Quantitative Studies of the Growth Response of the Krebs Ascites Tumor

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The application of ascites tumors to the study of growth and related phenomena has received considerable attention (1). Until recently, however, there was comparatively little information concerning the quantitative aspects of ascites tumor development. The nature of the growth response has been investigated independently by Patt, Blackford, and Drallmeier (6) for the Krebs ascites and by Klein and Révész (3) for the Ehrlich ascites. The present paper will be concerned with a further analysis of the growth pattern of the Krebs tumor, particularly in terms of the relationships between tumor cell number, volume of ascites, body weight change, and survival time.

METHODS

The ascites tumor[1] used in this investigation was derived originally from the Krebs-2 solid carcinoma (2). Female CF-1 mice 10–12 weeks of age were weighed and then injected intraperitoneally with 0.2 ml. of freshly obtained ascites diluted with Tyrode's solution to give the desired tumor cell concentration. Tumor cells were generally obtained from mice bearing 7-day-old ascites tumors.

In one series of experiments, mice were sacrificed by cervical fracture at intervals after inoculation of 1.1, 9.4, or 19.4 $\times 10^6$ cells. The animals were weighed, and the volume of ascites and the number of tumor cells were determined according to the procedure described by us previously (6). In brief, this consists of the intraperitoneal injection of a known volume of Evans blue dye (2 ml. of 0.05 per cent solution) and the withdrawal of an aliquot after an interval of 2 minutes during which the animal is gently rotated to insure mixing of the dye and peritoneal fluid. Ascitic volume is calculated from the dye dilution determined spectrophotometrically at 620 mμ on the cell-free supernate and from the percentage of cell-free fluid determined by centrifugation in Wintrobe tubes. Total cells are enumerated in a hemocytometer, and differential cell counts are made on air-dried smears stained with Feulgen reagent and light green. As seen in Table 1, there is a close correspondence between tumor cells estimated from stained preparations and large cells (greater than *ca.* 12 μ in diameter) enumerated in the hemocytometer. Large cell counts are consistently greater than the tumor cell estimates by about 3 per cent, which agrees with the percentage of large nontumor cells as determined from smears. In view of this correspondence, large cell counts were employed routinely in place of differential analyses of stained material.

[1] Kindly provided by Dr. T. S. Hauschka.

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A second series of experiments was concerned with measurement of ascitic volume and of wet and dry weights of the total body. Mice were sacrificed at intervals after tumor inoculation, weighed, and the volume of ascites estimated by dye dilution. The animals were then macerated in a Waring Blendor, and the residue was dried at 105° C. to constant weight. The percentage of water in skin and subcutaneous tissue, skeletal muscle, liver, and kidney was also determined in some instances.

In a third series of experiments, mice were inoculated with 1, 5, 10, or 19 $\times 10^6$ tumor cells and weighed daily until death. Noninjected animals of the same age served as controls.

RESULTS

Krebs ascites tumor cells multiply rapidly for a variable period, depending on the inoculum dose, and then increase at a progressively declining rate to an asymptote of about 10^9. There is little, if any, detectable delay in growth onset with inocula of 10^6 to 20 $\times 10^6$ cells. The period of slowed growth or leveling is apparent when the cell population reaches 2 $\times 10^8$, which corresponds to an interval of 3–5 days with the inocula used. The data are presented in Table 2.

TABLE 1

COMPARISON OF TUMOR CELL AND LARGE CELL COUNTS

(Each value represents the mean $\times 10^6$ of duplicate determinations on five mice)

Days after inoculation (21×10^6 cells)	Stained preparation: Tumor cells* (T)	Stained preparation: Tumor and large† nontumor cells (TNT)	Hemocytometer: Large cells† (L)	Per cent difference: TNT:T	Per cent difference: L:T
1	47.6	50.4	48.9	6.0	2.7
2	122.1	126.3	126.5	3.4	3.6
3	252.3	258.2	258.5	2.3	2.5
4	457.9	463.1	471.8	1.1	3.0
7	863.7	880.0	889.3	1.9	3.0

* Estimated from total and differential cell counts.
† >12 μ in diameter.

