The ewes which were given the supplement gave birth to 1484 living lambs and of these 35 or 2.3%, showed clinical symptoms of ataxia. The 566 control ewes gave birth to 612 living lambs and of these 101 or 16.3% showed clinical symptoms of the nervous disorder. Stillborn lambs are not included in the figures because of the practical difficulties in bringing the lambs to the laboratory quickly enough for post mortem examination.

In view of the above results, we think that the nervous disorder found in Icelandic lambs born by ewes fed on seaweed during pregnancy is similar to "swayback" or enzoötic ataxia in lambs as described in other countries(1,2).

Summary. 1. A brief description is given of a nervous disorder in lambs born by ewes which feed on seaweeds during pregnancy. Pathologically the disease is characterized by a demyelination confined to the cerebrum. The copper intake in affected areas does not seem to be low, but the ewes in these areas show low values for blood copper, *i.e.* less than 0.2 mg Cu/litre. The copper content of livers from affected lambs is very low. 2. A field experiment showed that the disorder can be largely prevented by giving a supplement of copper to the pregnant ewes.

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Growth Characteristics of the Krebs Ascites Tumor. (20403)

HARVEY M. PATT, MARGARET E. BLACKFORD, AND JEAN L. DRALLMEIER.

From the Division of Biological and Medical Research, Argonne National Laboratory, Lemont, Ill.

Ascites tumors, which consist essentially of a suspension of neoplastic cells growing in the peritoneal cavity, have been the subject of numerous investigations since the classical observations of Lowenthal and Jahn(1). The suitability of ascites tumors for study of the chemistry and physiology of growth has been emphasized by Klein(2-4), Lettré(5) and others. A number of tumors have been shown to have the capacity for growth as ascites tumors and certain of their chemical and biological characteristics have been described (3,4,7,8). Takes may occur after intraperitoneal injection of a single neoplastic cell (6,9). It is known from earlier work with the Ehrlich ascites tumor of mice that the survival time and body weight increase bear some relationship to the extent of tumor growth. Under certain controlled conditions, survival time is dependent on the number of tumor cells inoculated and the weight increase reflects ascites development(2,4,5,10). While such indices are useful in a general way, a more direct and quantitative measure of the growth characteristics is desirable. This has been attempted in the present experiments by estimating the volume of ascites by dye dilution and the concentration of tumor cells at intervals after intraperitoneal inoculation of the Krebs-2 carcinoma in mice. These studies are preliminary to an investigation of the radiosensitivity of the ascites tumor and related phenomena.

Methods. The ascites tumor* employed in these experiments was derived originally from the Krebs-2 solid carcinoma. The Krebs ascites tumor is closely related cytologically and chemically to the Ehrlich ascites tumor (11). Female CF-1 mice 10 to 12 weeks of age were injected intraperitoneally with 0.2 ml of freshly obtained ascitic fluid diluted with Tyrodes solution to give the desired tumor cell concentration. Animals were sacrificed by cervical fracture at intervals after tumor inoculation. Evans blue dye, usually 1.5 ml of a 0.05% solution, was injected i.p. immediately after sacrifice, care being taken to inject the entire volume directly into the peritoneal cavity. The dye solution was prepared in cell free ascitic fluid obtained by

* Kindly provided by Dr. T. S. Hauschka.

high speed centrifugation and repeated freezing and thawing. Two minutes were allowed for mixing of the dye and ascitic fluid by gently rotating the animal before an aliquot was removed with a glass pipette through a small incision in the peritoneal wall. This interval was adequate for complete mixing. The sample was divided into 4 portions: (1) for determination of the percentage of cell free fluid by centrifugation in Wintrobe tubes, (2) for measurement of dye dilution on the cell free supernate, (3) for estimation of cell concentration in a hemocytometer, (4) for preparation of air-dried smears stained with Feulgen reagent and methyl green. The degree of dye dilution was determined spectrophotometrically at 620 μ on a 1:25 dilution of the cell free supernate. The dye concentration was determined in the usual manner by comparison with the transmission of standard solutions; the latter was remarkably constant from one experimental period to another. Since dye uptake by the cells during the several minutes between injection and centrifugation is negligible, dye dilution is a direct measure of the volume of ascitic plasma. The total volume of ascites may be calculated from the dye dilution and the percentage of cell free fluid.

$$\text{Ascites in ml} = \frac{100\,(C_i/C_d)\ (\text{ml dye})}{\%\ \text{cell free fluid}} - \text{ml dye inj.}$$

where C_i = dye concentration inj.
C_d = dye concentration determined.

