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IN VIVO STUDIES OF RADIATION POTENTIATON BY IODOACETAMIDE AND OBSERVATIONS ON TUMOR TRANSPLANTATION IMMUNITY

W. Robert Richards* and Lola S. Kelly

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LAWRENCE RADIATION LABORATORY
UNIVERSITY of CALIFORNIA BERKELEY

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ABSTRACT

Iodoacetamide has been shown by others to be a radiation sensitizer for bacteria and for certain mammalian cells tested in vitro. This work describes an examination of the effectiveness of iodoacetamide used in vivo. Survival of ascites tumor cells maintained in the peritoneal cavity of mice was used as an indicator of sensitization. Survival was assessed using TD_{50} and total tumor cell population determination methods. A comparison of results obtained by these methods is made. The effects of oxygen tension and radiation dose rate upon results was examined. Iodoacetamide was found to be effective as a radiation sensitizer under all conditions although to a lesser degree than that reported by others for in vitro experiments with bacteria.

Radioactive tracer studies indicate that iodoacetamide has rapid and total access to most if not all tissues of the body. This fact coupled with the observation of a sensitization in an in vivo system where the anoxia so prevalent in well developed tumors was present, suggests the possibility of clinical usefulness of iodoacetamide in cancer radiation therapy.

Certain observations are reported on the effect of various cell and host treatment procedures upon cell population growth kinetics seen subsequent to inoculation of hosts with the cells. A hypothesis is presented which can account for the observations made by the author and also for those made by some others who report that large inocula, i.e., greater than 10 cells, are required to give rise to a lethal tumor in isologous hosts of the strain of tumor origin. The hypothesis may also account for what is known in the literature as the "Hybrid Effect."

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CHAPTER I

INTRODUCTION

Radiation Modifiers

The search for modifiers of the actions of electromagnetic and particulate radiation began only a few months after the discovery X-rays. Walsh (1) wrote his observations on the matter of prevention of radiation sickness, mentioning the efficacy of lead shielding. Interest in means of modification other than the purely physical ones of shielding, geometry, etc. also began soon after X-rays came into regular use. In 1909, Schwartz (2) observed that ischemia served to protect the skin from radium and X-radiation effects. Crabtree and Cramer (3) examined in some detail the effects of anoxia and other means of altering respiratory mechanisms. This work was done in an effort to discover any relationship between what is now known as the Crabtree effect and radiosensitivity. These authors found that anaerobiosis decreased radiation sensitivity while treatment with HCN or low temperature increased sensitivity.

Chemical Protection

The work of Dale (4) beginning in 1940 with experimental observations on X-ray inactivation of enzymes showed that the resultant

inactivation was not only proportional to radiation dose but also was concentration dependent. This same investigator in collaboration with other workers showed that enzymes and other molecules are protected in aqueous solution if other compounds are also in the solution at the time of irradiation (5, 6, 7). (Protection will be defined for the purposes of this discussion as a lessening of the deleterious effects of ionizing radiation due to the presence of the protecting agent at the time of irradiation. It will not include beneficial effects derived from any post irradiation treatment.)

The work of Dale logically stimulated a search for protective chemical compounds and classes of compounds. There were very practical reasons for such a search. It was hoped that people might be protected from radiation sickness and mortality following large doses such as might result from nuclear warfare or from clinical treatment of tumors. The possibility existed that substances capable of protecting normal tissue while not protecting cancerous tissue might be found. Latarjet and Ephrati (8) looked at the efficacy of various compounds in protecting a bacteriophage against X-ray inactivation and found that compounds of two classes, sulphydryl and amino, were effective. Investigation by Patt (9) and collaborators showed that the amino acid cysteine was quite effective as a protective substance in aqueous solution whereas cystine, which is the disulfide formed from two cysteine molecules, is quite inactive. Bacq (10) and

coworkers synthesized β-Mercaptoethylamine (cysteamine) in 1951 by removing the carboxyl group from cysteine and found that this compound was more active than cysteine. In addition, it was found that the disulphide (cystamine) was also active in contradistinction to the facts with cystine.

The Mechanism of Chemical Protection - Inhibition of the Oxygen Effect

A number of hypotheses have been set forth to account for chemical protection (11, 12, 13, 14, 15) and the list seems to grow with the list of protective agents. A few of the more popular ones will be discussed in a cursory way here. If one idea could be considered to be the most widely held, it would likely be the supposition that protection is due to a reduction or elimination of the oxygen effect. The term, oxygen effect, refers to the observation that biological systems which are irradiated while well supplied with oxygen are more radiosensitive than the same systems irradiated while in an anoxic state. In cellular systems, the magnitude of this effect is such that two to three times as much radiation may be required to result in a specific surviving fraction under anoxia as that required in an oxygenated state. Perhaps protective chemicals somehow prevent damage to cellular constituents which occurs due to the presence of oxygen. This hypothesis is easily tested. If protection is in fact partial or total abolition of the oxygen effect then (1) no protection should be

observed when anoxic systems are irradiated with the protective substance being present, and (2) protection should never exceed the magnitude of the oxygen effect.

Of the many chemical protectors examined for the above mentioned criteria, a large number are found to conform. It is found that there are important exceptions, however. A number of sulphydryl compounds protect anoxic systems and the compounds cysteamine and cysteine give dose reduction factors which are considerably larger than the maximum obtained with anoxia (16).

The Mechanism of Chemical Protection - Toxicity

A second hypothesis is that protective chemicals function because of their toxicity. The idea being that protection results when some normal cellular processes are inhibited. It is true that protectors seem to be toxic without exception but it is not true that all toxic compounds protect.

Of interest is the observation that radiation sensitivity is a function of the phase of a cell cycle in which the irradiation is done (17, 18). With certain mammalian cells, a variation of about the same magnitude as that of the oxygen effect is observed with cells being most sensitive in G1, the time preceding DNA synthesis, decreasing in sensitivity through the S phase to a minimum in G2, the post synthetic phase. Sinclair (19) has recently shown that cysteamine

tends to abolish this variation and that the survival of the cysteamine treated cells is always superior to the survival of the most radioresistant stage of the untreated cells. It then appears unlikely that protectors could by some toxic action cause cells to revert to some radioresistant stage thereby effecting protection (20).

The Mechanism of Chemical Protection - Free Radical Inactivation

A third hypothesis has been that protective compounds somehow prevent reactive species formed by ionizing radiation from reacting with sensitive target molecules. The existence of such free radical formation has been known for some time (21). Dale (22, 23, 24) showed that the presence of molecules of a type other than that to be protected, produced competition for the radicals thereby protecting the molecule of interest. But this idea is of greater scope than simple competition. Eldjarn et al. (25) found that the inactivation of free radicals by cystamine in a cell free environment was considerably less than that necessary to produce the protective effect observed in vivo and concluded that the in vivo protection, if due to free radical inactivation, required selective concentration in organs, cells and subcellular structures most probably on the surface of the target proper. Of course there are vast differences between living and nonliving systems, and such a selective concentration remains to be shown. The above mentioned conclusions may for these reasons be unwarranted.

It would seem that the existence of an underlying mechanism common to all chemical protectors has yet to be shown. Perhaps there is none. By analogy, there are many ways to kill cells, e.g., radiation, starvation, mechanical disruption, non-physiological temperature, pH, osmotic pressure, etc. It certainly is difficult to imagine a mechanism common to all these methods. In view of the fairly wide spectrum of protectors, it would not be surprising to also find a plurality of mechanisms of protection and as will later be discussed, a plurality of mechanisms for sensitizers.

Chemical Sensitization

Some definition of just what constitutes radiosensitization or a radiosensitizer is in order. For the purposes of this discussion, radiosensitization is the condition in which the results of exposure to ionizing radiation are increased. A radiation sensitizer is defined to be an agent which when present at the time of irradiation results in the above defined condition. In general, a further restriction is applied to the class known as sensitizers. It is that the presence of a sensitizer at the time of radiation results in a greater effect than the simple sum of the effects of radiation and the sensitizer administered alone. That is to say, sensitizers are not simply additive in their action, but are synergistic.

There are considerably fewer chemical sensitizers than protectors

known (26, 27). Representative compounds and their classification are shown below:

1. Oxygen
2. Antibiotics
   Actinomycin D, Mitomycin C
3. Synkavit (2-methyl-1, 4-naphthohydroquinone diphosphate) and related compounds
4. DNA Base Analogs
   5 Iododeoxyuridine, 5 Bromodeoxyuridine, 5 Flurouracil
5. Sulphydryl Poisons
   N-ethyl maleimide, ethylmethane sulphonate, P-chloromecuribenzoate, Iodoacetamide
6. Halogen containing compounds
   Iodoacetamide, Iodoacetic acid, Potassium Iodide, Chlorohydrate, Trifluroacetaldehyde, P-chloromecuribenzoate.

1. Oxygen. The first classification consists of oxygen in reference to the well-known oxygen effect where the presence of oxygen at the time of irradiation results in more radiation induced damage than will occur in anoxic conditions. The magnitude of the effect is such that the result of a radiation dose in the presence of oxygen may be equivalent to that obtained with two to three times the radiation dose used under anoxia. The effect is clearly a sensitization since the oxygen may be present in the physiological condition where it produces no toxicity of its own.

2. Antibiotics. The antibiotic Actinomycin D has been shown to react with DNA (28, 29) and to sensitize cells to X-irradiation. The

mechanism appears to be an interference with repair processes (30). Elkind et al. (31) have shown the effect to be a function of cell age with the greatest interaction between Actinomycin D and X-rays occurring in late S phase. Interaction results when X-irradiation precedes Actinomycin or vice versa. Another antibiotic which has been reported as a sensitizer is Mitomycin C (32). The use of this agent either prior to or immediately following X-irradiation produced a much greater decrease of mitotic index for a rat sarcoma carried interperitoneally than that due to either X-rays or Mitomycin C alone. It appears then that both of these agents act to inhibit repair of radiation damage and are not sensitizers in the sense of increasing the effectiveness of the radiation in producing damage.

3. Synkavit. Synkavit is a compound closely related to the K vitamins. It and related compounds have been extensively investigated by a large group led by J. S. Mitchell. These investigators have found Synkavit to be an effective sensitizer in both laboratory and clinical trials using a variety of endpoints for comparison (33, 34, 35, 36). Synkavit was found to be of low toxicity and to selectively concentrate in some types of tumor cells. The latter property led Mitchell and his coworkers to prepare tritium labeled Synkavit and to use it to selectively irradiate tumor cells in clinical trials. This has resulted in some success. Synkavit seems to have a complex biochemistry,

one interesting aspect of which is its interference with aerobic glycolysis in tumor cells thereby forcing them to increase respiration if possible. Warburg has shown a high ratio of aerobic glycolysis to respiration to be a common if not universal distinguishing feature of tumor cells (37, 38, 39).

4. DNA Base Analogs. The class of radiation sensitizers which are DNA base analogs seem to act in a singular manner. These analogs are incorporated into the DNA molecule where they replace bases normally present (40). The mechanism of sensitization to X or Gamma irradiation is not known positively at present. However, there is reason to suspect sensitization occurs by either or both of the following mechanisms: (1) weakening of the sugar phosphate backbone of the DNA strand, or (2) inhibition of repair (41, 42). The analogs differ in their dimensions from normal bases and their presence could put some considerable stress on the backbone. The resulting strain could result in an increase in the frequency of lesions. This same strain might render repair enzymes of systems incapable of operating in the region of the base analogs.

5. Sulphydryl Poisons. The class of compounds called sulphydryl poisons or sulphydryl binding agents has been found to contain members which are true sensitizers. Recall that sulphydryl compounds were found to be radiation protectors. It then would seem

likely that antagonists might at least abolish protection and perhaps act as sensitizers. While sulphydryl poisons have indeed been found to sensitize, the mechanism of action has not been clearly elucidated. There is evidence that sensitization by these compounds may not be intimately linked to their sulphydryl binding properties (43). More will be said about the mechanism involved in the conclusions section of this work.

Studies in a Whole-Animal System

A fairly large amount of investigation into this class of compounds has been done. A number of assay methods have been used and results confirming radiosensitization have been the rule. Perhaps the earliest report on a member of this class of compounds was that of Patt et al. (44) in which the effect of administration of p-chloromecuribenzoate (CMB) upon radiation lethality of mice given whole body X-irradiation is discussed. The authors found an additive effect only. They also found that the portion of the lethality due to CMB alone is prevented by the presence of cysteine before or after administration of CMB. Other investigations using lethality of a whole animal as an endpoint have been done by Moroson and Spielman (45). These authors tested a number of compounds finding sodium iodoacetate (IAA), iodoacetamide (IA), hydroxymecuribenzoate (HMB), and p-chloromecuribenzoate (CMB) to increase mortality over that due to radiation only, but little

difference due to use of N-ethylmaleimide (NEM). The last compound was used in a lesser quantity because of its greater toxicity. Moroson and Spielman also performed experiments to ascertain whether the drug and radiation effects were synergistic or just simply additive. It was found that postirradiation injection of CMB or IAA produced no significant increase in mortality over that of post-irradiation saline injected controls. It is to be inferred then that for the conditions of this experiment at least, CMB and IAA are radio-sensitizers.

The system chosen for study by the above mentioned authors is a very difficult one to use for quantitative purposes. The death of an animal following an injury such as is inflicted by radiation has many possible contributory causes which can complicate any quantitative interpretations one might wish to make. Many workers have chosen to reduce these complications by using cellular systems.

Studies with Bacteria

Two groups have examined sulphydryl binding agents for radio-sensitization using the very radioresistant organism, Micrococcus radiodurans. Lee et al. (46) tested NEM and IAA on this organism finding no effect by NEM but a decrease in the D_{37} (dose of radiation resulting in 37% survivors) for the organism from about 300 kilorads to 100 kilorads, giving a dose modifying factor DMF of 3 when IAA was

used. The concentration of the chemicals was 10^{-4} molar in both cases and irradiation was carried out in air at atmospheric pressure. Dean and Alexander (47, 48) tested iodoacetamide on this same organism. They obtained a DMF of 7 using IA at 3×10^{-4} molar and a DMF of 90 when the IA was used at 10^{-3} molar concentration. It should be noted that Lee et al. (49) found NEM and IAA to be toxic to Micrococcus radiodurans when used in concentrations above 1.5 and 3.5×10^{-4} molar respectively and when incubated for a period of 4 hours. Dean and Alexander (50) observed no toxicity due to IA at 10^{-3} molar for 1 hour and exposed cells to this compound for a maximum of 2 hours. It may be expected, however, that the threshold of toxicity was approached by these authors. Dean and Alexander (51) did use NEM at 10^{-3} molar in tests with Micrococcus sodenensis in a comparison with other sulphydryl poisons at the same concentration. No statement as to chemical toxicity was made. The DMF's estimated from these data at 0.1% survival are given below with reservations as to the contribution to them due to drug toxicity.

Quite obviously iodoacetamide is the best sensitizer if the DMF's are relatively free of the influence of drug toxicity. Recall that a DMF of 3 is about the best that may be expected with the oxygen effect and note that IA is the only compound in Table 1 which gives an appreciably greater DMF.

TABLE 1

X-ray Dose Modifying Factors for Some Radiation Sensitizers of Micrococcus Sodenensis

Compound	DMF
NEM	1.4
Divinyl Sulphone	2.1
CMB	3.0
Iodobenzoic Acid	3.6
IA	18.0

Studies with Mammalian Cells In Vitro

Two groups of workers have worked with in vitro mammalian cell systems. Bianchi et al. (52) examined the effects of some sulphydryl poisons upon rabbit erythrocytes. Two endpoints used were: potassium loss as measured immediately after irradiation and hemolysis as measured 22 hours after irradiation. The results as obtained at 158 Kr of 200 Kvp X-rays are presented in Table 2. The relative potassium loss and hemolysis are with respect to values obtained without the presence of the compound. These values are not dose modifying factors. The effect of the compounds themselves upon potassium loss is said to be negligible as used. No statement as to effect upon hemolysis is made.

TABLE 2

The Effect of Some Sulphydryl Poisons on Potassium Loss and Hemolysis of Irradiated Rabbit Erythrocytes

Compound	Concentration (Molarity)	Relative loss of K	Hb
Iodoacetic Acid	1.3×10^{-4}	5.6	2.0
	6.5×10^{-4}	10.3	4.6
Iodoacetamide	1.3×10^{-4}	9.6	3.0
	6.5×10^{-4}	21.6	4.7
	1.3×10^{-3}	21.5	4.4
Bromoacetic Acid	6.5×10^{-4}	15.7	2.0
	1.3×10^{-3}	18.7	2.6
	6.5×10^{-3}	21.5	3.2

These data suggest iodoacetamide to be the most effective of the three substances at 6.5×10^{-4} molar, the only concentration at which all three were tested. The data also indicate that the maximum effect with iodoacetamide was obtained at 6.5×10^{-4} molar; no further increase resulted from doubling the concentration.

