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The Influence of In Vitro Holding Media on the Surface Charge, Histocompatibility, and Viability of TA3 Ascites Tumor Cells

Authors

Tenforde, Tom
Richards, William R
Kelly, Lola S

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The Influence of *In Vitro* Holding Media on the Surface Charge, Histocompatibility, and Viability of TA3 Ascites Tumor Cells

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Tom Tenforde, William R. Richards[1], and Lola S. Kelly

Donner Laboratory, Lawrence Berkeley Laboratory
University of California, Berkeley, California 94720

Running Title:

Influence of *In Vitro* Holding Media on TA3 Cells

SUMMARY

The degenerative processes that occur during *in vitro* maintenance of TA3 ascites tumor cells have been examined. TA3 cells held in 100 volumes of saline or nutrient medium were found to exhibit both a rapid alteration in histocompatibility properties and a reduction of proliferative capacity in LAF_1/J hosts. The modification in tumor histocompatibility was demonstrated by comparing both TA3 growth kinetics and the tumor dose for 50% mortality (the TD_{50}) in normal and pre-irradiated hosts. A 30% decrease in the net negative cellular surface charge was also observed within two hours when TA3 cells were held in a saline solution at room temperature ($\sim$23°C). Blocking cellular metabolism by maintaining the holding medium at 4°C was found to prevent a reduction in surface charge, and to slow the onset of degenerative processes leading to decreased transplantability of the TA3 cells. Addition of the metabolic blocking agent KCN to a 23°C saline holding medium also protected against a rapid decrease in cellular surface charge, but greatly reduced the proliferative capacity of TA3 cells in LAF_1/J hosts.

INTRODUCTION

Experimental procedures with tumors frequently require *in vitro* maintenance of the cells under conditions that represent only an approximation to the physiological milieu. Examples of such procedures include the preparation of inocula for tumor transplantation and immunological studies on tumors in a cell culture system. Another example is the *in vitro* - *in vivo* cytotoxicity test, in which the infectivity of tumor cells is studied following incubation with a chemotherapeutic agent *in vitro*. As a means of defining the degenerative processes that occur when tumor cells are maintained *in vitro*, we have examined the influence of holding media on the surface charge properties, histocompatibility, and viability of TA3 ascites tumor cells.

MATERIALS AND METHODS

Suppliers. Female adult LAF_1/J mice (Jackson Laboratories, Bar Harbor, Maine) were used in all experiments. Saline was obtained from the Cutter Laboratories (Berkeley, California), and Medium 199 from Microbiological Associates (Bethesda, Maryland).

Transplantation and TD_{50} Measurements. The TA3 tumor line was maintained in LAF_1/J carriers by the weekly intraperitoneal injection of 10^6 cells in a 0.1 ml volume of cold saline. Tumor doses for determination of a TD_{50} were prepared by serial twofold dilutions starting from a stock solution previously adjusted to the highest dose level. In all cases, the highest dose level was chosen to produce 100% mortality. Each tumor dose in a 0.1 ml volume was injected intraperitoneally into 10 mice. The TD_{50} was calculated from mortality levels observed over an eight week period following tumor inoculation. As a test of reproducibility, TD_{50} determinations were performed twice.

Tumor Growth Kinetics. Tumor growth kinetics were assessed by measuring the total number of TA3 cells in the peritoneal cavity as a function of time after tumor cell inoculation. The total population of TA3 cells was determined from the product of peritoneal volume and tumor cell concentration. Volume was determined by the isotope dilution technique using ^{131}I-labelled human serum albumin (Mallinkrodt, St. Louis, Mo.). Cell concentrations were measured with a Model B Coulter Counter (Coulter Electronics, Hialeah, Florida). Each point of a tumor cell growth curve was determined from the average population of TA3 cells in the peritoneal cavities of 10 mice.

Cell Viability. The viability of TA3 cells was determined by their ability to exclude nigrosin dye. Cells were examined immediately

following addition of 0.5% (wt/vol) nigrosin (Matheson, Coleman, and Bell, Norwood, Ohio). The superior staining characteristics of this vital dye with ascites tumor cells has been discussed by Kaltenbach et al. (3).

Host Irradiation. Irradiation of LAF_1/J mice was performed with a 1400 Ci ^{60}Co air source. The exposure dose rate was 20 r/min.