The error of this procedure, estimated from injection of known volumes of ascitic fluid (2-5 ml) and their recovery by dye dilution, is 4.9 $\pm$ 1%. Cell counts were made in a Spencer Bright Line hemocytometer using a binocular microscope and a magnification of 270. For this purpose, 0.1 ml of cell suspension was diluted with a suitable volume of Tyrodes solution containing 1% Eosin Y (National Aniline). Resistance to eosin-staining may be assumed to be an index of viability(3,12,13). Determinations were made in duplicate and at least 1000 cells, exclusive of erythrocytes, were counted in each instance. It was possible from the wet counts to classify the cells both as to size (greater or less than ca 12 μ in diameter) and eosin resistance. An attempt was made to differentiate the various cell types on the Feulgen-methyl green stained smears. Differential counts were made in duplicate; a minimum of 1000 cells was enumerated on each smear using a magnification of 540. Cells were classified as tumor cells, polymorphonuclear leucocytes, lymphocytes, large nontumor cells (presumably mainly mesothelial cells and histiocytes), and small cells other than polymorphonuclear leucocytes and lymphocytes. The frequency of tumor cells in mitosis was also determined. Although there are no absolute cytological criteria to distinguish a tumor cell from a nontumor cell, it was possible to differentiate the neoplastic cells with reasonable reproducibility from one slide to another on the basis of cell size and the large and variable nucleus with one or more large nucleoli. These cells are present in abundance relative to the other cell types and differ from the cells found in normal or inflammatory peritoneal fluid.

Results. The tumor cell population increases exponentially with a doubling time of 19 to 20 hours during the first 3 days after inoculation of 20 x 10^6 cells. After the initial log phase, the total number of tumor cells suspended in ascitic fluid increases more slowly and approaches an asymptote of about 10^9 cells at one week. These data are depicted in Fig. 1, which also reveals the close correspon-

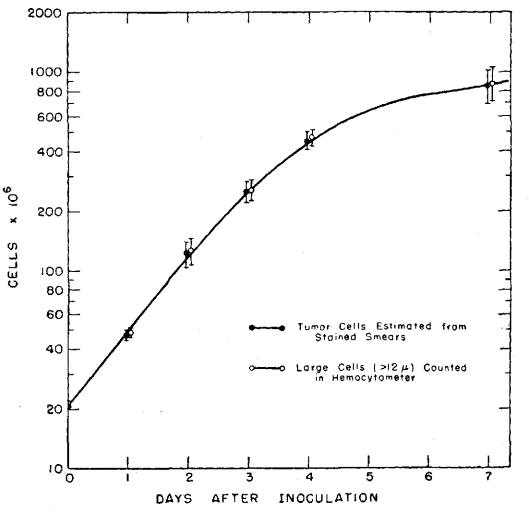

FIG. 1. Number of tumor cells as a function of time after inoculation. (Each value represents the mean of duplicate determinations on 5 mice; vertical lines designate stand. error of the mean.)

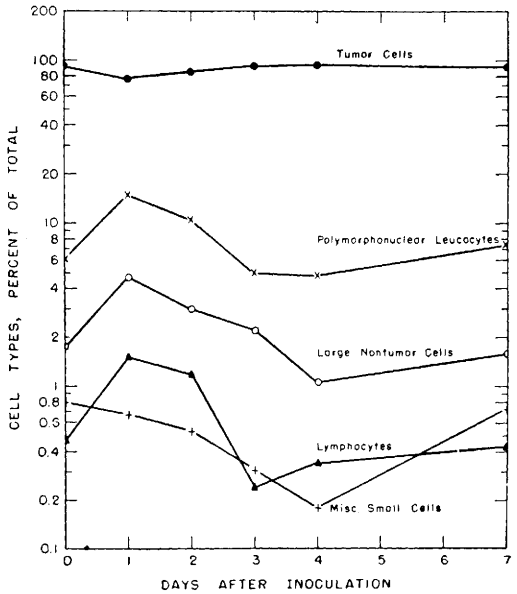

FIG. 2. Changes in composition of ascites after inoculation of 20 × 10^6 Krebs-2 tumor cells. (Each value represents the mean of duplicate determinations on 5 mice.)

dence between tumor cells estimated from the Feulgen-stained smears and the large cells (> ca 12 μ) counted in the hemocytometer. The percentage of large eosin-stained cells is relatively constant during the entire course of tumor development. The frequency of mitosis ranges from 2 to 2.8% during the period of logarithmic growth and is 1.2% seven days after tumor cell inoculation. The period of slowed growth or leveling corresponds to the time of death of mice inoculated with 20 x 10^6 Krebs ascites tumor cells (50% fatalities at 5 days; 100% fatalities at 10 days).

Differentiation of the various cell types is shown in Fig. 2 where it will be noted that the percentage of tumor cells in the total population is decreased from the inoculum value of 92% to 78% on the first day. The decrease in relative tumor cell concentration corresponds to the apogee of an inflammatory reaction as manifested by an increase in polymorphonuclear leucocytes, lymphocytes, and large nontumor cells, presumably mainly mesothelial cells and histiocytes. The percentage of tumor cells returns to a rather constant level of about 90% on the third day, which coincides with the decline of the inflammatory cells. The differential cell counts at 7 days are remarkably similar to the cell population of the original inoculum.

The volume of ascitic fluid increases progressively during the course of tumor development. In general, the rate of increase parallels that of the tumor cells during the initial log period, but exceeds the latter during the more terminal period (Fig. 3). Body weight is also increased during tumor growth, but this parameter is apparently a more complex function of the ascites and its sequelae. The data are summarized in Table I.

Discussion. The methods employed in these studies provide a convenient and reasonably quantitative measure of certain of the growth characteristics of the ascites tumor. The problems inherent in the diagnosis of individual tumor cells in general and in ascitic fluids in particular have been discussed by Klein(3). Since the Krebs-2 ascites consists of an almost pure culture of cells which differ from the cells normally found in peritoneal exudates, the over-all errors in discrimination may be comparatively small. Diagnoses of duplicate smears in the present experiments agree within 5%. The close correspondence between tumor cells estimated from the stained preparations and large cells enumerated in the hemocytometer is of interest in this connection. Since each determination was independent of the other, the wet counts may suffice for many routine purposes. While differential counts provide useful information relative to tumor development, the additional parameter of ascitic volume enables a more

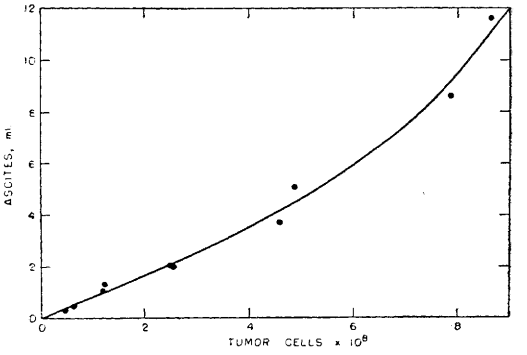

FIG. 3. Relationship between number of tumor cells and volume of ascites. (Each value represents the mean of 5 mice.)

TABLE I. Number of Cells, Ascitic Volume and Body Weight as a Function of Time after i.p. Inoculation of 20 × 10^6 Krebs-2 Tumor Cells. (Each value represents the mean ± S.E. of 11 mice except for % mitosis, based on 5 mice each.)

Days after inoculation	Total cells × 10^6	Tumor cells × 10^6	Tumor cells % eosin stained*	Tumor cells % mitosis	Ascitic vol, ml	Body wt increase, g
1	73.4 ± 4.7	57.2 ± 3.9	1.6 ± .24	2.2 ± .15	.4 ± .04	−.1 ± .21
2	142.3 ± 10.9	120.9 ± 9.9	2.0 ± .31	2.8 ± .17	1.2 ± .16	1.4 ± .40
3	266.7 ± 23.4	250.4 ± 22.2	2.0 ± .27	2.0 ± .23	2.0 ± .20	5.0 ± .19
4	507.0 ± 34.1	474.6 ± 31.6	1.7 ± .18	2.0 ± .16	4.5 ± .40	9.2 ± .20
7	1010.2 ± 98.0	913.6 ± 87.5	2.8 ± .61	1.2 ± .18	10.2 ±1.35	11.7 ±1.5

* Based on large cell counts in hemocytometer.

complete analysis of the growth function. The conventional dye dilution technic is applicable in this instance. The injection of dye solution prior to sampling has the additional advantage of providing a more uniform aliquot for analysis, particularly during the first day or two after tumor inoculation.