Growth is essentially exponential over the range from 10^6 to 2 $\times 10^8$ cells. This is shown in Chart 1, in which the data for the various inoculum groups have been transposed to a single curve. The time axis was transformed by allowing the mean of the natural logarithms of the tumor cell numbers for a given group to fall on a line with the

indicated slope.[2] The slope of the least squares line is 1.04 ± 0.11 and represents the mean weighted slope of regressions derived from the first three determinations after inoculation of 1.1 and 9.4 × 10^6 cells. The slopes of these lines were not about 16 hours. The available data suggest that the initial growth rate of the Krebs ascites does not vary appreciably with increase in the inoculum dose from 10^6 to 20 × 10^6 cells. More information is required to establish this, however.

TABLE 2

GROWTH OF KREBS ASCITES TUMOR CELLS WITH TIME AFTER INOCULATION

(Each value represents mean ± S.E. of duplicate determinations on number of mice designated in parentheses)

Days after inoculation	Cells × 10^6	Cells × 10^6	Days after inoculation	Cells × 10^6
0	19.4	9.4	0	1.1
1	54.3 ± 2.8 (11)	30.7 ± 2.5 (5)	2.8	17.3 ± 2.9 (10)
2	122.4 ± 7.1 (16)	93.9 ± 6.5 (10)	3.8	64.0 ± 11.5 (10)
3	254.5 ± 16.5 (16)	207.6 ± 14.9 (10)	4.8	158.9 ± 16.6 (10)
4	491.2 ± 28.4 (16)	354.3 ± 18.5 (15)	5.8	341.4 ± 16.3 (5)
5		430.9 ± 30.7 (5)	6.8	474.1 ± 28.9 (10)
7	821.7 ± 81.5 (11)		9.8	751.0 ± 108.8 (8)

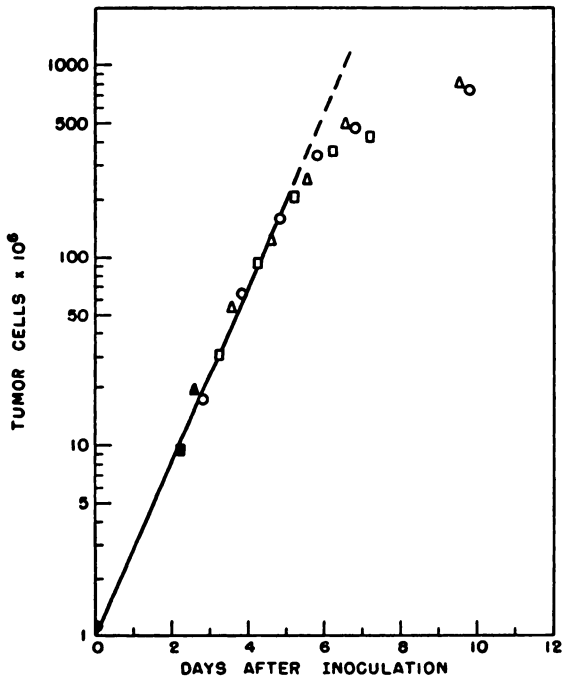

CHART 1.—Exponential relationship between tumor cell numbers and time after inoculation. (The data shown in Table 2 have been transposed to a common time axis as indicated in text footnote 2. Solid symbols represent inoculum values.)