Microelectrophoresis. Microelectrophoretic studies were performed with a Zeiss Cytopherometer (Carl Zeiss, N.Y.) using a $Zn/ZnSo_4$ electrode assembly (7). The rectangular microelectrophoresis chamber was immersed in a circulating water bath maintained at either $4 \pm 0.1°C$ or $23 \pm 0.1°$ C. The electrophoretic mobility was determined from a series of velocity measurements at each of the two stationary levels within the microelectrophoresis chamber. All measurements were made with the polarity of the applied field in alternate directions, thereby cancelling out any effect of fluid drift on the net electrophoretic velocity.

Surface charge densities (σ) of TA3 cells in a saline medium were calculated from the electrophoretic mobility (μ) by means of the Gouy-Chapman relation (6):

$$\sigma = 0.1171\ C^{1/2} \sinh(0.25\mu)$$

Here C is the molar concentration of the uni-univalent NaCl solution, the mobility μ has conventional units of micron/sec/V/cm, and the surface charge density σ is expressed in units of coulomb/m^2. The validity of this equation for cells in physiological media has been discussed in detail by Tenforde (6).

Sialic Acid Measurements. A determination of the amount of neuraminidase-susceptible sialic acid at the surface of TA3 cells was per-

formed in the following manner. The TA3 cells were suspended in 100 volumes of saline at either 4°C or 23°C for a prescribed length of time. The cells were then spun down at 300 x g for 5 min, and subsequently washed four times (300 x g, 5 min) in 15 volumes of saline at the same temperature as the holding medium. All erythrocytes were carefully removed from the top of the cell pellet. The concentration of TA3 cells was determined by counting in a hemocytometer, and the cells were then pelleted by centrifugation at 800 x g for 5 min. The packed cells ($\sim 10^8$ cells) were mixed with 0.7 ml of a 500 Units/ml V. cholerae neuraminidase preparation (Behring Diagnostics, Woodbury, N.Y.), and incubated for 30 min at 37°C. One unit of neuraminidase activity is defined by the supplier as the amount of enzyme that releases 1 μg of N-acetylneuraminic acid from human α_1-glycoprotein in 15 min at 37°C. After incubation, the cells were spun down at 1100 x g for 5 min, and the supernatant removed for analysis. Enzyme and residual cells in the supernatant were flocculated by heating at 70-80°C for 10 min. The supernatant solution was then clarified by two serial centrifugations at 1100 x g for 5 min, and the sialic acid content determined on 0.2 ml aliquots by the method of Warren (8). Crystalline N-acetylneuraminic acid (Sigma, St. Louis, Mo.) was used as a standard. Appropriate controls and blanks were processed in an identical manner.

RESULTS

Surface Charge. The surface charge properties of TA3 cells held in 100 volumes of saline at 4°C and 23°C are shown as a function of time in Chart 1. At 4°C, the surface charge density gradually increased from an initial value of approximately 0.014 coul/m^2 to 0.018 coul/m^2 during a period of four hours. This result was found to be reproducible in each of three experiments. A different result was obtained with TA3 cells washed four times in 15 volumes of 4°C saline (300 x g, 5 min), and resuspended in 100 volumes of saline at this temperature. In two experiments, the net negative surface charge density of washed TA3 cells was found to be 20% higher than that of unwashed cells, and was observed to remain constant for a period of four hours. These results indicate that ascitic fluid mucoids are slowly desorbed from the native cell surface in a 4°C saline holding medium, and that centrifugal washing serves to rapidly elute these substances from the cell periphery.

When TA3 cells were held in 100 volumes of saline at 23°C, the net negative surface charge density decreased by 40% from an initial value of 0.020 coul/m^2 to 0.012 coul/m^2 in a period of three hours. Addition of 1 mM KCN to a 23°C saline holding medium prevented this large reduction in surface charge, although a 10% decrease in surface charge density did occur in a period of four hours. When KCN was removed by washing and resuspending the cells in 23°C saline after a two hour incubation period, the surface charge decreased by 30% during the following two hours. These results suggest that the integrity of the TA3 cellular surface in a saline holding medium at room temperature is lost under conditions where the cells are actively metab-

olizing. Introduction of a metabolic block through addition of KCN prevents the onset of major time dependent changes in surface properties. A similar observation has previously been reported by Glaeser (2). As discussed in the preceding paragraph, the process of reversibly blocking cell metabolism by suspending TA3 cells at 4°C also prevents the reduction in surface charge observed for cells held at 23°C.

The surface charge properties of centrifugally washed TA3 cells were measured as a function of time in a 23°C saline holding medium. Following four washes in 15 volumes of room temperature saline (300 x g, 5 min), TA3 cells exhibited a time-dependent reduction in surface charge identical to that shown in Chart 1 for unwashed cells. Although a forced desorption of ascitic fluid mucoids results from suspension of TA3 cells in a large volume of saline, it appears that this process is extremely rapid at room temperature and is not facilitated by centrifugal washing. Consequently, the time dependent changes in surface charge observed in a 23°C saline holding medium cannot be attributed to the loss of an adsorbed sialomucin coat.

Another possible explanation for the reduction in surface charge observed when TA3 cells are suspended in a 23°C medium would be a loss of components rich in sialic acid from the native membrane surface. A major contribution of sialic acids to the surface charge of washed TA3 cells has previously been demonstrated by Weiss and Hauschka (9). After desorption of ascitic fluid mucoids, a loss of sialomucin material may occur at the outer membrane surface. We therefore determined the amount of neuraminidase-susceptible sialic acid as a function of time in both 4°C and 23°C holding media. The amount of sialic acid released by neuraminidase from TA3 cells that had previously been

washed four times with 23°C saline (300 x g, 5 min) was 0.56 μmole/10^9 cells. After a two hour incubation in 100 volumes of 23°C saline followed by four washes, the neuraminidase-susceptible sialic acid content of TA3 cells was 0.63 μmole/10^9 cells. When 4°C saline was used, the sialic acid released by neuraminidase from washed TA3 cells was initially 0.60 μmole/10^9 cells, and after a two hour incubation was 0.62 μmole/10^9 cells. These results demonstrate that the sialic acid content of TA3 cells does not change appreciably during two hours in a room temperature saline medium, and that the amount of sialic acid released by neuraminidase is not influenced by lowering the temperature of the holding medium to 4°C. This finding is in sharp contrast to the observed difference in surface charge properties of centrifugally washed TA3 cells held in 4°C and 23°C saline.

An attempt was also made to measure the neuraminidase-susceptible sialic acid content of unwashed TA3 cells in 4°C and 23°C saline holding media. These experiments were unsuccessful because of interference by sialic acid released enzymatically from ascitic fluid mucoids.

TD_{50}'s and Viability. When TA3 cells were held prior to transplantation as a concentrated suspension in ascitic fluid at 4°C, the tumor dose for 50% mortality (TD_{50}) in LAF_1/J hosts was 18 cells. TD_{50}'s were also determined in irradiated and non-irradiated hosts after maintenance of the cells in 100 volumes of saline at either 4°C or 23°C. The results of these studies are summarized in Table 1.

TA3 cells maintained in 100 volumes of 4°C saline for two hours were found to have a TD_{50} of 1200 cells in non-irradiated hosts, and 310 cells in hosts given 500 rad two days prior to transplantation. These TD_{50}'s indicate a substantial decrease in the proliferative

capacity of TA3 cells following incubation in a 4°C saline holding medium. Lethal cellular damage was not indicated, however, by uptake of nigrosin dye. The significantly lower TD_{50} observed for irradiated relative to non-irradiated hosts demonstrates that an alteration occurs in the histocompatibility properties of TA3 cells held in 4°C saline, and that this factor plays a role in the decreased transplantability observed following in vitro maintenance of the tumor.

TA3 cells held in 100 volumes of 23°C saline for two hours prior to transplantation were found to have a TD_{50} of 3750 cells. This value is three times larger than the TD_{50} observed when TA3 cells were held for two hours at 4°C, even though dye exclusion indicated only a 9% decrease in the number of viable cells at 23°C relative to 4°C. The TD_{50} for cells held at 23°C was identical in irradiated and non-irradiated hosts, thereby indicating that the decreased transplantability of these cells results from a reduced proliferative capacity in LAF_1/J hosts. An alteration in histocompatibility properties also occurs when TA3 cells are held at 23°C (as demonstrated below), but cannot be detected by measurement of the TD_{50}. From data presented in Table 1, it is also apparent that the TD_{50} of cells maintained in 23°C saline increases as a function of incubation time. Addition of 1 mM KCN to a 23°C saline holding medium served only to increase the TD_{50} in both irradiated and non-irradiated hosts, indicating an irreversible effect of this metabolic blocking agent on the proliferative capacity of TA3 cells.