The data reveal that ascites tumor development after inoculation of 20 x 10^6 cells consists of an initial log phase of several days duration followed by a period of less rapid growth during which the cell population approaches an asymptote. Estimates of the mitotic time (25 to 30 minutes) based on the mitotic index and the interval required for a doubling of the cell population during the log phase agree with the values reported for other types of cells(6). The apparent decrease in rate of growth with time may be related to the moribund state of the animals. It may also be recalled that the mitotic index is decreased during this period. It is likely, moreover, that the number of cells removed from the effective ascitic pool by adherence or organized growth on peritoneal surfaces increases with time and the magnitude of the ascites(4,10). Qualitatively-similar growth curves have been observed with a less concentrated inoculum, *e.g.*, 1 x 10^6 tumor cells. In this instance the log phase persists for about one week and the period of decelerated growth commences when the development of ascites is comparable to that observed with the more concentrated inoculum. It is not known whether a brief lag or latent period intervenes during the first hours after tumor inoculation. This may perhaps be resolved by inoculating a large volume of undiluted ascites initially to minimize sampling errors due to the small amount of fluid formed during the first day, or by re-inoculation of the same animals during the midpoint of logarithmic growth.

The differential cell counts are suggestive of a transient inflammatory reaction during the first day or two after inoculation. In general, a similar host reaction has been described after intraperitoneal injection of several varieties of ascites tumors and of cell-free ascitic fluid(3,4,7). An important difference in the relative frequency of the tumor cells may be noted, however. A pronounced decrease in the percentage of Ehrlich ascites tumor cells (from an initial value of about 80% to less than 5%) has been observed during the first 24 hours after inoculation(3). This is followed by an almost equally abrupt increase in tumor cell frequency on the second day. The present experiments with the Krebs ascites differ markedly in the extent of the initial decline in the relative frequency of tumor cells even though the values from 3 days on are in essential agreement. The initial discrepancy may be due in part to the difficulty in differentiating tumor cells from the large mononuclear cells during the inflammatory reaction and possibly to the host response, which is known to differ from one strain to another(3,4). It is unlikely, however, that these factors are mainly responsible for differences of this order. It may be recalled that the volume of ascites is quite small during the first 24 hours and it is well, therefore, to consider the methods of sampling in the two cases. It seems reasonable to assume that the prior injection of Evans blue solution and subsequent mixing by rotation of the animal will assure a more uniform sample for withdrawal than simple puncture and aspiration of the ascites during the early growth period. The injection procedure, employed

in the present experiments, should serve also to free tumor cells adhering to peritoneal surfaces because of the small volume of ascites. Klein(3) has, in fact, intimated that a mechanism of this sort may account for part of the large decrease in relative numbers of tumor cells observed by him during the first 24 hours. These considerations are obviously germane to the question of the early fate of the injected cells and, therefore, to the matter of a lag period in tumor development.

Ascitic fluid volume appears to be a linear function of the number of tumor cells during the log phase, but increases somewhat more rapidly during the terminal period. Ascitic fluid volumes of 20 ml or more are not uncommon during the second week of tumor growth. Ascitic fluid obtained from mice with the Ehrlich tumor contains 2.5 to 3% protein, about 60% of which is albumin(14). The absorption spectrum of the Krebs fluid resembles that of the Ehrlich ascites. Fluid obtained during the first few days is usually free of erythrocytes; however, a bloody ascites is frequent by the end of the first week and may be correlated with peripheral anemia. The results are generally suggestive of an effect on capillary permeability by the products of tumor cell metabolism or breakdown, but this remains to be demonstrated. Edema of skin and muscle is evident several days after tumor inoculation and may perhaps be attributed to the loss of plasma protein and consequent decrease in oncotic pressure. There are also indications of an absolute decrease in dry weight of the whole animal during the later stages of ascites development. The nature of the relationships between body weight, the volume of ascites and the number of tumor cells will be reported elsewhere.

Summary. Experiments are reported in which the growth of an ascites tumor in mice has been followed by estimating the volume of ascites by dye dilution and the concentration of tumor cells from total and differential cell counts. The methods afford a reasonably quantitative picture of the growth pattern, which, after inoculation of 20 x 10^6 Krebs-2 ascites tumor cells, consists of an initial log phase of several days duration followed by a period of leveling to an asymptote of about 10^9 cells. The period of apparent less rapid growth parallels the moribund condition of the animals. The number of tumor cells doubles in 19 to 20 hours during the log phase. The mitotic index is around 2.3%, which is suggestive of a mitotic time of 25 to 30 minutes. It is not possible from these experiments to determine the extent, if any, of a lag or delay period in growth immediately after tumor inoculation. The volume of ascites and body weight increase progressively during the course of tumor development. However, the relationships between these parameters and the tumor cell population apparently depend on a number of factors. The results are discussed in connection with related observations on the growth characteristics of ascites tumors.

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