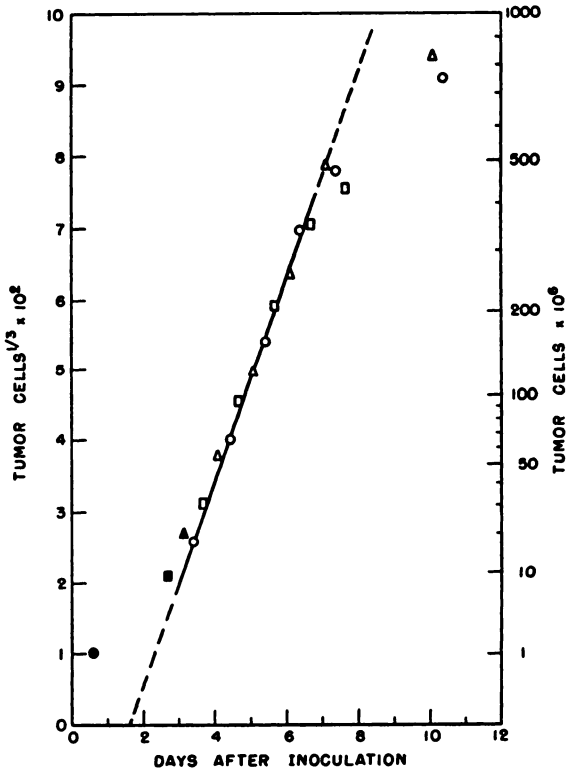

CHART 2.—Relationship between cube roots of tumor cell numbers and time after inoculation. (The data shown in Table 2 have been transposed to a common time axis as indicated in text footnote 3. Solid symbols represent inoculum values.)

significantly different, and their intercepts were in essential agreement with the initial inoculum values. The doubling time during the log phase is

[2] $T = \bar{x} - (\bar{y} - a/b)$, where T = transformation in days, $\bar{x}$ = mean of sampling times in days, $\bar{y}$ = mean log tumor cell numbers, a = log arbitrary intercept = 6, and b = slope = 1.04.

T = 0.06, 2.24, and 2.55 days for inocula of 1.1, 9.4, and 19.4 × 10^6 cells, respectively.

The relationship between tumor cell numbers and time after inoculation may also be expressed in part by a cube root transformation as shown by Klein and Révész (3). These data have also been transposed to a common time axis by the pro-

cedure indicated previously, except that the cube roots of the tumor cell numbers were used in place of logarithms.[3] The slope of the least squares line is 1.37 ± 0.05 and represents the mean weighted slope of regressions derived from the first four determinations after inoculation of 1.1, 9.4, and 19.4 × 10^6 cells. These slopes were not significantly different, which is also suggestive of a comparable growth rate for the different inocula. As noted in Chart 2, the linearity between the cube roots of tumor cell numbers and time extends over a more limited range (10^7 to 4 × 10^8 cells) than the exponential fit. The upper limit of the former

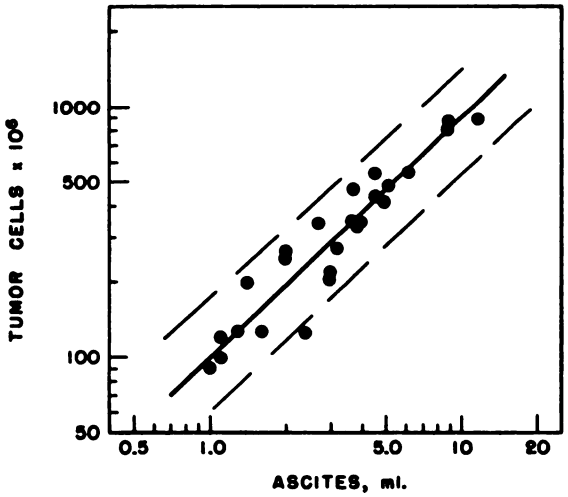

CHART 3.—Relationship between number of tumor cells and volume of ascites. (Each value represents the mean of determinations on five or six mice. Dashed lines designate 95 per cent confidence interval.)

is higher than that of the latter, which is consistent with the onset of a detectable decrease in growth rate during ascites tumor development. The cube root transformation fails to account, however, for the entire course of slowed growth. It is apparently not so applicable as the exponential function to the initial growth period of the Krebs ascites tumor.

Ascitic volume increases progressively during the course of tumor development. The linear relationship between tumor cell number and the volume of ascites is shown in Chart 3. The slope of the least squares line is 0.94 ± 0.07, and the mean cell concentration is 94.3 ± 4.6 × 10^6 cells/ml. The correspondence between ascitic volume and body weight is depicted in Chart 4, in which it will be noted that the former increases exponentially with the latter (k = 0.2 ± 0.01). Estimation of ascitic volume from body weight change or of tumor cell number from ascitic volume is subject to a large error. For a 95 per cent confidence interval, this amounts to ± 50 per cent for the body weight-ascitic volume and for the ascitic volume-cell number relationships over the ranges shown in Charts 3 and 4.