When all of the above mentioned studies with sulphydryl binding agents are considered, it is seen that several are sensitizers but that iodoacetamide seems to be superior in this respect.

6. Halogen Containing Compounds. Many of these have already been discussed, especially under the class of sulphydryl poisons. For this reason, the consideration of the class of halogen containing compounds will be limited to those that are not members of one of the preceding groups except to compare them with compounds already discussed.

Dean and Alexander (53) examined the effect of some halogen compounds on the radiosensitivity of Micrococcus sodenensis. Values of the DMF for three of them are given in Table 3. Drug concentration is 10^{-3} molar in each case. Compare these values with those in Table 1 which were computed using data obtained under identical

TABLE 3

X-ray Dose Modifying Factors for Some Halogen Compounds Used with Micrococcus sodenensis

Compound	DMF
Trifluoroacetaldehyde	1.0
Chloral Hydrate	1.3
Potassium Iodide	3.0

circumstances by the same authors. It is seen that the simple salt, potassium iodide is a fairly good sensitizer, as good or better in fact than some of the sulphydryl agents. Note also that possession of a

halogen atom does not necessarily confer the radiosensitizing property(ies) upon a compound nor can it explain the remarkable sensitization produced by iodoacetamide under identical circumstances.

The Choice of an Assay System

A number of systems have been used to assay radiation sensitization. The list includes the following:

1. Cell free systems (54, 55, 56)
2. Bacteria (57, 58, 59)
3. Whole Animals (60, 61, 62)
4. Mammalian cells in vitro (63, 64, 65)
5. Mammalian cells in vivo (66, 67, 68).

1. Cell-free Systems. Cell free systems are, of course, the simplest of systems to use. However, data obtained from the use of such systems is of limited use in the study of biological problems since biological systems are so much more complex.

2. Bacteria. Bacteria offer a useful means of studying radiosensitization. The results obtained from bacterial systems are of value both as an aid to understanding the mechanism of action of radiosensitizers and, of course, may be of immediate practical use in solving problems such as sterilization. But if one is interested in extrapolating conclusions to mammalian situations, one must be aware

of the considerable differences between bacteria and mammalian cells, three of which may be easily observed. They are: the lack of a well defined nucleus and the presence of a more impermeable membrane in the bacteria and the difference in cell cycle times between typical bacteria and mammalian cells.

3. Whole Animals. Whole animal systems are at the opposite pole of complexity when compared with cell free systems. Because of this, data obtained using such systems can be extremely difficult to interpret, a fact which diminishes the value of such data.

4. Mammalian Cells In Vitro. The in vitro mammalian cell system offers the same desirable features of ease of handling, relative simplicity and amenability to quantitation as possessed by bacterial systems and in addition offers the relevancy of responses of mammalian cells to clinical problems. Certain difficulties arise when one wishes to extrapolate results to the situation existing in an intact animal, however.

5. Mammalian Cells In Vivo. In vivo systems have the greatest potential for providing the experimenter with quantitative clinically relevant data, but some of the systems used do not yield quantitative data. An example of this is the assay of radiation sensitization by linear dimensions or mass of solid tumors (69). In addition to the

difficulty of measuring a body of irregular shape, there are the problems of estimating the portion of the mass which consists of tumor cells rather than fibrous tissue, and the well-known fact that solid tumors become necrotic in their interiors thereby altering growth characteristics in some complex way.

The ascites tumors do not share the above mentioned disadvantages (70). These tumors are a free floating suspension of cells maintained in and through the agency of ascitic fluid formed in the peritoneal cavity of the host. They may be thought of as in vivo cell cultures.

Measurement of Ascites Tumors

TD_{50}

There are at least two means of obtaining quantitative data from ascites tumors; the first is determination of the TD_{50} (71, 72) and the second is determination of tumor cell number in a recipient (73, 74). TD_{50} determination consists of finding how many cells are needed in an average tumor dose to give 50% incidence of tumors arising in a large group of mice. For tumors which have arisen in highly inbred animal strains and have been transplanted in same, the TD_{50} can be quite small, e.g., 2 or 3 cells. Tumors which differ genetically from their hosts may have TD_{50} values of several thousand or more since the host's immune system is able to recognize the tumor as foreign and act to reject it. Hence the fact of tumor development in such

systems is the outcome of a race between tumor cell production and cell removal by immune mechanisms. It is desirable to avoid this last complication unless one wishes to study immunity.

If tumor cells are treated with lethal agents, the TD_{50} obtained from the recipient animals will be increased. The increase in the required number of tumor cells is due to a decrease in the fraction of competent cells. As an example, consider a control TD_{50} of 3 cells and a TD_{50} for treated cells equal to 9 cells. If we state that a tumor will arise from one competent cell, then one out of three cells produced the tumor in the controls. This is often expressed as a competence efficiency (C. E.) which in this case is 0.33 or 33%. The C. E. for the treated cells is one in nine or 11%. The surviving fraction, a term referring to the relative number of treated to control cells and denoted as S_x/S_o, can be computed as the ratio TD_{50_o}/TD_{50_x} or as $C.E._o/C.E._o$. In this example, S_x/S_o is 3/9 or 0.33. The above calculations are based on two assumptions. First, it is necessary that the number of competent cells be independent of the number of incompetent cells present; and second, it is necessary that the shape of the cumulative mortality curves for both control and treated cells reflect Poisson sampling statistics.

Hewitt and Wilson (75) tested the first point by adding radiation killed cells to competent cells and observing the TD_{50} as a function of the ratio of killed to competent cells. They found that there was no

significant change in TD_{50} when this ratio of killed to competent cells varied from 6.4×10^3 to 6.4×10^6 indicating that the competence of cells was not significantly influenced by immune, nutritional or physiological factors. This work was done with genetically isologous mice and tumor cells. Such a result may well not be obtained under other conditions.

The second condition insures that the mortality results only from the innoculation of one or more competent cells. When the shape of the mortality curve reflects Poisson sampling statistics for treated as well as control cells, the surviving fraction is equal to the ratio of the TD_{50}'s as stated above (76).

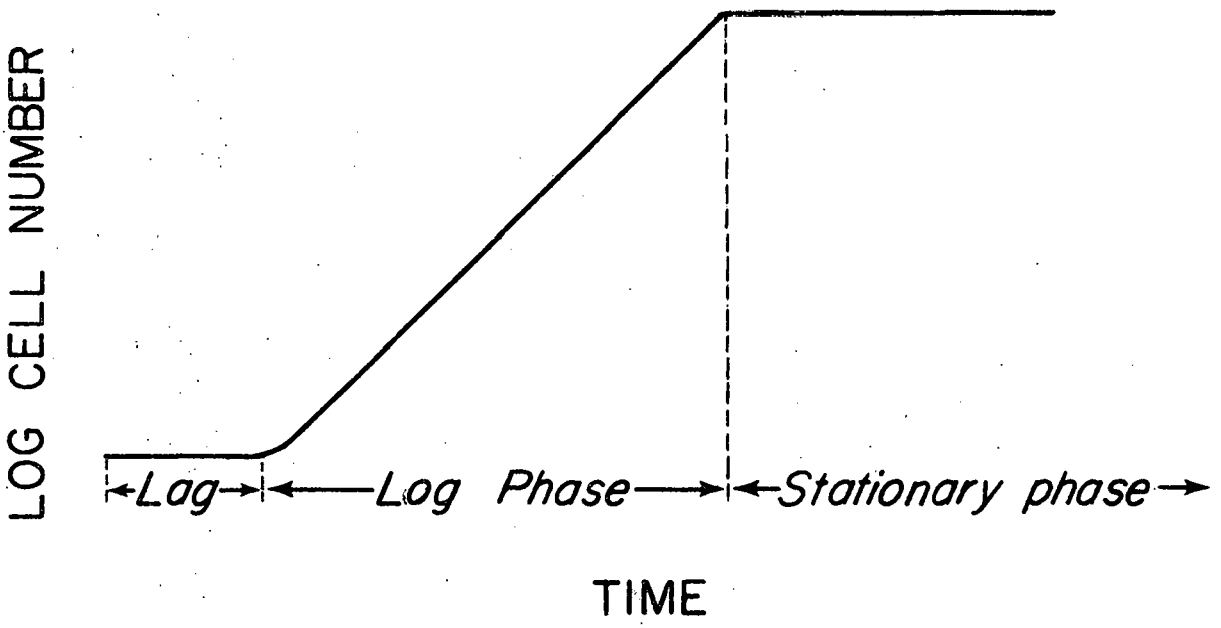
TD_{50} determination has been widely used. Here at the Donner Laboratory, Jose Feola et al. have used it extensively for assessing the effects of various radiations upon tumor cells (77, 78, 79). It seems to give reliable and reproducible results. But the method does not give any information about events involving the tumor cells and their hosts between the time of tumor innoculation and the external manifestation of a fully developed tumor. Some such information may be obtained if one assays the total tumor cell population as a function of time.

Measurement of Ascites Tumors - Determination of Tumor Cell Population

Consider the following typical growth curve of cells in culture.

There is often an initial lag phase with nearly constant population following the introduction of cells into a medium, particularly if the initial concentration is low.

Figure 1: Growth Curve of Cells in Culture



The log phase follows the lag phase, if any, and is characterized by logarithmic population growth with time, i.e.,

$$N(t) = N_o e^{kt} \qquad (1)$$

Where: $N(t)$ is the cell population at time t

N_o is the initial ($t=0$) cell population

e is the base of natural logarithms

k is a proportionality constant which has units of reciprocal time and is a measure of the rate of change of N with respect to time.

t is time elapsed since $t = 0$ in log phase.

Note that equation (1) describes a condition where N(t) is directly proportional to N_o for any given value of time with the proportionality constant being e^{kt}. That is to say, the value of N(t) will be found to be a fixed multiple of N_o for any given time and is independent of the value of N_o. The fixed multiple is equal to e^{kt}. Experimentally, t is easily determined as the time lapsed between the formation of the initial population N_o and the measurement of N(t). N(t) is measured by certain techniques to be described. The value of k may be obtained by measurement of N(t) at times t_1 and t_2 since:

$$N(t_1) = N(t_2)e^{k(t_2 - t_1)} \qquad (2)$$

If the values of t, N(t) and k are known, N_o may be calculated using equation (1). In the case where all of the original cells survive, the value of N_o obtained from the use of (1) will be equal to the actual number of cells initially present. This last quantity can be determined at the time the initial population is formed. However, when the initial population is given some treatment such that only a fraction of the cells survive and grow exponentially, then use of (1)

will yield a value of N_o which is not equal to the initial population of cells but rather is equal to that number of the initial cell population which survived treatment and produced progeny capable of exponential growth. These populations are related by equation (3).

$$SN_o = N_o' \tag{3}$$

Where: S is the fraction of N_o surviving treatment

and N_o' is the number of cells in the initial population which survived treatment

In log phase growth:

$$N(t)' = N_o' e^{kt} \tag{4}$$

Where: $N(t)'$ is the population at time t arising from N_o'.

Using (1) and (4):

$$\frac{N(t)'}{N(t)} = \frac{N_o' e^{kt}}{N_o e^{kt}}$$

or

$$\frac{N(t)'}{N(t)} = \frac{N_o'}{N_o} = S \tag{5}$$

So it is seen that the surviving fraction is unchanged with time and may be assayed at any t by obtaining the ratio $N(t)'/N(t)$. In fact, any initial population modifying factor will be preserved allowing one to select the time and population on the log phase growth curve at

which he assays. Consider the condition where one starts with a population to be treated that is A times that of the control population. Then the treated population will always be A times that which would have resulted from a population equal to the control population and this may be normalized to the control population by dividing N(t)' by A yielding:

$$\frac{N(t)'}{N(t)} = AS \tag{6}$$

An alternate and useful means of accounting for differing initial populations is conversion to per unit dimensions. This is accomplished by rearrangement of (1) and (4) to:

$$\frac{N(t)}{N_o} = e^{kt} \tag{1a}$$

$$\frac{N(t)'}{N_o} = e^{kt} \tag{2a}$$

The use of per unit quantities is an aid in constructing a growth curve for cell population where, for practical reasons, the population assayed must not vary much even though assay takes place over a time in which populations will increase by orders of magnitude. This is effected by adjusting initial populations of cultures to be assayed at later times downward with respect to those to be assayed soon after time equal to zero.

Measurement of Ascites Tumors - Some Special Considerations

Figure 1 indicates that the log phase is followed by the plateau or stationary phase. This is a phase in which population growth is inhibited by space and/or nutritional limitations. Commonly, a population will decline somewhat from a maximum attained on entering the plateau and will remain relatively constant thereafter. It is difficult to assess the effects of treating cells in a quantitative manner when they enter this phase.

The ascites tumor lends itself quite nicely to a total cell assay as it is in effect an in vivo cell culture using an undefined but nutritious and adequate medium. The cells are uniformly dispersed in a fluid, hence the total population may be obtained by finding the cell concentration and the total fluid volume, then multiplying these factors together. Certain complications arise in the carrying out of such measurements. For example, we estimate that a free peritoneal cell population of about 10^7 cells normally exists in the LAF_1 mouse. This value is substantiated by Kornfeld and Greenman (80). These authors found an average of 2.4×10^6 macrophages and 5.4×10^6 lymphocytes present in the peritoneal cavity of LAF_1 mice. These cells will result in a high background concentration unless one of two procedures is possible. First, one can somehow discriminate between tumor and normal cells by size, shape, stained color, etc., or,

secondly, one can arrange the experimental conditions so that the ratio of tumor to normal cells is high, thereby making the background contribution as small as possible. In practice, it turns out that the first option can be difficult or tedious to do, especially if the only method available is the making of differential cell counts from a smear preparation. The second option has a rather low practical limit to its usefulness since the tumor cell population in the mouse will enter stationary phase at a value of about 10^8 cells thereby ending the system's usefulness as a quantitative assay system. But by exercising both options, a situation is obtained that is of practical value.

Statement of Purpose

With the information presented above in mind, the author set about to study in detail the action of radiation sensitizers in general and iodoacetamide in particular in the in vivo mouse ascites tumor system. That which follows is an account of the observations made and the methods used to obtain them. Briefly, it may be stated that iodoacetamide was found to be a sensitizer when present at the time of irradiation. This conclusion was reached by two methods of determination; TD_{50} and total tumor cell assay. While the magnitude of the effect is not as large as that observed in bacterial systems, it is not negligible. Surviving fractions of cells pretreated with iodoacetamide were commonly reduced by a factor of two over those given

iodoacetamide as a post-irradiation treatment. In the course of measuring the growth of cells some observations were made of factors effecting such growth. The factors involve the cell holding temperature and medium during transplantation and the condition of the host.

CHAPTER II

METHODS

Animals and Their Care

All animals were female LAF_1 (C57L ♀ x A/He ♂) obtained from the Jackson Laboratory, Bar Harbor, Maine. Animals were housed 10 to a cage and were given chlorinated water and food pellets ad libitum.

Tumor Types and Transplantation

Two tumors were used, the L2 Lymphoma and the TA_3 Mammary Carcinoma. Both were obtained from Mr. Jose M. Feola of this laboratory. The original source of the L2 tumor is Dr. Emma Shelton (81) of the National Cancer Institute, Bethesda, Maryland and that of the TA_3 tumor is Dr. T. S. Hauschka (82) of Roswell Park Memorial Institute, Buffalo, New York. The L2 Lymphoma was found in 1946 in a 6-month old strain A female that had been exposed to 400 R whole body X-radiation on the date of birth. It was carried by subcutaneous transplantation of tissue in strain A or CAF_1 hybrids for more than 100 generations before conversion to ascites form in the 1950's. Hauschka found the TA_3 mammary adenocarcinoma in a strain

A female in 1949. It was carried as a solid tumor for 34 transplant generations in A mice before conversion to ascites form in 1951.

Since these tumors arose in the strain A mouse they are therefore compatible with that strain and all F_1 hybrids thereof. Tumors were carried in the LAF_1 mouse. Carriers were given an interperitoneal injection of approximately 10^6 cells in a 0.1 cc volume. After a period of growth, usually 6 to 7 days, carriers were harvested for use from the peritoneal cavity by means of a 1 cc tuberculin syringe inserted into the cavity. The gut was held aside when necessary to provide a collection site using a device which we have called a Gut Paddle. The paddle may be described as a small flat version of the straining spoon so prevalent in household kitchens.

It was not important to know the number of cells given to carriers with any more accuracy than about a factor of 2 which was obtainable by an educated guess, but when cells were given to experimental animals, a much better knowledge of cell number was required. In these circumstances, the cell concentration was obtained using a Model B Coulter Counter.* This device and its use will be discussed presently.

General Chemical and Radiation Treatment Procedures

Most experimental animal groups were given an interperitoneal (IP) tumor inoculation. The volume of the inoculum was generally

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*Coulter Electronics, Hialeah, Florida.

0.10 cc and was injected using a 0.25 cc glass syringe and a 25 g x 5/8" needle. The number of cells given to animals was identical within an experimental group but varied from group to group depending upon the treatment to be administered and also the time elapsing until assay of the tumor cell population. In general, animals to receive chemical and/or radiation treatment were given more tumor cells than controls. Animals which were examined later in the experiment were given fewer cells than those examined earlier. The absolute number of cells to be given was determined by a measurement of cell concentration in ascites fluid pooled from several carrier donors. The cells were enumerated with a Coulter Counter. Once the cell concentration of the pooled donor ascites fluid was known, it was a simple matter to make dilutions such that 0.10 cc of the inoculum contained a known appropriate number of cells for any given group of recipient animals.

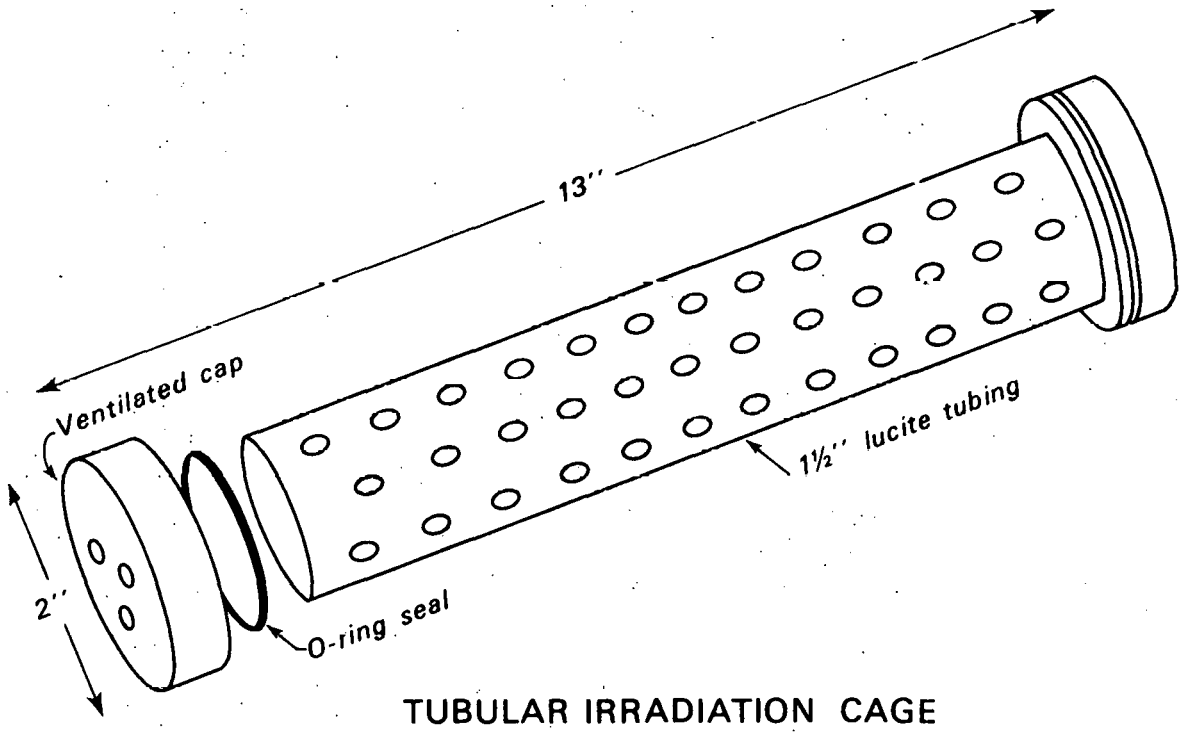
After a time lapse of from minutes to days following inoculation, the animals were given chemical and/or radiation treatment. Five groups of animals were created. First, tumor controls were given a 1.00 cc IP injection of isotonic saline. Secondly, a group of animals was usually created to measure drug effects on the tumor cells. These animals received a 1.00 cc injection of the drug in appropriate concentration using isotonic saline as the diluent. The third group of mice was used to measure the effect of the radiation only on tumor

cells. These animals received a 1.00 cc IP injection of saline prior to radiation treatment. Two other groups were usually included; one which received a drug injection prior to radiation exposure, and one which received a drug injection after radiation exposure.

All irradiation was done with the 1400 Ci Co^{60} air source located in Building 74 of the Lawrence Radiation Laboratory, University of California at Berkeley. This source is nearly a point source, at least as viewed at distances greater than 10 cm, and provided an exposure dose rate of about 30 roentgen per minute at a radius of 1 meter from the source. Dose rates of from 20 to 40 R/min were used in these experiments. Such dose rates were high enough that the well known Dose-Rate Effect was not a consideration in evaluating the results obtained at differing dose rates (83). Mice were confined within tubular cages held in a vertical cage rack. Eight cages were used; each cage usually containing 5 mice. The design of the individual cage is shown in Figure 2. No food or water was provided for the animals during irradiation since the total time involved was less than one hour.

General Assay Procedure

Experimental groups usually consisted of 10 individuals which were examined in subgroups of 5. A subgroup was consigned to a jar containing anesthetic ether (Squibb). Upon the death of the animals,



TUBULAR IRRADIATION CAGE

DRT. 709-5896

Figure 2

they were individually laid out on absorbent paper. Each animal was given an interperitoneal injection of 1.00 cc of a solution made up of isotonic saline + 4% by weight of Bovine Serum Albumin (BSA) fraction V (Pentex Inc.) + 1 μc of human Radio-Iodinated Serum Albumin (RISA by Mallinckrodt). Injections were given using a 1 cc syringe with a 25 g x 5/8" needle.

The body of each mouse was gently massaged for 20-30 seconds to mix the injected fluid with that already present in the cavity. Next, a 70% ethyl alcohol solution was applied to the abdomen for the purpose of holding the fur in place as an incision was made. Surgical scissors were then used to make an opening along the midline of the abdomen. While the incision was held open, a 1 cc disposable syringe without a needle was inserted into the cavity and a volume of 0.2 - 1.0 cc of fluid obtained and transfered to a test tube. Mice were, at this point, disposed of.

When smears were required, they were prepared at this time. A drop of the fluid contained in a test tube was transfered by means of a disposable pipette to a clean glass slide. After spreading the drop with another glass slide, the slide was dryed as rapidly as possible with a hair dryer. Methyl alcohol was used as a fixative. After 5 minutes in the fixative, slides were rinsed in tap water and placed in a stain bath. The stain used was Giemsa blood stain, original azure blend type (Scientific Products). About 5 minutes in the stain was

usually adequate. In the event that destaining was required, it was done with methyl alcohol.

Twenty microliters (μl) of the fluid in the test tubes were pipetted into a 1/2 x 2" plastic vial (Lermer Plastics) containing 1 ml of distilled water. An additional 20 μl was pipetted into a 7 dram plastic vial (Armstrong Plastics) which contained 10.0 ml of isotonic saline (Cutter Laboratories) + 4% by weight of BSA. BSA served to increase fluid viscosity thereby markedly reducing settling of the larger cells. At this point, a new subgroup of 5 mice was consigned to the ether jar for use about 10 minutes in the future. Mixing of the contents of the 7 dram vials within the individual vials was effected as uniformly and gently as possible by simultaneous repeated inversion of the vials by hand.

Next, the cell concentration of the suspension in the 7 dram vials was obtained using the Coulter Counter. Two readings were obtained; one with each polarity. On occasion, several trials were required owing to aperture blockage by debris. When a prolonged time was required, vial contents were mixed to resuspend any settled cells.

After all groups were examined, the contents of the 1/2" x 2" vials were counted to find their I^{131} content compared with that of standard vials into which 20 μl of the RISA containing injection solution had been pipetted. Counting was done using a well type NaI cystal

scintillation counter. Once tumor cell concentration data from the Coulter Counter and data giving the RISA concentration of the fluid in the peritoneal cavity were known, total cell numbers were obtained as the product of the cell concentration and the volume as calculated on the basis of I^{131} content of the 20 µl samples of fluid as compared to that of the I^{131} content of the standard.

Accounting for Normal Cells

Since ascites tumor cells are resident in the peritoneal cavity of mice, and as has been mentioned, the population of cells normally present in the cavity is of the order of 10^7 cells, any determination of cell number necessarily must include a discrimination between tumor and normal cells. The resident cell population in both number and type of cell, depends upon the treatment given the host. The resident cells present in sufficient numbers to affect cell number determination under any of the circumstances studied are given in Table 4.

Kornfeld and Greenman (84) found that the normal peritoneal population of LAF_1 mice consisted largely of 2.4×10^6 macrophages and 5.4×10^6 lymphocytes. Approximately half or 2.7×10^6 of the lymphocytes were of the small type. Their data indicate that exposure to 90-590 R of X-rays does not alter the macrophage population appreciably for at least 2 weeks; a period of time longer than

that of interest in the present studies. However, these authors found that the lymphocyte population changed markedly in a few hours following radiation exposure. The greatest effect was on the small lymphocytes. These cells decreased to a few percent of normal levels for X-ray exposure of 290-690 R. At the same time, the medium lymphocyte levels show no change at 290 R but decrease to about 30% of non-irradiated levels at a dose of 690 R. Lymphocyte levels begin to return to normal about 2 weeks after irradiation. We concluded that macrophages especially would present a problem regardless of radiation treatment of the hosts. Lymphocytes could be expected to cause less difficulty with animals given radiation exposure.

TABLE 4

Major Constituents of the Free Peritoneal Cell Population

Cell Type	Range of Cell Diameter (Microns)	Mean Diameter (Microns)	Volume of Cell Having Mean Diameter (Cubic Microns)
L2 Lymphoma	18-25	20	4180
TA_3 Carcinoma	22-32	27	10300
Macrophage	22-27	23	6340
Granulocyte	10-12	11	696
Small lymphocyte	6-7	6	115
Medium lymphocyte	8-10	9	380

Accounting for Induced Granulocytes

In the course of examining peritoneal cell smears, it was discovered that iodoacetamide administration produced a remarkable increase in peritoneal granulocyte levels, and that this increase occurred independently of treatment other than iodoacetamide administration. Such cells are rare in normal animals but seemed to constitute about a third to a half of the cell population when at maximum levels in iodoacetamide treated animals. The maximum granulocyte population was reached about 2 days post-treatment and returned to low levels by 4 days. Assuming that the population of cells normally present in the cavity was not changed by iodoacetamide administration, the maximum granulocyte level was between 4 and 8×10^6 cells. While radiation alone does not mobilize these granulocytes, it must affect the granulocytic response to iodoacetamide. The magnitude of this effect is unknown.

Differentiation of Cell Types

Table 4 indicates that a way of discrimination between the various cell types is by size. Diameter alone is enough to enable one to discriminate between the tumor cells and the granulocytes and lymphocytes which are all considerably smaller cells. In addition, if one prepares a smear, utilizing appropriate staining techniques, various other morphological characteristics aid in discrimination.

Determination of Cell Concentration

While an examination of a slide will yield data on the relative abundance of cells, it can yield only very uncertain data on cell concentrations. Such data may be obtained using the hemocytometer. However, the errors inherent in the use of this device due to physical effects and the statistical error that is associated with the practical limit on the number of cells to be counted, are quite large (85, 86, 87). Probable errors of 10-15% may be expected. The method is tedious and if applied in an experimental situation where cell concentrations from a large number of animals is required, it becomes the limiting factor in the experiment.

Cell concentration determination can be accomplished with greater accuracy and speed using an electronic cell counter. The particular apparatus used in these experiments was a Model B Coulter Counter.* The principle of operation is quite simple. Intact cells have an electrical resistance that is orders of magnitude greater than that of commonly used bathing media such as isotonic saline. Cells are made to pass through a small orifice (100 microns in our usage). This orifice serves as the limiting fluid and electrical impedances in a circuit from the outside to inside of a glass tube containing the orifice. Passage of a cell through this orifice results in a

*Coulter Electronics, Hialeah, Florida.

momentary increase in circuit impedance. The pulse change of electrical impedance is capacity coupled to amplifying and pulse height discrimination circuitry. Inasmuch as it is the rate of change of impedance rather than the change of impedance which is seen by the electronics, it is necessary to eliminate velocity of the cell as a variable. This is accomplished in the Coulter Counter by driving the fluid through the orifice with a constant pressure head during the time of measurement.

The pulse height obtained with the Coulter Counter is proportional to the volume of a cell. Table 4 indicates that volume discrimination is better than discrimination by diameter. Macrophages still present a problem, especially with the use of the L2 tumor. The TA_3 tumor cells are usually somewhat larger than the macrophages. Experimentation with discrimination between TA_3 cells and macrophages showed that discriminator settings which allowed count of a relatively small and constant number of macrophages were easily obtainable thus making the preparation and scanning of smears unnecessary. For this reason, most of the data presented here was obtained using the TA_3 tumor.

Standard procedure using the Coulter Counter was to place 20 µl of the extracted fluid from the peritoneal cavity of a mouse into 10 cc of the diluent and mix the two. Cells contained in 0.500 cc of this mixture were automatically counted hence the cell count obtained

was equal to that in 1 μl of peritoneal fluid. Cell concentrations were converted to units of cells/cc simply by multiplying the Coulter Counter reading which was in cell/μl x 10^3 μl/cc (the small difference between units of ml and cc being ignored). The Coulter Counter discrimination circuitry was used to eliminate the counting of debris and red blood cells. In the case of the TA_3 tumor, it was possible to discriminate against most of the lymphocytes as well. It may be assumed that a certain fraction of tumor cells were not counted due to small size although a good correlation between Coulter Counter cell numbers and those obtained with a hemocytometer was obtained. However, if the size distribution of cells remains fairly constant, a fixed fraction of the tumor cells will be counted and comparison of cell numbers is not impaired. The important consideration then, is the reproducibility of cell counts rather than accuracy in measuring absolute cell numbers.

A test of the reproducibility of cell counts was made by obtaining one count each from each of 20 samples prepared by pipetting 20 μl of tumor fluid from one animal into 10.0 ml of diluent. The result was a mean cell count of 13026 and a standard error of 178, thus the error in reproducibility attributable to pipetting and counting with the Coulter Counter is about 1.4%.

Determination of Tumor Volume

The datum desired from a mouse was the total tumor cell

population in its peritoneal cavity. As has already been mentioned, this information is obtained by measuring the volume of the peritoneal cavity and the cell concentration. The volume measurement is indirect, since direct measurement of this complicated geometry is impractical. The method used was the isotopic dilution method first used by Kelly et al. (88) for this purpose. In this method, a known volume of a radioactive fluid is added to the unknown volume. After mixing the known and unknown volumes thoroughly, a quantity q of the mixed volume, X, is removed. The specific activity, SA_x of q may be expressed as:

$$SA_x = \frac{A_i}{V_x} \tag{1}$$

and since $$V_x = V_i + V_u \tag{2}$$

Where: A_i is the total injected activity

V_i is the injected volume

V_u is the unknown volume

It follows that $$SA_x = \frac{A_i}{V_i + V_u} \tag{3}$$

Now, $$A_i = V_i SA_i \tag{4}$$

So that, $$SA_x = \frac{V_i SA_i}{V_x} \tag{5}$$

or $$\frac{SA_i}{SA_x} = \frac{V_x}{V_i} \tag{6}$$

Since V_i is known, V_x or V_u may be found if the ratio of specific activities can be found. This is easily accomplished in practice by obtaining the count-rate from a sample quantity q and an equal sample volume of the fluid used for injections. If counting geometry is identical,

$$SA = k \text{ Count-rate} \tag{7}$$

Where k is a constant

so (6) becomes

$$\frac{\text{Count-rate}_i}{\text{Count-rate}_x} = \frac{V_x}{V_i} \tag{8}$$

With the apparatus and animals used, it was convenient to have A_i approximately equal to 1 μc of RISA, V_i = 1.00 cc, q = 20 μl washed into 1 cc of distilled water. The count-rates in (8) were corrected for background. Coincidence correction was not a consideration with our counting system at the count rates used. It is imperative in any dilution technique that the substance to be quantitated not be capable of leaving the unknown volume by diffusion or any other means of transport. If this occurs, results will be in error (high values for volume). RISA is ideal for this purpose in biological systems since the radioactive atom, I^{131} is bound to albumin; a large molecule which is not easily transported across biological boundaries. Evidence for this and for the accuracy of RISA injection, pipetting and counting technique is seen in the result of injecting 20 normal mice with 1.00 cc

of RISA solution and obtaining V_x for these animals. V_x was found to be 1.005 cc with a standard error of 0.010 cc. The standard error is then only about 1% with this technique. The result of this measurement also indicates that the fluid volume of the peritoneal cavity of a normal mouse is essentially zero since equation (2) yields $V_u = 1.005 - 1.00$ cc.

Recall that the total cell number was obtained by multiplying the volume V_x obtained from equation (8) by the cell concentration in cells/cc. Since the errors in these procedures are independent, the standard error expected for the product should be $1\%^2 + 1.4\%^2 = 1.7\%$. Such a low standard error due to measurement technique gives some confidence that the larger standard errors often found for data obtained from a group of animals is largely due to biological variation rather than to measurement technique.

CHAPTER III

RESULTS OF IA STUDIES

1. Whole Body Effects of Iodoacetamide and Radiation

A study of the toxicity of iodoacetamide when given to the LAF_1 mouse was made. Lethality was the endpoint. Table 5 presents the results obtained when iodoacetamide was given IP in 1.00 cc of isotonic saline. Results of combining iodoacetamide with 600 R Co^{60} treatment are also shown. Mouse survival was measured at 30 days after treatment. The data of Table 5 indicate that lethality increased when iodoacetamide and 600 R of Co^{60} gamma rays were used. Six hundred R of Co^{60} gamma alone produces no acute lethality.

Table 6 presents data obtained in a second test for lethality. In this experiment, radiation exposure dose was varied while iodoacetamide dose was held constant. The data show the small lethality due to 0.370 mg iodoacetamide used with gamma radiation and also indicates that the LD_{50}, i.e., the dose of radiation which is a lethal dose to 50% of the recipients, is between 7 and 9 hundred roentgen exposure. A better estimate of this value is obtained in Section 3

TABLE 5

Long Term Survival of Treated Mice

Treatment	Survivors at 30 days	Mean Time of Death for Decedents (days)
0.555 mg Iodoacetamide	10/10	-
0.648 mg Iodoacetamide	10/10	-
0.740 mg Iodoacetamide	8/10	5
0.833 mg Iodoacetamide	1/10	2
0.555 mg Iodoacetamide + 600 R	9/10	9
0.648 mg Iodoacetamide + 600 R	6/10	7
0.740 mg Iodoacetamide + 600 R	4/10	2
0.833 mg Iodoacetamide + 600 R	0/10	2

TABLE 6

Long Term Survival of Treated Mice

Treatment	Survivors at 30 days	Mean Time of Death for Decedents (days)
700 R	5/10	25
800 R	6/10	23
900 R	3/10	20
1000 R	0/10	19
1100 R	0/10	13
600 R + 0.370 mg Iodoacetamide	9/10	19
700 R + 0.370 mg Iodoacetamide	8/10	29
800 R + 0.370 mg Iodoacetamide	6/10	25
900 R + 0.370 mg Iodoacetamide	3/10	19
1000 R + 0.370 mg Iodoacetamide	0/10	18

which follows. An LD_{50} as high as this is testimony to the good physical condition of the mice and assures one that the complicating effects of infection should not affect data obtained with these and like animals.

2. Whole Body Effects of Iodoacetamide and Radiation Upon Tumor Bearing Animals

The effect of treatment on the survival of animals which carry a well developed tumor was examined. Results are presented in Table 7. All tumor bearing animals were given 10^7 L2 tumor cells just before treatment. Radiation and iodoacetamide were administered as indicated in the table. The various non-tumor bearing controls were included as indicators of the lethality of the treatment procedures alone. It is seen that use of 0.648 and 0.740 mg of iodoacetamide in conjunction with 600 R Co^{60} whole body radiation were the only treatments of those given that were effective in prolonging survival of mice inoculated with this large dose of L2 tumor. It must also be noted that these treatment combinations seem to be approximately LD_{50}'s for non-tumor bearing mice. It is not necessary to nearly kill the host with the treatment in order to inhibit tumor growth. The treatments given were whole-body in scope. Had the radiation been localized to the tumor site, the untoward effects on healthy tissue could have been reduced. The lethal contribution of the gamma irradiation alone cannot be deduced from the results presented in

TABLE 7

Effect of Treatment on the Short Term Survival of Mice Given L2 Cells

Treatment	Survivors at Indicated Day Post Treatment														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
10^7 cells No treatment	5/5							3/5		1/5	0/5					
10^7 cells + 0.648 mg Iodoacetamide	5/5			4/5				3/5	1/5	0/5						
10^7 cells + 0.740 mg Iodoacetamide	5/5	3/5	2/5						1/5	0/5						
10^7 cells + 0.833 mg Iodoacetamide	1/5								0/5							
10^7 cells + 0.555 mg Iodoacetamide + 600 R	5/5								1/5	0/5						
10^7 cells + 0.648 mg Iodoacetamide + 600 R	5/5									4/5	3/5		1/5			0/5
10^7 cells + 0.740 mg Iodoacetamide + 600 R	5/6										4/6		1/6			0/6
10^7 cells + 0.833 mg Iodoacetamide + 600 R	5/6	1/6											0/6			

Table 7. Such information is obtainable from the data in Table 8.

Table 8 presents the results from an experiment in which the TA_3 mammary carcinoma was used. All groups initially contained 10 animals. Iodoacetamide and Co^{60} irradiation were as indicated. The last horizontal entry in Table 8 indicates that 600 R Co^{60} was not of itself, acutely lethal. When given in combination with iodoacetamide in various dosages, lethality of non-tumor bearing animals was observed as before. Lethality began to appear at an iodoacetamide dosage of 0.555 mg and was quite in evidence at 0.740 mg. This finding correlates well with the data of Table 7.

The inoculation of 10^7 cells resulted in 50% survivors at 5 days post-injection and 0% survivors at 6 days. The correspondir.g times for the L2 tumor were 9 and 11 days respectively and this in spite of the fact that the L2 cells exhibit a doubling time of about 11 hours when in logarithmic phase as compared to 15 hours for the TA_3 cells.

Table 8 shows that the time to which 50% of tumor bearing animals survived was increased by 2 days when animals received 600 R and that the 0% survivor time was increased by 7 days using this treatment. The effect of giving 0.370 mg of iodoacetamide was an increase of 2 days for 50% survival and 3 days in the timc to 0% survival. Thus this dose of iodoacetamide was approximately the equivalent of 600 R in its effectiveness at prolonging life of the animal. Table 8 indicates that the administration of iodoacetamide followed by

TABLE 8

Effect of Treatment on the Short Term Survival of Mice Carrying the TA_3 Tumor

Treatment	Survivors at Indicated Day Post Treatment																												
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
10^7 cells No treatment	10			10	5	0																							
10^7 cells + 0.370 mg Iodoacetamide + 600 R	10	10	9									9	8	6	6	6	4	0											
10^7 cells + 0.555 mg Iodoacetamide + 600 R	9	9										9	8	6	5	0													
10^7 cells + 0.740 mg Iodoacetamide + 600 R	8	6													6	4	4	2	1									1	0
10^7 cells + 0.925 mg Iodoacetamide + 600 R	10	5	4	4	4	3							3	2				2	1					1	0				
10^7 cells + 0.370 mg Iodoacetamide	10				10	9	7	1	0																				
10^7 cells + 0.555 mg Iodoacetamide	9	8	8	7				7	4	3	3	0																	
10^7 cells + 0.740 mg Iodoacetamide	9	7						7	5	1	0																		
10^7 cells + 0.925 mg Iodoacetamide	9	5	4						4	3	1	0																	
10^7 cells + 600 R	10				10	9	6	2	1			1	0																
No tumor 600 R	10	10																										10	

600 R Co^{60} resulted in an increase of time to 50% survival by 11 days and the time to 0% survivors by 12 days. Using either time measure, the result of using both agents is considerably greater than the sum of the effects of each agent alone. The time of survival for 50% of the animals over that of controls is expected to be 4 days if effects were simply additive whereas it was actually 11 days when both agents were used. In similar fashion, one would expect 0% survivors to be attained some 10 days later than controls but the result of using both treatments was an increase of 12 days. It should be recalled that the tumor is a very rapidly dividing one. Then the significance of an increased survival time is more fully appreciated.

The data of Table 8 indicate that the greatest increase in 50% survival time of treated animals is obtained using 0.370 mg of iodoacetamide in conjunction with the 600 R Co^{60} exposure dose. However, longer survival times of a few animals in groups which received 0.740 mg or 0.925 mg of iodoacetamide followed by 600 R were observed. Table 7 indicates that use of 0.648 or 0.740 mg of iodoacetamide with 600 R was optimal for the L2 tumor bearing animals. Whether these differences are tumor specific is not known. The mean lethal dose of iodoacetamide given alone to healthy LAF_1 mice is about 0.8 mg as indicated in Table 5. It is usually desirable to maintain as high a therapeutic ratio as possible yet obtaining a beneficial effect. With this thought in mind, the largest part of the studies to be

described in which the tumor cell surviving fraction was obtained were done with 0.370 mg iodoacetamide per 20 gm mouse.

3. Whole Body Effects Assessed by the TD_{50} Method

The data presented in Section 1 of this chapter may be subjected to statistical analysis in order to determine the mean dose of an agent or combination of agents required to result in animal mortality. Such a dose is termed a LD_{50} (lethal dose to 50% of animals), an ED_{50} (mean effective dose) or in the case where the agent is in the form of an inoculum of tumor cells, the dose is termed a TD_{50} (mean tumor dose). The determination of this parameter was done with the semi-graphical method described by Litchfield and Wilcoxon (89).

When iodoacetamide was administered IP in 1 cc of isotonic saline, the resultant mortality seen over an 8 week period of observation was as presented in Table 5. Analysis of this data yields a mean lethal dose of 0.79 mg of iodoacetamide with a 95% confidence interval of 0.76 to 0.82 mg. Table 5 also shows the toxicity of a combination of 600 R of radiation and a dose of iodoacetamide. Under these conditions, the mean lethal dose is reduced to 0.69 mg with a 95% confidence interval of 0.62 to 0.77 mg.

Table 6 presented data on radiation lethality of mice with and without a pre-irradiation injection of 0.370 mg of iodoacetamide. The LD_{50} of mice given iodoacetamide is found to be 830 R with a 95%

confidence interval of 740 to 930 R. The corresponding values for radiation exposure only are 760, 660 and 880 R. Quite plainly, these LD_{50} values may be considered to be identical. Hence, the effect of 0.370 mg iodoacetamide upon radiation lethality is negligible.

4. Iodoacetamide Distribution

An examination of the distribution of iodoacetamide given by 2 routes was made using I^{131} labeled iodoacetamide. The iodoacetamide was given either interperitoneally or intravenously as indicated in Table 9. Administration was accomplished in the latter case using a 27 gauge x 1/2 inch needle with a 0.25 cc syringe containing 0.10 cc of 4.5×10^{-2} molar iodoacetamide in isotonic saline. Injection was made into one of the lateral tail veins of the mouse. IP injections were of the same volume and concentration as the IV injections. Animals were sacrificed by cervical dislocation at certain times after injection. Table 9 contains data obtained by counting several organs of the animals. The values given are expressed as a percent of the injected dose. (The standard was a 20 cc saline solution containing an amount of labeled iodoacetamide equal to that given the 20 gm mice. The standard was contained in the same type of vial as were the mice and their organs.)

The data presented in Table 9 may be subject to some considerable error since each measurement presented represents data on only one mouse. However, some qualitative conclusions might be drawn

TABLE 9

Distribution of I^{131} Labeled Iodoacetamide in the LAF_1 Mouse as a Function of Time After Injection

Iodoacetamide Injection Route	Time after Injection	I^{131} Activity as a Percent of Standard						
		GI Tract	Liver	Spleen	Thyroid	Femurs	Tumor	Remainder of Carcass
IV	5 min	9.7	5.9	0.7	0.2	0.6	2.8	83.3
IV	12 min	9.4	4.9	0.7	0.1	0.4	3.0	76.7
IV	20 min	11.2	4.8	0.7	0.3	0.8	2.5	71.3
IV	30 min	10.9	4.6	0.6	0.2	0.6	2.8	72.9
IV	90 min	13.5	2.9	0.5	0.2	0.4	2.0	54.5
IV	120 min	13.1	4.9	0.9	0.2	0.6	2.0	90.6
IV	240 min	19.7	4.9	0.5	0.6	0.5	1.9	63.5
IP	1 min	24.7	9.8	0.8	0.1	0.3		63.1
IP	2 min	16.2	11.0	0.8	0.3	0.7		76.1
IP	5 min	13.7	8.7	0.6	0.4	0.7		85.5
IP	10 min	12.2	5.4	0.7	0.5	1.0		91.5
IP	20 min	13.6	6.9	0.6	0.2	0.8		83.1

from the data. It may be concluded that iodoacetamide is probably retained in the body considerably longer than 4 hours. The iodoacetamide is well distributed in a few minutes after injection by either route. The animals given an IV injection had been given a subcutaneous injection of 10^6 L2 cells in the neck some 10 days previously and had, at the time of iodoacetamide injection, a solid tumor of about 1 gm mass. The data show that iodoacetamide reached the tumor in less than 5 minutes and remained there. The thyroid data are also of interest. They indicate that the iodine atom was firmly bound to the iodoacetamide molecule for if this were not the case, this organ would have contained a large fraction of the I^{131}. This would be the case since the thyroid of a mammal typically contains from 10 to 100 times as much iodide as is present in the blood and also concentrates each day an amount of iodide approximately equal to the total amount found in the blood.

The data of Table 9 indicate that iodoacetamide is widely distributed. However, since the organs listed are of various masses, one can say nothing as to the relative concentrations of iodoacetamide in differing organs. Table 10 presents the specific activity of the labeled iodoacetamide in liver, spleen and tumor tissue of the same animals from which the data of Table 9 were obtained. The data indicate that the concentration of iodoacetamide probably becomes nearly constant throughout the animal soon after administration and

TABLE 10

I^{131} Specific Activity in the LAF_1 Mouse as a Function of Time After Injection

Iodoacetamide Injection Route	Time after Injection	Specific Activity (CPM/gm)		
		Liver	Spleen	Tumor
IV	5 min	420	459	464
IV	12 min	370	500	599
IV	20 min	304	441	468
IV	30 min	340	381	619
IV	90 min	222	329	360
IV	120 min	397	442	667
IV	240 min	435	514	734
IP	1 min	1084	1529	
IP	2 min	886	1140	
IP	5 min	797	922	
IP	10 min	516	1010	
IP	20 min	624	911	

that the concentration in the subcutaneous tumor was equal to or greater than that in the liver and spleen.

5. The TD_{50} of Tumor Cells Treated with Radiation and Iodoacetamide

The experiments described below were done in collaboration with Jose Feola of this laboratory. L2 lymphoma cells were injected IP into female LAF_1 mice. Dilutions were made into Medium 199 with Hanks balanced salt solution buffered with $NaHCO_3$ (Microbiological Associates, Bethesda, Md.). The medium was cooled in an ice-water bath before and during use. Results of an experiment in which 0.37 mg iodoacetamide and 250 R Co^{60} exposure were administered to tumor bearing hosts are shown in Table 11. The hosts were sacrificed immediately after treatment and cells were harvested, diluted and injected into recipients within approximately 1 hour of the treatment. Cells were injected in a 0.10 cc volume into experimental groups consisting of 10 animals. TD_{50} and the 95% confidence interval obtained from this data are as shown in Table 11. Table 12 gives the results of TD_{50} determination experiments for 0.37 mg iodoacetamide used with a 500 R radiation dose.

Table 13 gives a summary of the TD_{50} values of Tables 11 and 12 normalized to the TD_{50} of the control group for each experiment. The inverse of these values should be a measure of the surviving

TABLE 11

The TD_{50} of L2 Cells Treated with 0.37 mg Iodoacetamide and 250 R

Treatment	# Cells Injected	Survivors	TD_{50} (95% CI)
Control	0.8	8	
	4.0	2	3 (1 - 8)
	20	2	
	100	0	
IA	5	3	
	25	3	12 (6 - 25)
	125	4	
	625	1	
250 R	2	7	
	10	5	10 (5 - 20)
	50	2	
	250	1	
250 R + IA	60	6	
	300	4	135 (64 - 285)
	1500	0	
	7500	0	

TABLE 12

The TD_{50} of L2 Cells Treated with 0.37 mg Iodoacetamide and 500 R

Treatment	# Cells Injected	Survivors	TD_{50} (95% CI)
Control	0.4	10	
	2.0	10	
	10	5	10 (3 - 30)
	50	1	
	250	0	
IA	0.4	10	
	2.0	10	
	10	8	110 (50 - 610)
	50	9	
	250	4	
500 R	4	10	
	20	7	
	100	3	70 (35 - 190)
	500	3	
	2500	0	
500 R + IA	8	10	
	40	10	
	200	9	1000 (330 - 3000)
	1000	5	
	5000	1	
	25000	0	

fraction of competent cells (see Introduction). The surviving fractions appear in the third column.

TABLE 13

Surviving Fraction Obtained by the TD_{50} Method

Treatment	Normalized TD_{50} (Cells)	Surviving Fraction
Control	1.0	1.000
IA	4.0	0.250
250 R	3.3	0.300
250 R + IA	45	0.022
Control	1.0	1.000
IA	18	0.056
500 R	8	0.125
500 R + IA	100	0.010

The data of Table 13 indicate that the presence of iodoacetamide at the time of radiation reduced the surviving fraction of cells by a factor of approximately 13 for both doses. It is also clear that iodoacetamide as used was of itself toxic to the L2 cells. There appears to be some considerable difference in the magnitude of this effect in these two experiments. We have found quite a variation in drug effect in other studies as well.

The data of Table 13 indicate the possibility of a synergistic effect between iodoacetamide and radiation. One would expect that the result of using two agents together which act independently would be a surviving fraction equal to the product of the surviving fractions which result from the use of each agent singly. On this basis, one would expect a surviving fraction of 0.075 for the use of 250 R + iodoacetamide and one of 0.007 for 500 R + iodoacetamide. The actual value for 250 R + iodoacetamide is some 3 times smaller and that for 500 R + iodoacetamide is 1.5 times larger than the values one might predict. Thus, at the least, the agents appear to be independent in their mode of action, and if the data at 250 R is the more typical of that to be expected, then a synergism exists. A further indication of synergism would be a smaller surviving fraction of cells resulting when cells are irradiated in the presence of iodoacetamide than results when the compound is supplied after irradiation. Such studies were not done with the TD_{50} method. They were done, however, with the total cell assay method as reported in the next section.

6. Results Obtained by Measurement of Total Tumor Cell Number

Growth Curves

Figure 3 shows some typical results of measurement of total L2 tumor cell number in LAF_1 hosts which received the indicated treatment. All cell populations are normalized to that expected from an initial cell population of 1.48×10^7 cells. This number of cells was

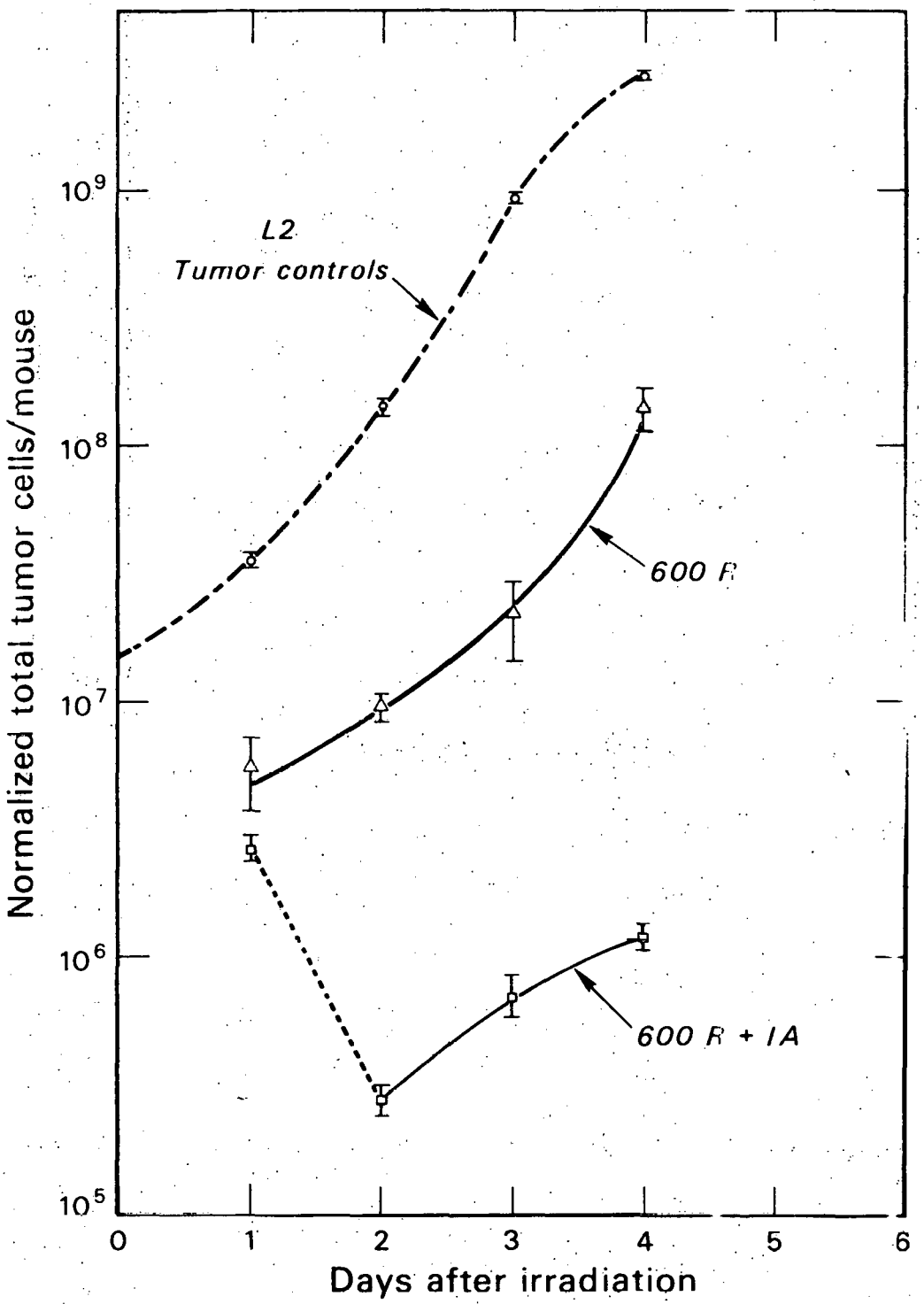


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Figure 3

Typical L2 Cell Growth Curves

in fact the number given to most experimental groups. Exceptions to this are groups that would be expected to yield populations in stationary phase at the time of examination if 1.48×10^7 cells had been the actual initial population. The control groups of mice yielding data on days 3 and 4 are typical exceptions. They were given 1.48×10^6 cells which grew to actual populations of 0.15×10^7 and 2.74×10^8 cells. These values are multiplied by 10 to estimate what would have been the result of exponential growth of 1.48×10^7 cells were it not for the limits set by the onset of stationary phase. In spite of this adjustment in initial population, the datum of control cell population at day 4 shows that those cells had begun entry into stationary phase.

Figure 4 shows data obtained with TA3 tumor cells. These data are normalized to those expected from an inoculum of 7.80×10^6 cells. A significant difference exists between data from animals given iodoacetamide before irradiation and data from animals given a post-irradiation injection of iodoacetamide. This is strong evidence of a synergism between radiation and iodoacetamide present at the time of radiation.

Survival Curves

If data such as that presented in Figures 3 and 4 are obtained at several radiation doses, one can plot survival curves such as those of

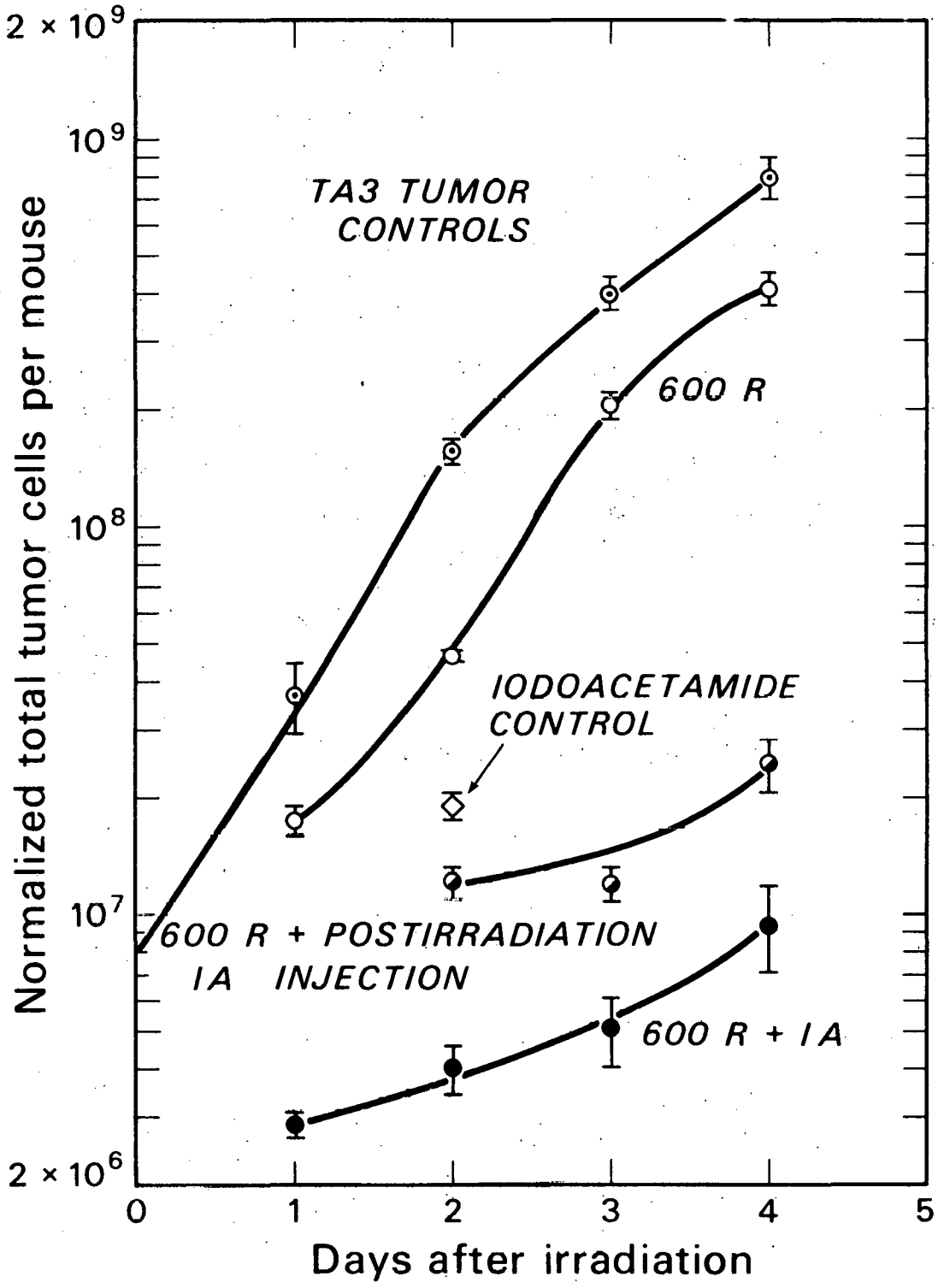

Figure 4

Typical TA3 Cell Growth Curves

Figure 5. These curves were constructed with L2 tumor cell data obtained 3 days after treatment. Three days post treatment is an especially good time to assess cell number because it appears that all cells killed by the treatment have been removed at this time and less adjustment of initial cell population is required at three days than is the case at longer times.

Figure 5 shows quite clearly that a synergism exists between iodoacetamide and gamma radiation. The administration of 2 μmoles (0.370 mg) of iodoacetamide some 20 minutes after irradiation reduces the surviving fraction by a factor of 5 to 20 over that due to radiation alone with the greater reduction occurring at higher radiation doses. A further reduction by a factor of 2 to 5 is obtained if iodoacetamide is supplied 20 minutes before radiation exposure.

It is of interest to compare the surviving fractions of Figure 5 with those obtained by the TD_{50} method as shown in Table 13 of Section 5. Table 14 contains a comparison of the results of these two methods. Values at 250 R from the total tumor cell assay are estimates from extrapolation of the curves where necessary.

Table 15 presents the percent survival of L2 cells for several treatments as measured over the first four days following treatment. Figure 5 was constructed from the data shown for day 3. Errors are standard errors.

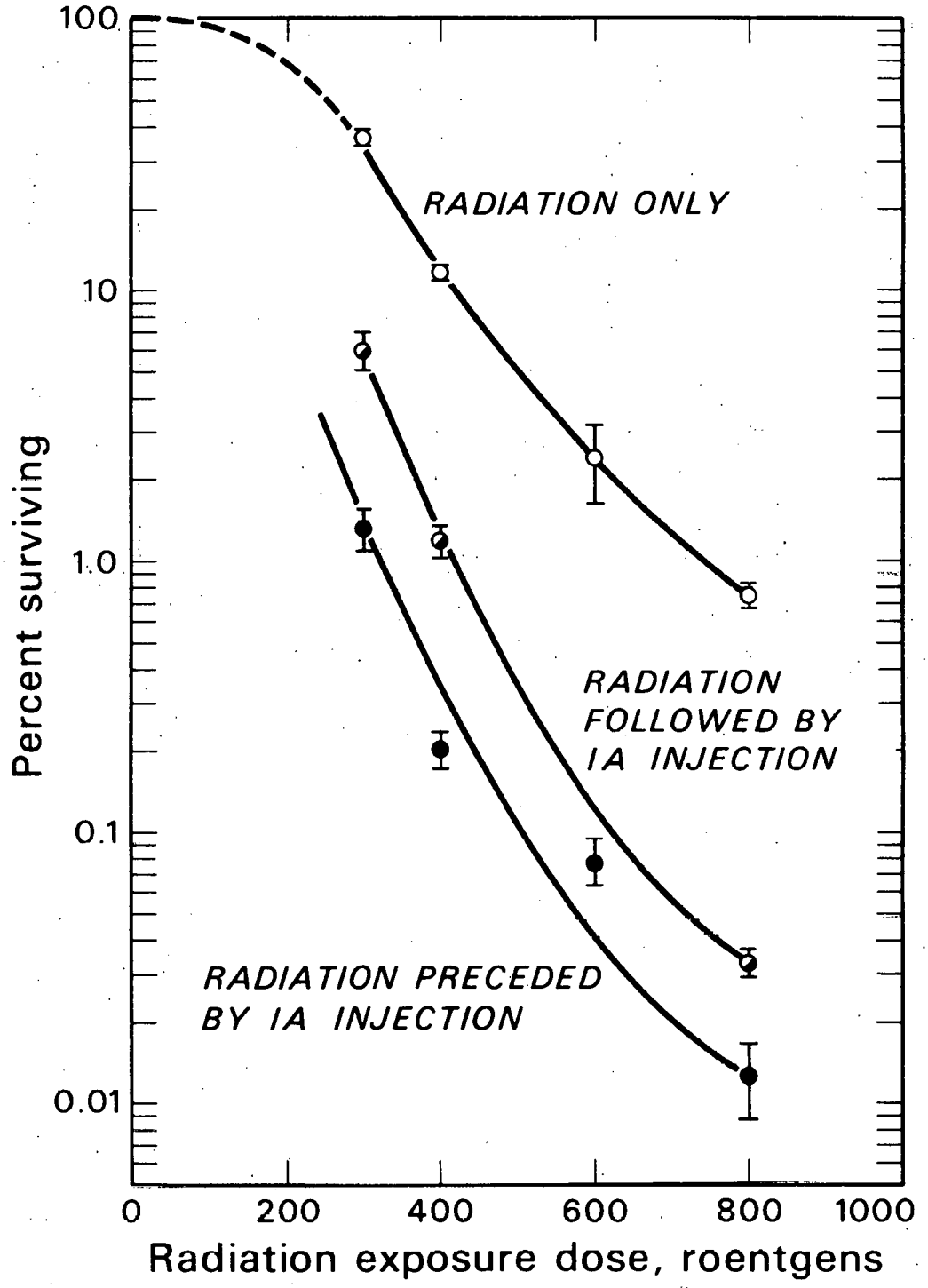

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Figure 5

L2 Cell Survival Curves

TABLE 14

A Comparison of Surviving Fractions Obtained by Two Assay Methods

Treatment	Surviving Fraction of L2 Cells	
	TD_{50} Method	Total Tumor Cell Method
Controls	1.000	1.000
250 R	0.30	0.5
500 R	0.125	0.047
250 R + IA	0.022	0.03
500 R + IA	0.010	0.010

TABLE 15

L2 Cell Survival as a Percent of Controls

Treatment	Percent Survival			
	Day 1	Day 2	Day 3	Day 4
300 R	63 ±4	50 ±3	36.8 ±2.8	45.2 ±3.7
300 R preceded by IA	16 ±1.5	2.3 ±0.3	1.32 ±0.23	3.96 ±0.20
300 R followed by IA	...	4.3 ±0.5	6.0 ±1.0	16.0 ±1.9
IA only	...	10.8 ±1.2	...	...
400 R	42.7 ±4.6	27.0 ±3.8	11.8 ±0.7	13.0 ±0.3
400 R preceded by IA	9.5 ±1.3	1.41 ±0.29	0.205 ±0.031	0.66 ±0.14
400 R followed by IA	...	1.88 ±0.19	1.20 ±0.16	3.42 ±0.23
IA only	...	9.82 ±1.88	22.4 ±1.9	35.9 ±4.0
600 R	15.2 ±4.6	6.72 ±0.86	2.41 ±0.83	5.06 ±0.98
600 R preceded by IA	7.47 ±0.86	0.201 ±0.032	0.0787 ±0.0154	0.044 ±0.005
600 R followed by IA	...	...	...	...
800 R	26.9 ±3.3	1.39 ±0.29	0.754 ±0.080	...
800 R preceded by IA	6.40 ±1.35	0.122 ±0.008	0.013 ±0.004	...
800 R followed by IA	...	0.570 ±0.244	0.034 ±0.004	...
IA only	...	8.30 ±1.99	11.4 ±8.13	...

The data of Table 15 show evidence of synergism between iodoacetamide and radiation at a variety of doses and times. Another item of interest is the consistent decrease in surviving fractions with time for all treatments but that of iodoacetamide only. The change with time seems to disappear by day 3. This may be attributed to the presence of a declining population of damaged or dead cells which are removed or disappear by the third day and make no further contribution to the total cell number. Cells treated with iodoacetamide alone exhibit a surviving fraction which increases with time in a trend which is opposite to that of all other treatments. An especially rapid removal of cells damaged by iodoacetamide might explain the precipitous initial decrease in surviving cells but not the subsequent increase in surviving fraction with time.

The Oxygen Effect - In Vivo Studies

One of the most famous phenomena in radiation biology is the oxygen effect. It is observed that cells are more sensitive to radiation while well supplied with oxygen than when in a state of anoxia. Tumor cells are usually anoxic when present in large numbers and are for this reason resistant to radiation therapy. Ascites tumors are typical in this respect. Table 16 presents evidence showing this to be the case. The growth of cells known to be anoxic is compared with normal tumor cells in this table. The notation, anoxic, refers to cells irradiated

TABLE 16

A Comparison of the Response of Anoxic and Normal Ascites Tumor Cells to In Vivo Treatment with Radiation and Iodoacetamide

Treatment	Population Multiple	Surviving Fraction
Normal Controls	46.3 ± 1.2	1.000
Anoxic Controls	50.5 ± 1.9	1.000
Normal 600 R	13.1 ± 0.4	0.353
Anoxic 600 R	13.8 ± 0.4	0.273
Normal 600 R preceded by IA	6.70 ± 0.92	0.145
Anoxic 600 R preceded by IA	7.95 ± 0.50	0.157
Normal 600 R followed by IA	10.8 ± 0.3	0.233
Anoxic 600 R followed by IA	11.3 ± 0.5	0.224
Normal IA only	11.7 ± 0.5	0.253
Anoxic IA only	11.0 ± 0.3	0.218

while resident in the peritoneal cavity of mice which were killed in an ether jar 30 minutes before irradiation. Normal cells are cells which were irradiated while resident in living hosts. In all cases the hosts contained a TA3 population consisting of more than 10^8 cells. The iodoacetamide dose was 0.370 mg in 1 cc of isotonic saline and the radiation dose was 600 R where indicated.

The second column contains cells population data which are normalized to the actual number of injected cells and which therefore represent the multiple of the initial population present at the time of measurement. Errors are standard errors. The last column gives surviving fractions relative to the control values. Measurements of the TA3 cell population were made on the third day after treatment.

The data of Table 16 show no difference in radiosensitivity between normal and anoxic cells indicating that the normal cells were indeed anoxic. The data also show that a synergism exists between radiation and iodoacetamide in an anoxic system since the surviving fraction of cells irradiated in the presence of IA is lower than that of cells given IA following irradiation. These data on TA3 cells give a surviving fraction of about 0.3 for cells receiving 600 R whereas Figure 5 indicates a value of about 0.03 for the same dose given L2 cells. An examination of Figures 3 and 4 will show that the response of TA3 and L2 cells are similar under identical conditions. The explanation for the factor of 10 difference in surviving fraction in these

two experiments lies with the oxygen effect. The dose at which a surviving fraction of 0.3 exists in Figure 5 is roughly 300 R. Then the dose modifying factor computed at a surviving fraction of 0.3 for the conditions of anoxia versus those of Figure 5 is 2. This value is rather typical of that obtained from a comparison of the radiation response of anoxic and oxygenated cells indicating that the data of Figure 5 was obtained under oxygenated conditions. The oxygenated state of cells used to obtain the data of Figure 5 is due o their small populations at the time of treatment. While the cell po ulations in animals used to obtain data for Table 16 was in excess of 10^8 cells, populations used for Figure 5 did not exceed 10^7 cells. Jose Feola (90) has informed me that the dose response curves of these cells undergo a change from that expected of oxygenated cells to that expected of anoxic cells at a population of about 10^7 cells.

The Oxygen Effect - In Vitro Studies

Table 17 presents data obtained on the subsequent growth of TA3 cells treated in vitro. Cells were obtained from host animals and were placed into plastic tubes at a cell concentration of 0.821×10^8 cells/cc in the case of cells which received either no IA or IA before irradiation and a cell concentration of 1.642×10^8 cel s/cc when the cells were to receive IA after irradiation. The cell concentration of groups receiving no IA was achieved by a 1:1 dilution of cells at

TABLE 17

In Vitro Studies with Oxygenated and Anoxic TA3 Cells

Treatment	Population Multiple	Surviving Fraction
Controls (in nitrogen)	46.6 ± 6.4	1.000
O_2 + 600 R	13.4 ± 0.9	0.287
N_2 + 600 R	29.9 ± 2.4	0.641
O_2 + 600 R preceded by IA	0.662 ± 0.10	0.0142
N_2 + 600 R preceded by IA	5.41 ± 0.77	0.116
O_2 + 600 R followed by IA	1.77 ± 0.23	0.0379
N_2 + 600 R followed by IA	6.91 ± 0.69	0.148

1.642×10^8 cells/cc with isotonic saline. The cell concentration of cells receiving IA before irradiation was achieved by a 1:1 dilution of cells with isotonic saline containing 2×10^{-4} molar IA. The result of dilution was a cell suspension containing IA at 10^{-4} molar. This concentration was selected since it simulates a 20 gram mouse with 0.370 mg (2×10^{-6} moles) of IA uniformly distributed throughout the body. (See Section 4 of this chapter for a discussion of IA distribution.) Following irradiation, cells which were to receive a post-irradiation exposure to IA were diluted 1:1 with isotonic saline containing 10^{-4} molar IA.

As has been already shown, ascites cells at high concentrations are anoxic. This is the case because their potential demand for oxygen is greater than that available to them. Cells added to the plastic vials were expected to quickly deplete their environment of oxygen. It was the purpose of this investigation to compare the effects of treatment of anoxic cells with those observed with oxygenated cells. Certain vials were therefore maintained in an anoxic state by means of nitrogen gas bubbled into the suspension at a rate of about 5-10 bubbles of 2 mm diameter per minute. Oxygenation of cells was accomplished by bubbling oxygen gas at the same rate into the appropriate vials.

After treatment, the cells were injected IP into groups of LAF_1 mice and allowed to grow for three days. Cell populations were assessed at 3 days after treatment. The radiation dose used for the

data of Table 17 was 600 R delivered in 26 minutes. All treatments were conducted at room temperature.

Table 18 presents data obtained with the methods used for the data of Table 17 but with a radiation exposure of 1000 R of Co^{60}. The radiation was delivered to the cells in 44 minutes. Populations were assessed 4 days after treatment.

The data of Tables 17 and 18 again show both the oxygen effect and that a synergism exists between IA and radiation. These data indicate that the magnitude of the synergism is larger with oxygenated cells than with anoxic cells but it is present in both conditions. Cells given IA only had a higher surviving fraction than observed in in vivo experiments. This may indicate that the distribution of IA is not actually uniform in a mouse but that the concentration in the peritoneal cavity (which is the injection site) remains higher than in other tissue thereby producing greater toxicity to the cells than would result with the presumed uniform 10^{-4} molarity.

There are two alternate possible explanations for the different IA toxicity that are also consistent with the data which indicates a uniform distribution of IA in the mouse. Firstly, it may be that the additional toxicity in the in vivo experiments is due to the initial molarity of 20×10^{-4} molar which is present in the 1 cc of isotonic saline injected. If so, the brief exposure to this concentration must be responsible for the additional cell death observed with IP injections. The second

TABLE 18

In Vitro Studies with Oxygenated and Anoxic TA3 Cells

Treatment	Population Multiple	Surviving Fraction
Controls (in nitrogen)	206 ± 13	1.000
O_2 + 1000 R	13.1 ± 0.7	0.0636
N_2 + 1000 R	33.6 ± 0.8	0.163
O_2 + 1000 R preceded by IA	7.04 ± 0.62	0.0342
N_2 + 1000 R preceded by IA	28.7 ± 0.7	0.139
O_2 + 1000 R followed by IA	11.7 ± 0.7	0.0568
N_2 + 1000 R followed by IA	26.0 ± 1.3	0.126
O_2 + IA	143 ± 5	0.694
N_2 + IA	103 ± 5	0.500

possible consideration is the fact that after being incubated in IA for times of about 1.5 to 2 hours, the cells treated in vitro were injected into host animals. A volume of 0.1 cc containing cells in 10^{-4} molar IA was injected. Subsequent to the injection, a dilution of the IA by a factor of up to 200 should have occurred thereby possibly reducing the toxicity of IA to the TA3 cells.

The Influence of Dose Rate

A comparison of the effects of IA at low and high dose rates yielded the data presented in Table 19. The dose rates selected were 1.0 and 36 R/min. Comparisons were made at a total exposure of 600 R. Table 19 presents the data obtained at 600 R in each case and also data obtained at 1000 R total exposure delivered at 1 R/min. L2 cell populations were assessed at both 2 and 5 days after treatment.

The data of Table 19 quite clearly show a dose rate effect. Delivery of 600 R at 1.0 R/min results in a surviving fraction which is about twice as large as that obtained at 36 R/min. This finding is in agreement with that of Berry and Cohen (91) obtained using the TD_{50} method.

Elkind and Whitmore (92), while expressing belief in the existence of a dose rate effect, took issue with the methodology of Berry and Cohen. Their objection was that apparently Berry and Cohen were irradiating a population which was becoming anoxic with time. This

TABLE 19

The Effects of IA at Low and High Dose Rates

Treatment	Dose Rate	Day of Examination	Population Multiple	Surviving Fraction
Controls		2	62.3 ± 6.0	1.000
600 R	36 R/min	2	7.59 ± 0.37	0.122
600 R preceded by IA	36 R/min	2	0.594 ± 0.263	0.00954
600 R	1.0 R/min	2	13.2 ± 0.8	0.212
600 R preceded by IA	1.0 R/min	2	3.13 ± 1.77	0.0502
1000 R	1.0 R/min	2	8.69 ± 0.72	0.139
1000 R preceded by IA	1.0 R/min	2	0.465 ± 0.104	0.00746
Controls		5	2500 ± 430	1.000
600 R	36 R/min	5	156 ± 14	0.0624
600 R preceded by IA	36 R/min	5	104 ± 18	0.0416
600 R	1.0 R/min	5	294 ± 10	0.117
600 R preceded by IA	1.0 R/min	5	181 ± 23	0.0724
1000 R	1.0 R/min	5	245 ± 8	0.0980
1000 R preceded by IA	1.0 R/min	5	78.1 ± 7.0	0.0312

problem arose due to the long exposure times during which the cell population was increasing. Exposure times were as long as 4.5 days and exposures were not begun until cell populations were at least 10^7 cells. Populations of this size and larger should exhibit evidence of anoxia.

The low dose rate data of Table 19 were obtained from cells which had received 600 R in 10 hours or 1000 R in 16.7 hours. L2 cells have a doubling time in our hands of about 10 to 11 hours. Hence, the population did not increase by more than a factor of 3 during exposure. Since radiation is known to inhibit mitosis, the actual increase could have been much less. In any case, changes in oxygenation should have been slight. The initial population of cells assessed on day 2 was 1.37×10^7 cells; a number large enough to indicate low and possibly decreasing oxygen tension during exposure. In the case of cells assessed on the 5th day after treatment however, the initial population was only 1.37×10^6 cells. This population should have been well supplied with oxygen throughout the irradiation exposure. A dose rate effect is evident with this data.

Table 19 shows that the presence of IA reduces cell survival in both low and high dose rate situations. The magnitude of the effect appears to be quite variable in these experiments. It is interesting that there is no trend evident which might indicate a metabolic process working to render IA non-functional in a time period comparable to the exposure times.

7. Discussion of IA Studies

We have shown iodoacetamide to be a radiation sensitizer in a mammalian in vivo system. This conclusion is reached with data obtained with both TD_{50} and total tumor cell assay methods. IA is effective under conditions which can reasonably be expected to duplicate those found in clinical situations. Iodoacetamide is effective with both anoxic and oxygenated cells and appears to be effective for both low and high dose rates. The compound has easy and rapid access to many if not all tissues of the body.

The underlying mechanism involved in the sensitizing action of IA is not known with certainty. However, Dewey and Michael (93) have made some observations which implicate free radical formation as of importance. These authors used a unique experimental set-up to study the time relationships involved in obtaining radiosensitization of bacteria by IA. They used a 1.8 Mev linear accelerator capable of delivering 3 to 4 kR in 2 μseconds. The results of their study were:

1. Addition of IA to the bacteria only 3 msecond before irradiation was as effective as addition of IA hours before exposure.
2. Addition of IA to the bacteria 3 msecond after irradiation resulted in no sensitization.
3. Addition of bacteria to IA only 3 msecond after irradiation of the IA produced an effect equal to that attributed to sensitization by IA when it is present with bacteria during irradiation.

4. Addition of cysteine within 10 msecond of irradiation of bacteria with IA present virtually abolished the effect. Some protection was seen when cysteine was added at 100 msecond after irradiation.
5. Irradiated IA retained its activity up to 100 seconds after irradiation.

These results suggest that irradiation of IA produces a free radical of long life which is effective at killing cells. The fact that cysteine protects against IA indicates that it is scavenging such free radicals. If free radical formation is the mechanism of sensitization, then one would expect some recombination to occur thereby reducing the magnitude of the effect from that possible in the absence of this reaction. Assuming that irradiation of IA yields 2 or more product radicals, one of which is ineffective at sensitization but which participates in recombination reactions, then removal of this radical should increase the effectiveness of IA as a sensitizer. Mullenger et al. (94) have studied radical competition with IA acting on bacteria. They found that KNO_3 produced an increase in sensitization by IA. Presumably, this is due to the known ability of nitrates to scavenge the solvated electron. Preliminary in vivo experiments by us indicate that KNO_3 may enhance radiosensitization of tumor cells. KNO_3 has the virtue of being quite free of toxic effects.

Further studies should be done with KNO_3 in vivo; but regardless

of the outcome of such experiments, IA has already been shown to have potential usefulness in clinical radiotherapy.

CHAPTER IV

SOME OBSERVATIONS ON IN VIVO TUMOR CELL POPULATION ASSAY METHODS

A typical tumor growth curve has been presented in Figure 1 of the Introduction. The curve shows the population of cells as a function of time. For the purposes of the research presented in this thesis, it was desirable to use only the logarithmic portion of the curve. At times, certain difficulties arose which frustrated attempts to work only with cells in log phase growth. This section is a report on the factors which were found to affect the position of the lower boundary of the log phase portion of the curve.

The Size of the Initial Inoculum

In the course of the performance of experiments of types already described, we found that cell growth kinetics were markedly altered if the initial inoculum was less than 10^5 cells in number. With inocula of this size, the cell population could not be predicted by an exponential extrapolation of the original cell number. Rather, the growth curves could be described as consisting of a lag phase followed by exponential growth. The duration of the lag phase was found to be a function of the cell number in the inoculum; increasing with decreasing cell number and usually resulting in essentially no takes when the inoculum was below 10^3 cells.

Differences in Technique

The failure of cells to produce takes for inocula of fewer than 10^3 cells is at variance with results obtained by Jose Feola (95) who has routinely obtained TD_{50}'s of less than 10 cells for both the L2 and TA3 cell lines and has tested the cells as carried by us with the same values being obtained. One salient difference in technique existed between us. Jose Feola was injecting cells which were held at approximately 0°C in Medium 199 with Hank's balanced salt solution and sodium bicarbonate (Microbiological Associates Inc., Bethesda, Md.). We, however, were injecting cells held at approximately 23°C in isotonic saline.

The Medium

We have investigated this matter by comparing the growth of cells held in saline with that found for cells held in Medium 199. Cells were held at room temperature (23°C). The results are shown in Table 20. In this table, the medium used is denoted by S if it was isotonic saline and by M if Medium 199. The term Day of Examination refers to the day on which the tumor cell population was assessed as reckoned from the time of injection of the initial population. Population multiple is the number of times the initial population had multiplied itself when assessed. In log phase growth, this value should increase as an exponential with time, and should be independent of the absolute

TABLE 20

The Influence of Holding Medium Upon TA3 Cell Kinetics

Number of Cells Given	Medium	Day of Examination	Cell Population $\pm$SE x 10^{-7}	Population Multiple
9.65×10^6	S	2	8.09 ± 0.22	8.45 ± 0.23
8.43×10^6	M	2	6.66 ± 0.17	7.91 ± 0.20
9.56×10^5	S	3	6.13 ± 0.36	64.1 ± 3.8
8.42×10^5	M	3	4.41 ± 0.35	52.1 ± 4.2
4.78×10^5	S	4	8.40 ± 0.24	176 ± 5.0
4.21×10^5	M	4	7.78 ± 0.18	185 ± 4.3
9.56×10^4	S	5	0.97 ± 0.52	102 ± 54
8.42×10^4	M	5	0.78 ± 0.28	93 ± 33
9.56×10^4	S	7	1.04 ± 0.64	109 ± 67
8.42×10^4	M	7	0.88 ± 0.42	104 ± 50
4.78×10^4	S	10	2.46 ± 1.91	515 ± 400
4.21×10^4	M	10	0 0	0 0
4.78×10^4	S	12	0 0	0 0
4.21×10^4	M	12	0 0	0 0

value of the initial population and all other variables including the type of medium used. Table 20 and Figure 6 which is constructed with data selected from Table 20 indicate that indeed, the medium used did not influence the result but that the growth was not exponential when inocula of less than 10^5 cells were given in either medium.

Figure 6 shows an approximately exponential growth for times up to 4 days after which the data markedly depart from an exponential curve. This departure is a response to the fact that animals examined after the fourth day had received less than 10^5 cells in their inocula. This fact may be more clearly seen in a comparison of the data of Table 20 obtained on day 5. The population of cells in animals which had received about 4×10^5 cells had grown to stationary values while the population in animals which had received one-fifth as many cells (about 9×10^4) had fallen short of the level expected in exponential growth by a factor of 10! All data obtained from animals given injections of still fewer cells also exhibited this failure to grow in an exponential manner.

The Effect of Temperature

After ascertaining that cell growth was not appreciably influenced by the nature of the injection medium, an investigation as to the effects of temperature was made. Isotonic saline was used as the medium for all injections. Groups of mice were given cells held at either $0^{\circ}C$

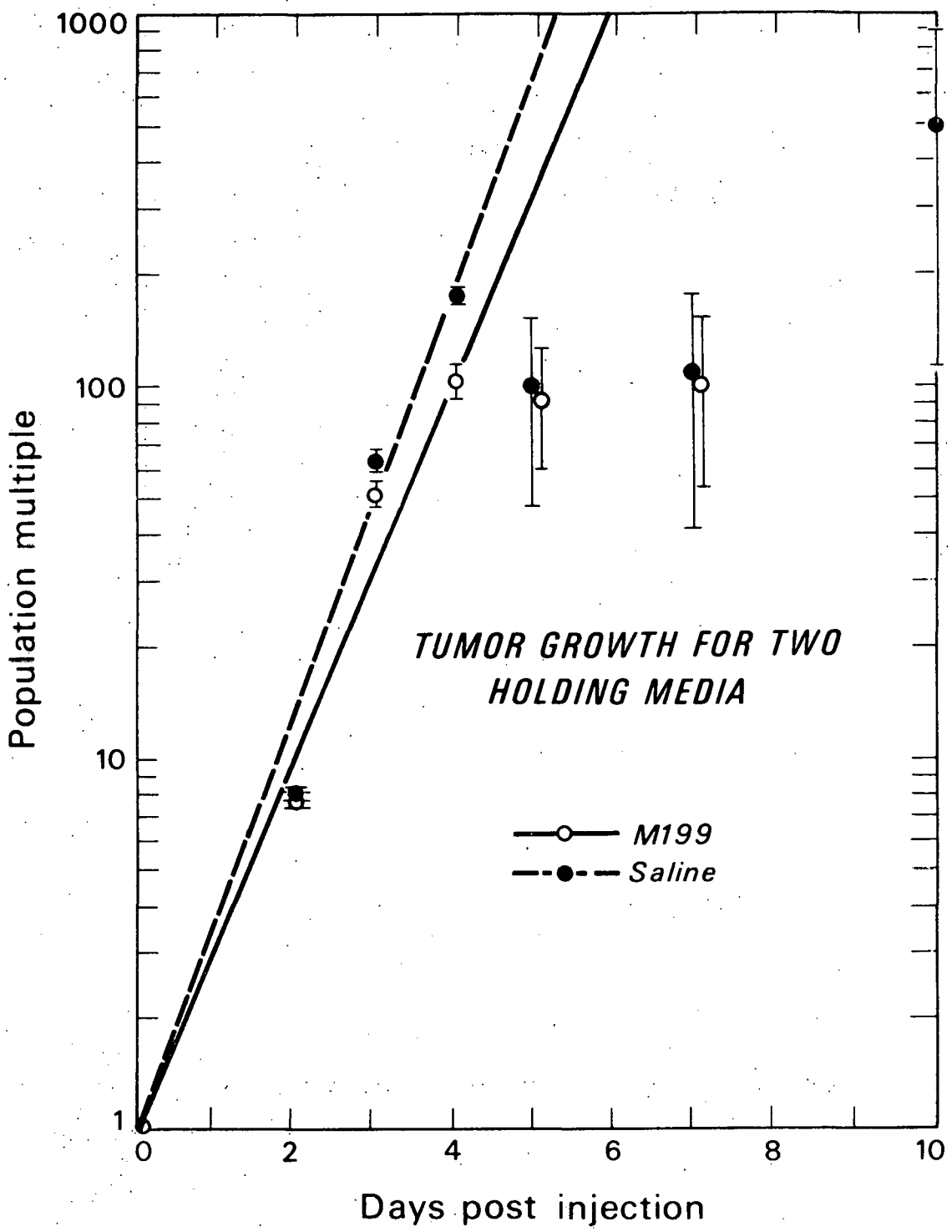

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Figure 6

Tumor Growth for Two Holding Media

(ice-water bath) or 23°C (room temperature). The results are given in Table 21 and Figure 7. There appears to be a departure from exponential growth evident in the data of days 10 and 13 obtained from cells incubated at 0°C. The initial cell numbers given were 544 and 54.4 cells respectively. These populations are considerably smaller than the population of cells incubated at room temperature for which a departure from exponential growth first becomes evident. In the case of cells held at 23°C, a departure is evident in the data of day 6. The initial population was 5.44×10^4 cells for this data. Hence holding cells at 0°C produced exponential growth from populations that were a factor of 100 times smaller than that required when cells were held at 23°C.

It might appear that the problem of obtaining exponential growth is solved simply by incubating cells at 0°C rather than room temperature. The temperature effect could be explained as being due to some deleterious metabolic process which occurs at room temperature and not at 0°C. However, cells do not normally function at 0°C in vivo but at 37°C which is approximated better by 23°C than by 0°C.

Pre-irradiation of the Host

An examination of the effect of host irradiation upon growth of TA3 cells injected one day after the 600 R irradiation yielded the data shown in Figure 8. All cells were incubated in saline at room

TABLE 21

TA3 Cell Population as a Function of Holding Temperature

Number of Cells Injected	Holding Temperature (Degrees Centigrade)	Day of Examination	Cell Population $\pm$SE x 10^{-7}	Population Multiple
5.44×10^6	0	2	4.63 ± 0.13	8.52 ± 0.24
5.44×10^6	25	2	4.29 ± 0.15	7.90 ± 0.28
5.44×10^4	0	6	10.26 ± 0.84	1890 ± 154
5.44×10^4	25	6	1.69 ± 0.88	311 ± 162
5.44×10^3	0	8	7.43 ± 0.83	$1.37 \pm 0.16 \times 10^4$
5.44×10^3	25	8	0 0	0 0
5.44×10^2	0	10	0.89 ± 0.34	$1.64 \pm 0.63 \times 10^4$
5.44×10^2	25	10	0 0	0 0
5.44×10^1	0	13	0 0	0 0
5.44×10^1	25	13	2.43 ± 1.17	$4.47 \pm 2.15 \times 10^5$

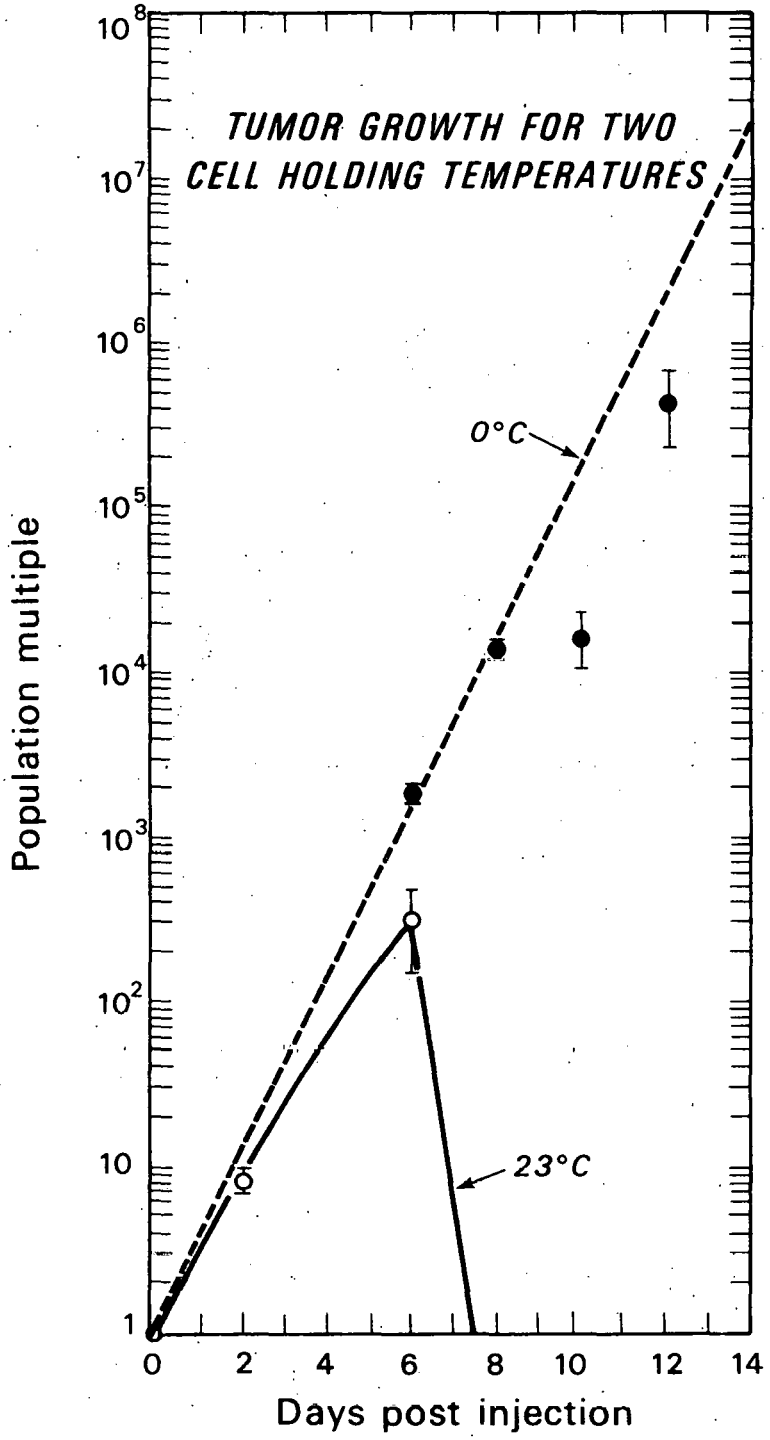

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Figure 7

Tumor Growth for Two Holding Temperatures

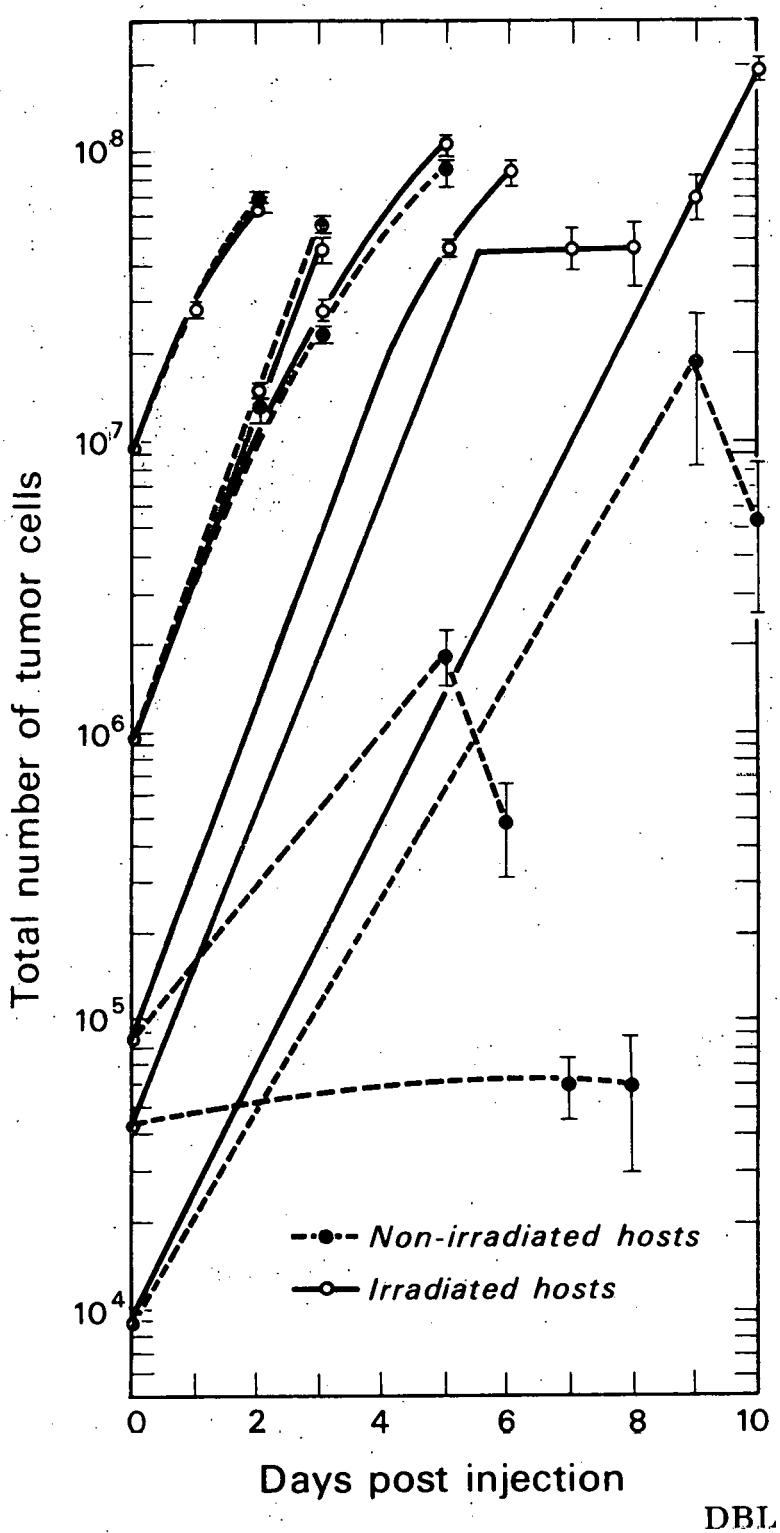

Figure 8

Growth of Cells in Hosts Given 600 R
One Day Before Inoculation

temperature for a time period ranging from about 30 minutes in the case of inocula of 8.7×10^3 cells to 240 minutes for inocula of 8.7×10^6 cells. The growth of cells in the two types of hosts is very similar for the two highest inocula. However, when inocula were below 10^5 cells, the difference in growth is striking.

When cells were injected into hosts immediately following irradiation, the results were as shown in Figure 9. In this case as well, the growth of cells in the irradiated hosts was much better than in the non-irradiated hosts for inocula of less than 10^5 cells.

Note the generally parallel slopes of all the growth curves obtained from cells in irradiated hosts. This indicates that the cell populations must have been growing at the same rate regardless of inoculum size, a necessary condition for exponential growth.

These data clearly show that incubation of cells at room temperature in saline is not of itself a sufficient condition to prevent exponential cell population growth. The effect of irradiation of the host upon subsequent cell growth was studied further as described below.

Ascites Fluids as Incubation Media

Proceeding on the theory that irradiation of the host might release some nutrient or nutrients which would be available to the TA3 cells and which would enable them to grow at lower initial populations than otherwise possible, a comparison of growth of cells incubated

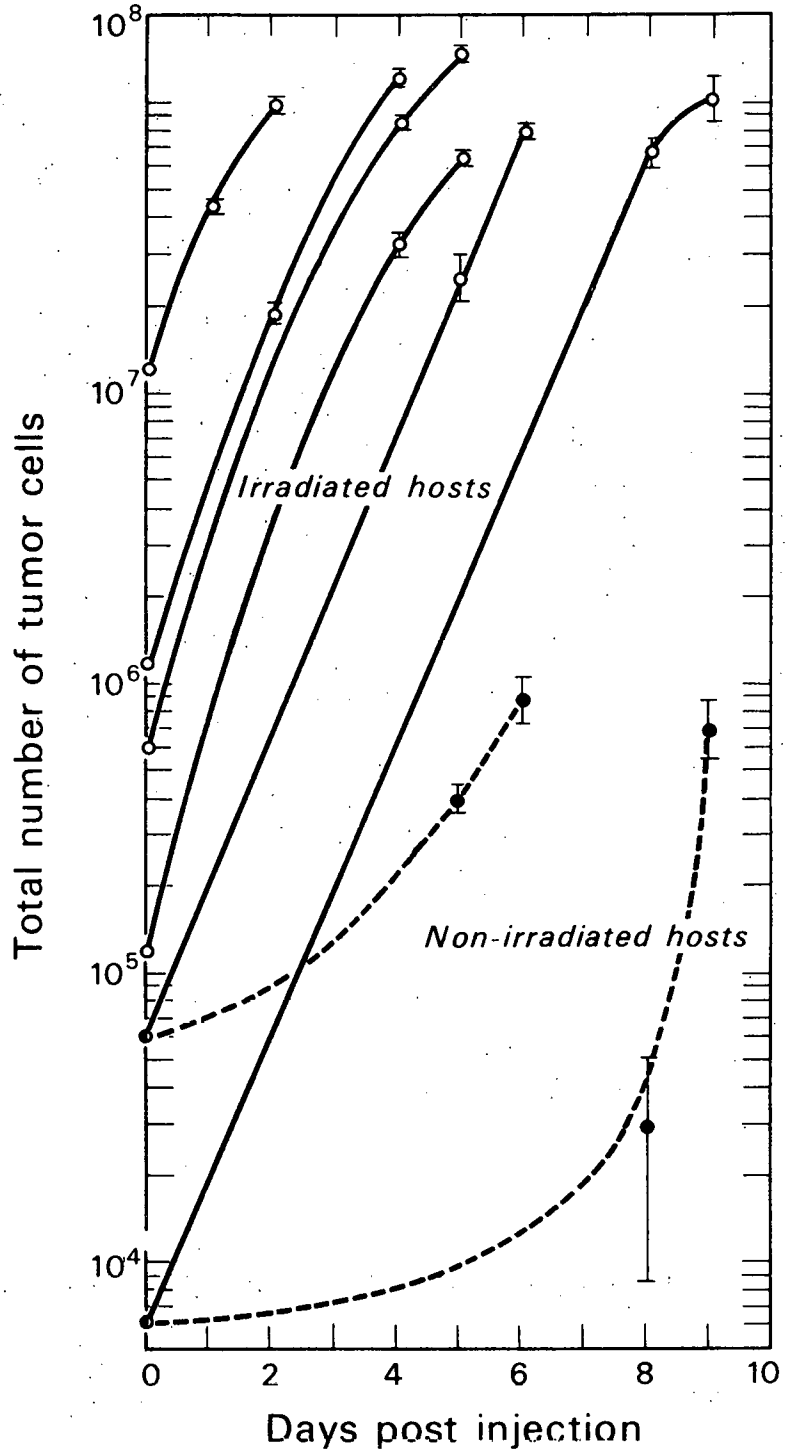

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Figure 9

Growth of Cells in Hosts Given 600 R
Just Before Inoculation

and inoculated in ascites fluid from irradiated hosts with that of cells incubated in ascites from non-irradiated hosts was done. The data appears in Figure 10. Ascites fluid was obtained from donors which had been given 10^7 cells IP six days before sacrifice. Some donors were exposed to 600 R one day prior to sacrifice. Cell free ascites fluid was obtained by 2 serial 20 minute centrifugations at 2500 g of the fluids obtained from the host animals. As is evident from Figure 10, when cells were then incubated in the ascites fluids at 23°C before inoculation, the result was that both fluids, the one from non-irradiated hosts and the other from irradiated hosts, were equally effective in promoting exponential growth of the TA3 tumor cells. Figure 11 presents the data of Figure 10 as the population multiple vs. time. Here is evidence of log phase growth over 5 decades of population.

Discussion of Factors Influencing Cell Growth

It would appear that a salt solution such as isotonic saline or Hank's solution is not an adequate medium for TA3 cells when they are incubated at room temperature. It is our impression that this condition is not peculiar to TA3 cells but is the rule for ascites tumors. The fact that a reduction in incubation temperature results in improved cell growth might be explained as being due to the reduced requirements for certain critical nutrients at lower metabolic rates.

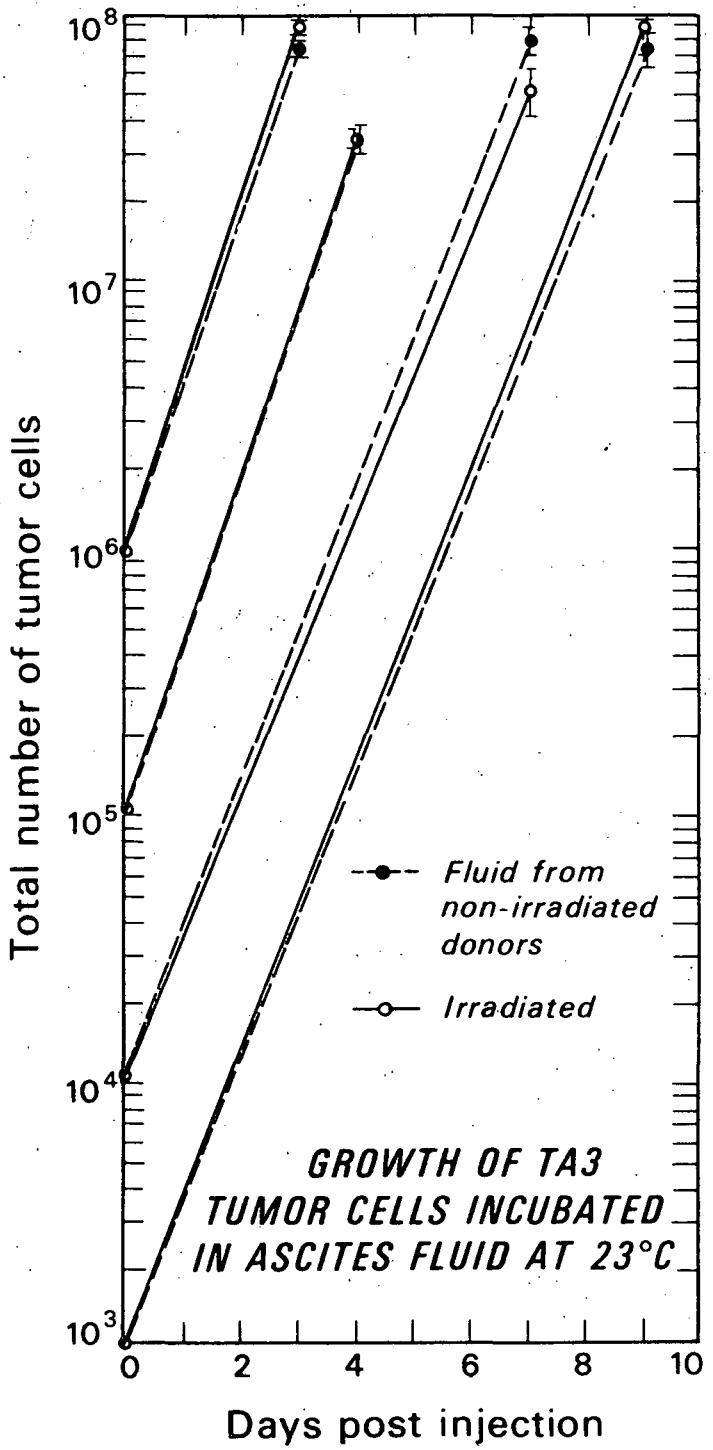

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Figure 10

Growth of TA3 Tumor Cells Incubated in Ascites Fluid at 23°C

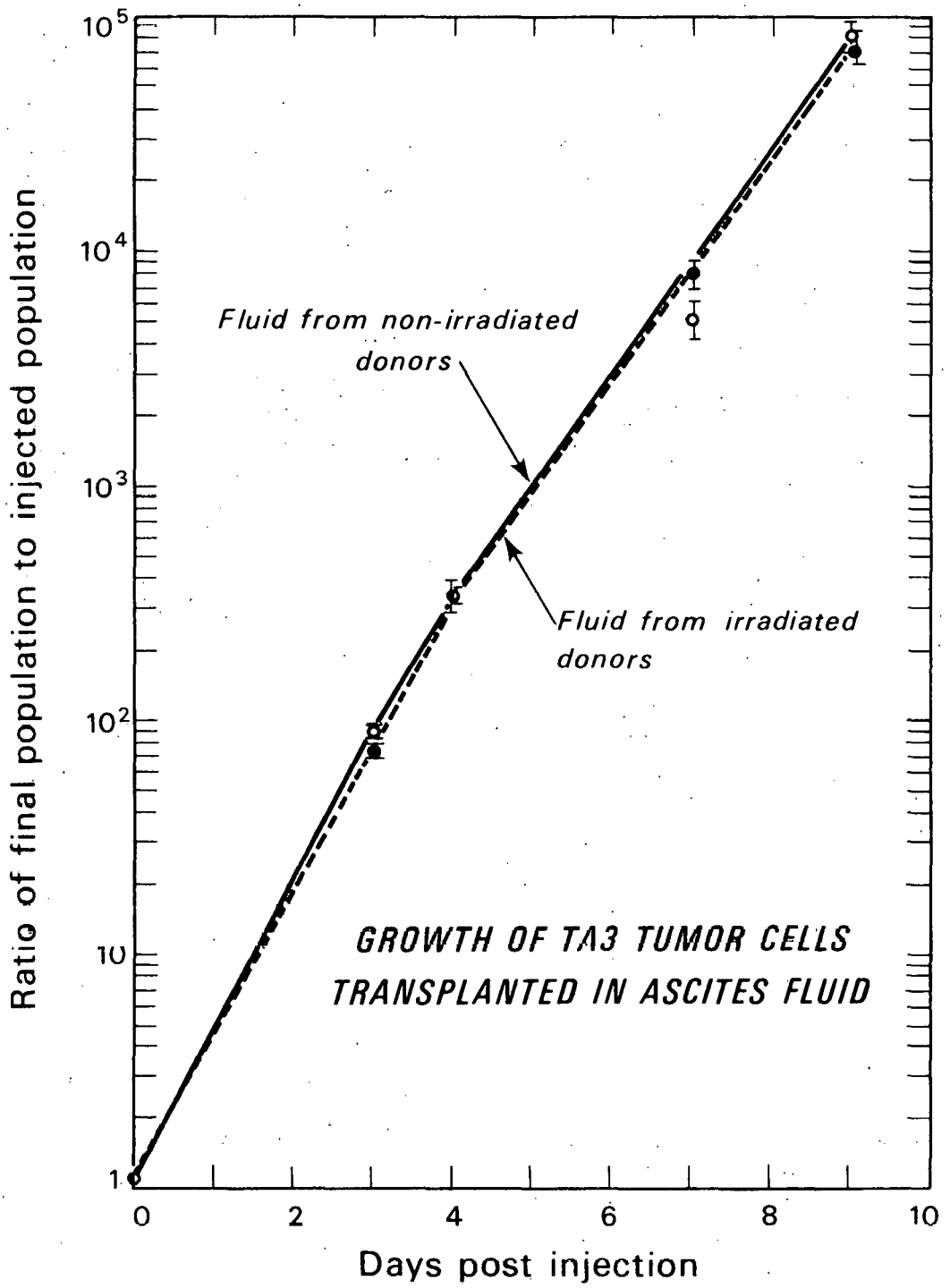

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Figure 11

Growth of TA3 Tumor Cells Transplanted in Ascites Fluid

However, we are inclined to think this is not the case. Pre-irradiation of the hosts results in excellent growth of cells incubated at room temperature and given in inocula below 10^5 cells. This fact indicates that a normal host can somehow recognize cells which are held at room temperature in a salt solution as being different in some way but that such cells are not deficient in their ability to carry on the metabolic processes requisite to growth and cell division. We believe that it is not likely that improved growth of cells in pre-irradiated hosts is due to a production of growth promoting substances as a result of host irradiation. If such were the case, we believe improved growth should have been manifest with cells incubated in ascites fluid from pre-irradiated donors over and above that seen for cells incubated in normal ascites fluid (Figure 10). No difference in growth was observed.

It is possible that our observations are related to those that have come to be called the "Hybrid Effect" (96, 97). Simply stated, the term "Hybrid Effect" refers to the observation that the minimum number of tumor cells required to produce a tumor in a F_1 hybrid host is greater than that required for tumor development in the parental strain in which the tumor arose. Several investigators have confirmed the existence of the effect and have speculated as to the mechanism involved. Hellstrom (98, 99) has examined the growth of a cell line which arose in the A x A.SW F_1 hybrid. He found that cells which were selected by one or more passages in either of the parental mouse

strains grew as well in the parental strain used for selection as had the unselected cells grown in the F_1 hosts. However, subsequent inoculation of F_1 mice with either of the selected lines revealed that the selected lines grew less well in the hybrid hosts than they did in the parental mouse strain used for selection. Hellstrom also found a "Hybrid Effect" for several cell lines of parental origin. Some of Hellstrom's coworkers have found no differences in the survival of skin grafts from homozygous mice transplanted to the same strain as compared to transplants to various genetically compatible F_1 hybrids (100).

Hellstrom reports that the difference between homozygous and F_1 hybrid mice was apparent both with regard to latency period preceding tumor appearance and total tumor frequency but that no certain differences were found in the growth rates of established tumors. In addition, Hellstrom found that exposure of mice to 540 R prior to tumor inoculation had no effect on the results of tumor inoculation.

Oth and coworkers (101, 102) did some similar experiments and generally confirmed the existence of the "Hybrid Effect" and Hellstrom's results. They found an exposure of 450 R to be relatively ineffective at influencing tumor growth in F_1 hybrids but found use of an exposure of 500 R or 550 R was effective in suppressing the "Hybrid Effect."

Sanford (103) has described the existence of a "Hybrid Effect" with a tumor of the Heston A mouse. The tumor used was the L#2

lymphoma. This tumor is in ascites form. