, i.e., one halving dilution of effector cells lysed an equal proportion of the standard targets. This observation again indicates the independence of NK susceptibility from fetal calf serum-induced surface antigens (41), since this susceptibility is still consistently present in these cultures. The other human lines are much less susceptible at 5 hr ("slow" targets), with a relative increase seen on overnight culture. As has been reported (42, 43), murine targets are apparently insensitive on short-term assay also, but their sensitivity is evident by 18 hr. It is interesting that the one target derived from normal cells (WI-38) was insensitive with the L/T ratios tested even on long-term culture. It is apparent from Table IV that

Table IV. Relative NK Susceptibility as a Function of K Values[a]

Cell line	Source	k_i/k_{std}[a] 5 hr	18 hr
K562/FCS-1	Erythroleukemia	1.00	1.00
K562/FCS-2[b]	Erythroleukemia	1.12	0.98
K562/HSA[c]	Erythroleukemia	0.38	0.46
Molt 4	T cell	0.94	1.75
A549	Alveolar cell, lung	0.14	0.39
Goodwin	Adenoca lung	0.02	0.51
2774	Adenoca ovary	0.23	0.36
HL-60	Promyelocytic leukemia	0.14	0.34
WI-38	Human embryonic lung	<0.013[d]	<0.008
P815X2	DBA/2 mastocytoma	0.02	0.35
EL-4	C57B1 T lymphoma	<0.013	0.06

[a]For purposes of this table, the A values of all cell lines were set as being equal to K562/FCS-1 as the standard. This happened to be the case where the data did reach plateau values. Individual k values (k_i) have been expressed relative to this standard (k_{std}).
[b]A K562 subline maintained independently of the original at the same time as K562/HSA.
[c]K562/HSA is a subline of K562 which has been maintained in complete medium without FCS but with 2% human serum albumin for 1 year.
[d]The minimum detectable cytotoxicity assuming that the next higher L/T ratio above that tested (40/1) would have caused a cytotoxicity of 5%.

the use of k values to describe target cells gives an accurate description of the NK sensitivity of the cells which is clearly more meaningful than the use of isolated chromium-release values at a single lymphocyte/target cell ratio. The technique is particularly useful when one wishes to categorize clones of target cells with reference to the parent cell line. To fully categorize a cell line this way, however, it is necessary to indicate both parameters, A and k, since it is theoretically possible that only a proportion of the target population may be susceptible to lysis.

DISCUSSION

In the present paper we have examined different methods of expressing the cytotoxicity of NK lymphocytes against ^{51}Cr-labeled tumor target cells. In doing this we have limited our studies to data derived from the conventional method of doing chromium-release assays to assess cell-mediated cytotoxicity, i.e., assays in which varying numbers of effector cells are mixed with a constant number of target cells. By far the majority of studies of cell-mediated cytotoxicity uses this methodology, and most of these report the data obtained in tabular form as percentage chromium release at a certain lymphocyte to target cell ratio. We believe that this practice is no longer acceptable, because it is severely limited by the fact that percentage chromium release is not linearly related to effector cell number, except at low lymphocyte to target cell ratios, making it impossible to draw conclusions as to the real relationship between different lymphocyte preparations in terms of effector cell function. To some extent this objection is overcome by the use of lytic units, providing that the method used to derive this value is correct. The majority of reports in which data are described in lytic units employs linear regression analysis of the points believed to be on the linear part of the sigmoid curve. In our experience this method is also inaccurate, the degree of error depending on the percentage cytotoxicity used to define the lytic unit as well as the level of cytotoxicity displayed by the lymphocytes. For the method to be precise, the data points used to compute the best-fit lytic parameter must be on the linear part of the curve, the cytotoxicity value arbitrarily chosen to define the lytic unit should also be on this part of the curve, and, for comparison purposes, the lines drawn for the different donors must be parallel. This can routinely be accomplished if an extensive and complete series of L/T ratios is assessed, such that no matter how high or low a particular donor may be, the entire curve can be plotted. In clinical studies, however, the limitation on the amount of blood available for testing, combined with the fact that many donors are lymphopenic, makes this goal unattainable. Thus, because the curve is frequently incomplete, it is difficult in practice to avoid including data at the top of the curve for high-activity donors or at the bottom of the curve for low-activity donors. As discussed above, this results in a lowering of the slope of the line in both cases, with a resultant incorrect extrapolation to the lytic unit value. Several investigators have tried to circumvent this error by using only those points which can be shown statistically not to deviate from linearity. Again, this is valid only if the majority of points used actually does lie on the linear part of the curve.

In view of these potential problems, we have defined what we consider to be the minimum requirements for a method of data reduction. These are (a) that the data can be reduced to a single numerical expression directly proportional to the effector cell activity and (b) that the data can be reduced to linearity irrespective of what portion of the curve is obtained experimentally. Both the simple exponential fit equation, $y = 1 - e^{-kx}$, and the von Krogh equation (21), $y = 1/[1 + (K/x)^n)$ can be shown to satisfy these requirements, providing the maximum cell-mediated lysis plateau is at 100%. However, even if detergent is used to obtain the value for maximum release (used in calculating cytotoxicity), the asymptote of the sigmoid curve is below 100%. Thus, the predicted curves from the formulas described above do not fit the experimental data as well as might be expected. To obtain the line which best fits the experimental data, and thus to obtain the most accurate estimation of the lytic unit, it is necessary to modify these equations by incorporating the value for the asymptote, A, to give the following: $y - A(1 - e^{-kx})$ and $y = A/[1 + (K/x)^n]$. The value of A may be estimated graphically or by computer iteration, and the values of k or K in these equations are proportional to the cytotoxic activity. From the data presented in this paper, we have concluded that both of these equations fulfill our requirements for a method of data reduction, and that the use of either of the two is preferable to expressing data either as simple cytotoxicity or as lytic units defined using only the linear portion of the sigmoid curve.

The use of these formulas is also limited to some extent, however. To be strictly comparable, either as relative k or K values or as lytic units, the A values must be the same for the lines under comparison. In our experience, this is usually the case if the "dose-response" curve is carried out far enough. However, it is a relatively simple matter to program the computer to indicate that an iterated A value for a particular line differs significantly from the A value chosen for the group as a whole. If this occurs, there is little that can be done except to report both the A value and the k or K values, in order to define the data. It is also possible that NK activity may be influenced by suppressor cells, resulting in a decrease in cytotoxicity with increasing cell number. Although we have not observed this within the series of L/T ratios we normally use, this type of data would require a different equation for its expression, such as a modification of that described by Clark *et al.* (44): $P = A(1 - e^{-N\alpha t e^{-N\gamma}})$ the modification being the usage of an iterated A value.

The extensive published experience of Miller *et al.* (36, 37) with the simple exponential fit equation (as $y = 1 - e^{-N\alpha t}$) and the modification of this equation to account for suppressor cell activity as described above (44) are compelling reasons for adoption of this equation instead of the von Krogh equation for routine use. The equation is also simpler to use, does not depend on the definition of the lytic unit for the accurate indication of relative cytotoxicity, and, as documented in the Results, can be used to describe the relative NK susceptibility of different target cells. In contrast to Miller and Dunkley (36), however, we have not used the expression $N\alpha t$, mainly because the relationship between the slope, k and time, t, was not readily apparent from our data using the longer incubation periods of 5 and 16 hr. This is a minor point, since at any particular time point the α ratio of two lines will of course equal the ratio of the k values for these lines. The use of these methods of data expression will depend to a large extent on the type of experiment being reported. There are numerous examples in the literature of comparisons *in vitro* between cell preparations before and after depletion of various lymphocyte subpopulations, or after treatment with agents such as interferon or cytotoxic drugs, and these types of data are readily amenable to being expressed as lytic units or as relative k values (45), since they are derived from the same experiment. On the other hand, the documentation of sequential studies over prolonged time periods requires the expression of the data for each donor relative to the mean of the control NK activity. This is necessary because the susceptibility of the target cells to lysis varies from assay to assay, associated to a lesser extent with individual donor variability (4, 29). The use of relative k values for each donor overcomes the error due to target cells to some extent since, as shown above, the result is not influenced to a large extent by variations in the value of the asymptote, A.

It should be noted that individual patient or control donor variability would not be eliminated by the use of these equations, which serve primarily to convert absolute cytotoxicity data into values *relative* to the control. As has been discussed previously, relative cytotoxicity values are fairly constant for any particular normal donor over the long term (4, 29) and the use of these values is preferable to the use of absolute cytoxicity. Because the equations also result in the best estimation of lytic unit, however, it would be expected that variability due to errors in this estimation would also be decreased. This methodology would complement other techniques to improve the consistency of results, such as the use of cryopreserved control lymphocytes or target cells (46, 47).

In conclusion, we have analyzed and compared several methods of data reduction, two of which offer appreciable advantages over methods commonly used at present. In spite of the use of those equations, or modifications of them by certain groups for some time (5, 17, 36, 37, 39, 40), the majority of investigators has not wholeheartedly adopted them. In view of the tremendous output of experimental data which is currently taking place in the field of NK cytotoxicity, it is apparent that some uniformity in data expression is necessary. We feel that the formulas described in this paper are ideally suited for this purpose, and that one or the other should be used in all such studies of cell-mediated cytotoxicity if the data are to be evaluable to the reader.

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