Growth Kinetics. TA3 growth kinetics were studied as a means of determining whether tumor cells maintained at 23°C undergo an initial alteration in histocompatibility properties. As shown in Chart 2,

tumor cells held in 100 volumes of 23°C saline for one half hour prior to transplantation immediately enter log phase growth only in pre-irradiated hosts. An immunologic response of normal LAF_1/J mice against the tumor cells is evident from the fact that a lag period of several days is observed prior to initiation of exponential growth in non-irradiated hosts. Inocula of less than 10^3 cells were found to be rejected in both irradiated and non-irradiated hosts. These results clearly demonstrate that TA3 cells held in a 23°C saline medium undergo an alteration in histocompatibility properties. This fact was not evident from TD_{50} measurements alone since an identical cell inoculum is required for successful tumor growth in normal and immunosuppressed hosts.

Growth kinetics were also measured for TA3 cells held at 23°C in Medium 199, a balanced salt solution containing glucose, amino acids, and other nutrients. The growth properties were found to be identical to those observed with a saline holding medium (4), indicating that the markedly decreased transplantability of cells maintained in 23°C saline is not attributable to a lack of metabolites in the medium.

DISCUSSION

On the basis of experimental results presented here, it may be concluded that suspension of TA3 ascites tumor cells in a large volume of saline or nutrient medium leads to a rapid alteration in the histocompatibility properties of these cells. In addition, there is a substantial reduction in the percentage of cells capable of dividing in recipient LAF_1/J hosts. The latter process occurs concurrently with the change in histocompatibility. The rate at which these degenerative processes occur can be minimized by maintaining the temperature of the tumor cell holding medium at 4°C, thereby introducing a reversible block in cellular metabolism. The rapid loss of proliferative capacity of tumor cells held at 23°C appears to result either from cellular autolysis or from the leakage of intracellular components, and cannot be countered by using a holding medium containing metabolites essential for long-term cell viability.

We propose the following mechanism for the observed alteration in histocompatibility properties. The dilution of TA3 ascites tumor cells with a large volume of saline forces the desorption of a sialomucin coat present at the cell surface *in vivo* (5). Comparative studies on the surface charge properties of washed and unwashed cells indicate that this desorptive process occurs instantaneously in room temperature saline. In a 4°C medium, however, complete desorption of the cellular mucoid coat requires several hours. Once the TA3 sialomucin coat has been desorbed, the cellular surface properties undergo rapid alteration. At 23°C, the surface charge decreases by 30% within a period of two hours. Introduction of a metabolic block through addition of KCN to a 23°C holding medium serves to prevent

this rapid reduction in surface charge. Similarly, cells maintained at 4°C do not exhibit a time-dependent reduction in surface charge. On the basis of these data, it appears that actively metabolizing TA3 cells held in vitro under conditions where their native surface coat is desorbed undergo a rapid rearrangement of charge-determining groups at the cell periphery. As a result, surface antigens defining the histocompatibility of the TA3 cell are altered. The possibility that antigen material is "lost" from the cell membrane appears unlikely since the content of neuraminidase-susceptible sialic acid -- a major antigenic determinant (1) -- does not decrease as a function of time in the holding medium.

The relationship of these changes in surface properties to the reduction in proliferative capacity of TA3 cells following in vitro maintenance is not clear, especially since a loss of cell viability is not indicated by uptake of the vital dye nigrosin. One possible explanation is that various forms of sublethal damage are incurred by TA3 cells in a holding medium, and that these effects subsequently limit their ability to undergo cell division in vivo. It would be reasonable to expect, for example, that the alteration in cellular surface properties that occurs in a holding medium may result in a leakage of ions and other cytoplasmic components from the cell. In addition, a limited degree of autolysis could severely restrict the proliferative capacity of TA3 cells. These forms of cellular damage may not be immediately detectable using dye exclusion as a criterion of cell viability.

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FOOTNOTE

[1]Present Address: Spectra Biologicals, Oxnard, California 93030.

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TABLE 1

The tumor dose of TA3 cells for 50% mortality (TD_{50}) in LAF_1/J hosts. The influence of *in vitro* holding media on the TD_{50} of TA3 cells is shown with normal LAF_1/J mice, and with tumor hosts immunosuppressed by 500 rad irradiation from a ^{60}Co source two days prior to transplantation.