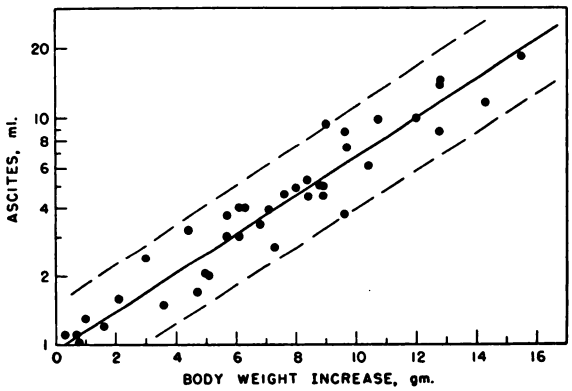

CHART 4.—Relationship between changes in ascitic volume and body weight. (Each value represents the mean of determinations on five or six mice. Dashed lines designate 95 per cent confidence interval.)

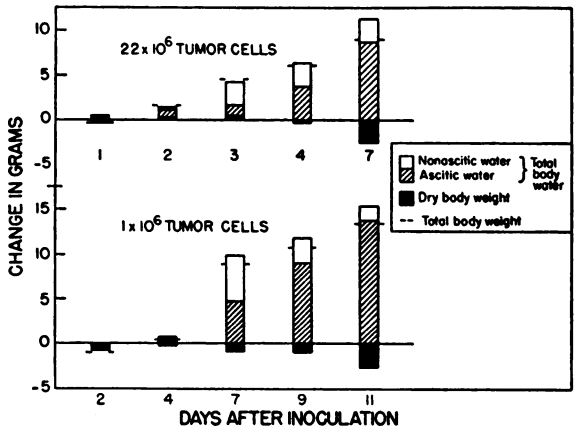

CHART 5.—Analysis of the body weight increase during tumor development. (Each value represents the mean of determinations on five mice.)

Analysis of the body weight increase in terms of ascitic water, nonascitic water, and dry weight is presented graphically in Chart 5. Changes in water content and in dry weight of the whole animal were computed from estimates of the initial values immediately prior to tumor inoculation. These were based on measurements on 27 control mice of the same age and body weight (per cent water = 65.3 with S.D. of 3.5). The increase in total body water less ascitic water was taken to represent the

[3] The following may be substituted in the transformation equation. $\bar{y}$ = mean tumor cell numbers$^{1/3}$; a = arbitrary intercept$^{1/3}$ = −2 × 10^2; b = 1.37; T = 0.60, 2.70, and 3.13 days for inocula of 1.1, 9.4, and 19.4 × 10^6 cells, respectively.

accumulation of nonascitic water or edema. A qualitatively similar picture is seen when the body weight gain is partitioned in this way in mice inoculated with 1×10^6 or 22×10^6 Krebs ascites cells. The more rapid increase in body weight than in ascites during the early phase of cell growth can be attributed to edema, particularly of skin and subcutaneous tissue and skeletal muscle (Charts 5 and 6). The ratio of body weight gain to ascites

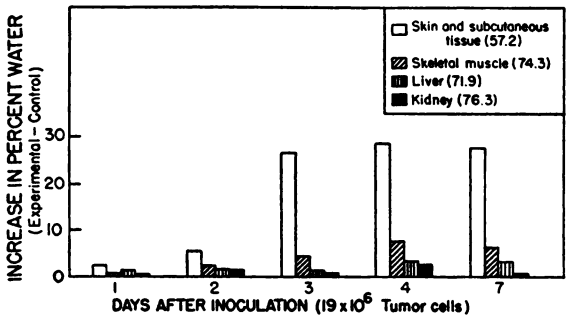

CHART 6.—Changes in tissue water content during tumor growth. (Each value represents the mean of determinations on six mice. Numbers in parentheses refer to control values obtained on ten mice.)

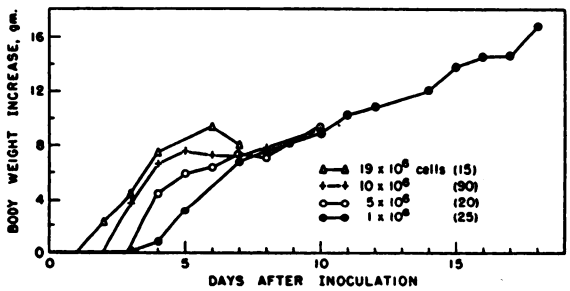

CHART 7.—Influence of inoculum size on the body weight increase of ascites tumor-bearing mice. (Values in parentheses refer to number of mice.)

decreases progressively during the subsequent growth period—from 2.95 on the 3d day to 1.04 on the 7th day with 22×10^6 cells, and from 1.94 on the 7th day to 0.94 on the 11th day with 1×10^6 cells. These effects can be related to the decrease in dry weight during the more terminal period and to the absence of further increase in nonascitic water.

The rate of increase in body weight during the early growth period is relatively independent of inoculum size. However, the time required for a standard weight increment may be related, within certain limits, to the initial number of tumor cells. Thus, an average increase of 5 gm. occurs on 3.2 days with 19×10^6 cells, 3.4 days with 10×10^6 cells, 4.4 days with 5×10^6 cells, and 6.0 days with 1×10^6 cells. The weight curves are shown in Chart 7. The average weight gains at 50 per cent mortality are 8.9, 7.5, 9.3, and 14.6 gm. in order of decreasing inoculum size. The time to 50 per cent mortality increases from 5.5 days with 10 or 19×10^6 cells to 10 days with 5×10^6 cells and 17 days with 1×10^6 cells.

DISCUSSION

The present experiments amplify our earlier observations on the growth characteristics of the Krebs ascites tumor (6). Tumor development has been shown to consist of an initial phase of rapid cell multiplication with little or no lag, followed by a period of progressively declining growth toward an asymptote. In contrast to the conclusion of Klein and Révész (3), who used the Ehrlich ascites tumor in similar studies, the initial growth pattern may be approximated by an exponential fit. In agreement with their observations, a portion of the growth curve may also be expressed linearly in terms of the cube roots of the tumor cell numbers. Thus, cell multiplication appears to be more nearly exponential when the number of tumor cells is comparatively small. With increasing cell numbers, the growth rate gradually declines, and it is this phase to which the cube root transformation is partially applicable. An intermediate area may be approximated with equal accuracy by both expressions. Neither the exponential nor the cube root transformation accounts for the entire growth pattern.

The course and rate of tumor growth is similar for inocula of 10^6 to 20×10^6 cells. There is no detectable latent period over this range with the Krebs ascites, unlike the findings with the Ehrlich tumor (3). This may be attributed perhaps to differences in the experimental methods, since sampling and other errors are considerable with low cell numbers (3, 6). The presence or absence of a lag in tumor development may also be a consequence of innate differences in the growth rates of the two tumors. It is noteworthy that the growth rate of the Krebs ascites tumor in CF-1 mice is over 40 per cent greater than that of the Ehrlich tumor in various inbred and F-1 hybrid strains. The slopes during the corresponding growth periods (cube root $\times 10^2$ vs. days) for a similar range of inocula are 0.97 for the Ehrlich tumor (3) and 1.37 for the Krebs tumor. This difference is borne out by the more rapid development of the various sequelae, e.g., body weight increase and mortality, with the latter.

The duration of the initial rapid growth of the Krebs ascites tumor is related to the size of the inoculum; leveling becomes apparent in each instance when a comparable number of tumor cells

has been attained. The entire pattern, though conditioned by intrinsic and extrinsic factors, may be a function mainly of the total number of free tumor cells. The leveling phase reflects a complex situation, which is brought about, at least in part, by the increasing competition among cells, the loss of cells by adherence or organized growth on peritoneal surfaces, and the progressive deterioration of the host. In this connection, it may be noted that the percentage of eosin-resistant and presumably viable cells is relatively constant during tumor development but that the mitotic index is decreased during the more terminal period (3, 6).

The volume of ascites is related linearly to the number of tumor cells with a slope approaching unity. This parameter, however, is subject to sufficient random variation to preclude its use as an accurate measure of cell multiplication. A similar proportionality between ascitic volume and tumor cell number has been described for the Ehrlich tumor (3). The volume of peritoneal fluid was calculated in the latter instance from the tumor cell concentration and the total number of tumor cells as determined by a rinsing procedure on a separate series of animals. The dye dilution technic used with the Krebs ascites affords a more convenient measure of these parameters in the same animal. The mean cell concentrations are rather similar, being $94.3 \pm 4.6 \times 10^6$ and $130.9 \pm 4.3 \times 10^6$ cells/ml for the Krebs and Ehrlich ascites tumors, respectively. The absorption spectra and protein contents of the two fluids are also comparable[4] (4). Although the mechanism of ascites formation is unknown, the data are generally suggestive of an effect on capillary permeability by the products of tumor cell metabolism or breakdown.

A relationship between body weight gain, ascitic volume, and tumor cell number has been inferred by Lettré (5) from analysis of the weight curves of ascites tumor-bearing mice. Klein (1), however, has concluded that the weight response is too variable for use as a quantitative index of tumor development. The results of the present investigation support both of these contentions. Body weight increases more rapidly than ascites during the initial growth period and less rapidly during the terminal period. The former can be attributed to edema, particularly of skin, subcutaneous tissue, and skeletal muscle. The latter is a consequence mainly of wasting as manifested by the decrease in dry weight, and of the absence of further edema in the face of continued and unimpaired ascites formation. The over-all relationship may be expressed as an exponential increase of ascitic volume with body weight. Prediction of the number of tumor cells from the body weight change is subject, however, to a large error of estimate. Although the weight gain may not be suitable for quantitative evaluation of the growth curve, it provides a useful parameter for comparative purposes.

Survival time has also been suggested as an indicator of tumor growth, since it is somewhat dependent on the inoculum dose (1). It is apparent, however, that differences in survival time may be considerably in excess of the time required for attainment of equivalent cell numbers. Fifty per cent mortality occurs by the 6th day with an inoculum of 10^7 cells and on the 17th day with an inoculum of 10^6 cells; yet, the corresponding cell growth curves are separated by only 2.3 days. Such differences may be related to the increased capacity of the host to adjust to the stress imposed by more prolonged development of a comparable ascitic volume.

[4] Unpublished observations by H. M. Patt and M. E. Blackford.

SUMMARY

Growth of the Krebs-2 ascites tumor of mice has been quantitated by estimating the volume of ascites by dye dilution and the concentration of tumor cells from total and differential cell counts. Tumor development has been shown to consist of an initial phase of rapid cell multiplication with little or no lag (doubling time, *ca.* 16 hours) followed by a period of slowed growth toward an asymptote. The course and rate of tumor growth are similar for inocula of 10^6 to 20×10^6 cells. Growth appears to be more nearly exponential when the number of tumor cells is comparatively small. With increasing cell numbers, the growth rate gradually declines. An exponential fit may be applied from 10^6 to 2×10^8 tumor cells, and a cube root transformation is applicable with cell numbers from 10^7 to 4×10^8. Neither expression accounts for the entire growth pattern, which, however, appears to be a function mainly of the total number of free tumor cells. Ascitic volume increases linearly with tumor cell number and exponentially with body weight. However, prediction from weight to ascites to cells is not suitable for quantitative evaluation of the growth curve owing to the large error of estimate. The body weight change is a complex function of tumor growth, reflecting not only the ascites but also the associated anasarca and wasting. Differences in survival time with varying inoculum size may be considerably in excess of the time required for attainment of equivalent cell numbers. The results are discussed in connection with related observations on the growth of the Ehrlich ascites tumor.

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