Holding Medium Conditions	Dilution Factor[b]	Incubation Time (Hrs)	% Viable Cells[c]	Host Irradiation (rad)	TD_{50}
4°C	1	0	97	0	18
4°C	100	2	97	0	1200
4°C	100	2	97	500	310
23°C	100	0.5	91	0	2400
23°C	100	0.5	91	500	2310
23°C	100	2	88	0	3750
23°C	100	2	88	500	3760
23°C + 1 mM KCN[a]	100	2	94	0	7000
23°C + 1 mM KCN	100	2	94	500	5630

[a] When KCN was present, the pH of the saline holding medium was adjusted to 6.5 with 0.15 N HCl prior to addition of TA3 cells.

[b] Saline dilution factors are expressed relative to the volume of ascitic fluid, and not packed cells.

[c] Based on nigrosin dye exclusion.

LEGENDS FOR ILLUSTRATIONS

Chart 1. The surface charge density of TA3 cells is shown as a function of time in three holding media: saline at 4°C and 23°C, and 23°C saline containing 1 mM KCN. Prior to the addition of ascitic fluid, the pH of the saline holding medium containing KCN was adjusted to 6.5 with 0.15 N HCl. In all cases, ascites fluid containing the TA3 cells was diluted with 100 volumes of holding medium. During the incubation period, the pH was maintained at pH 6.5 $\pm$ 0.1 by addition of 0.15 N HCl or 0.15 N NaOH.

Chart 2. The growth kinetics of TA3 cells are shown for inocula of 6×10^3 and 6×10^4 cells in normal LAF_1/J hosts, and in hosts given 600 rad irradiation from a ^{60}Co source immediately prior to injection.

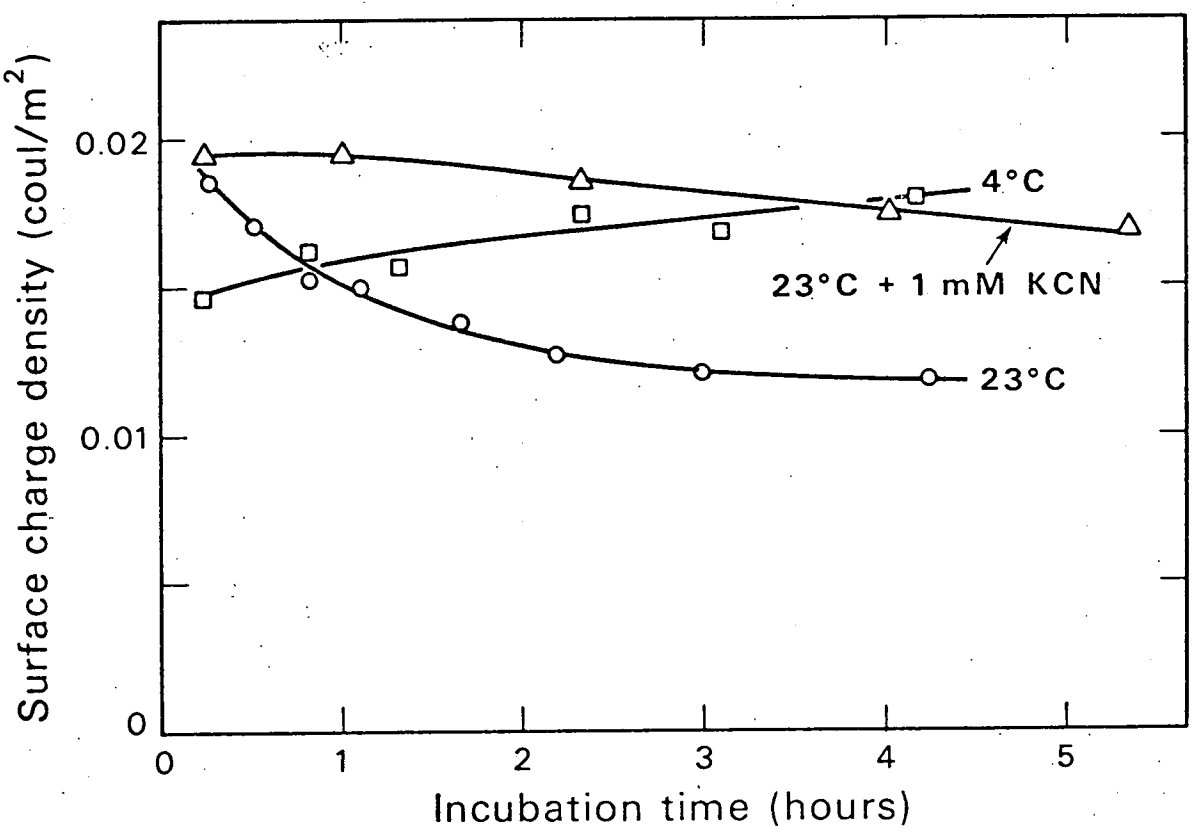

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CHART 1.

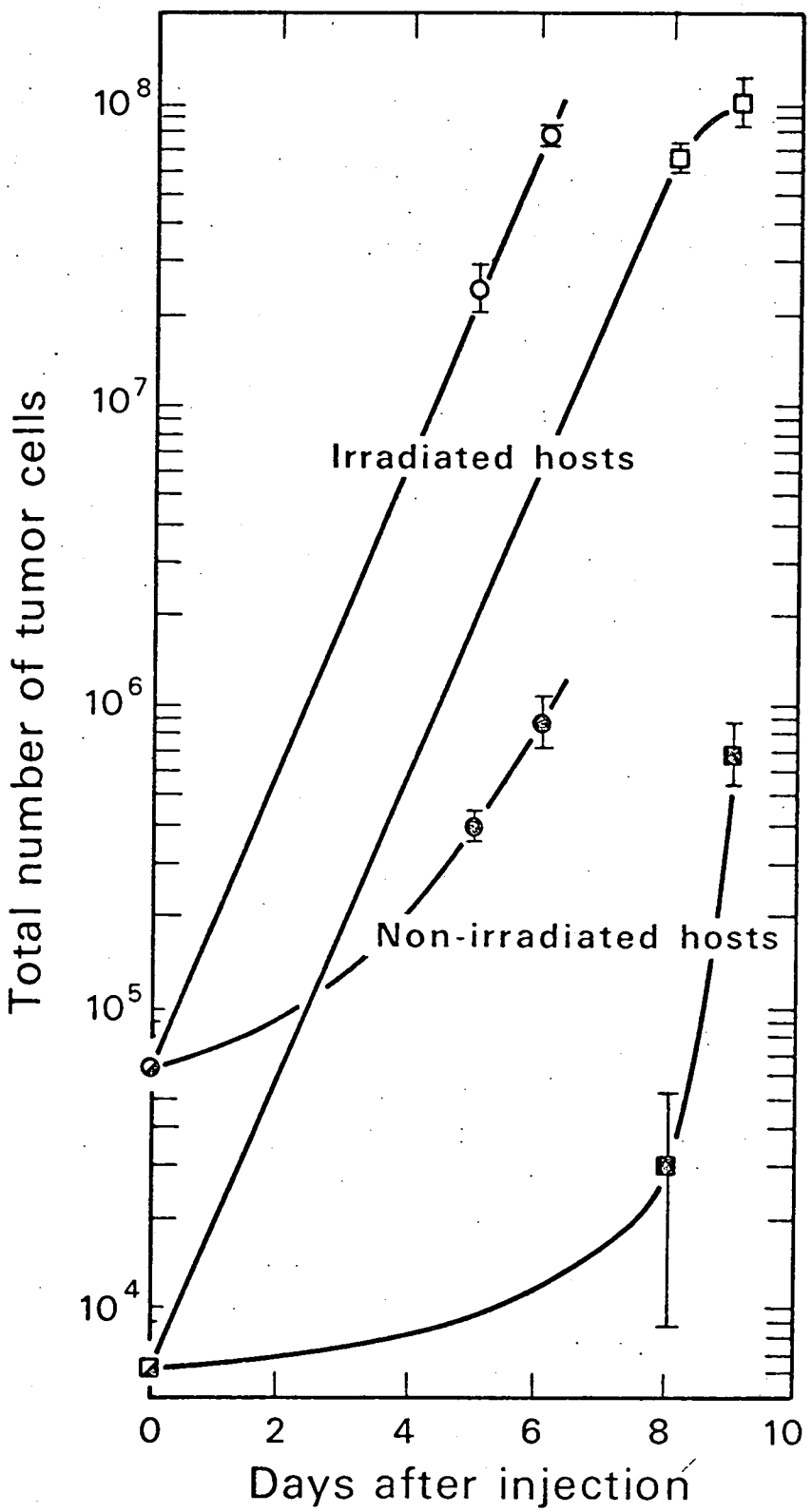

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CHART 2.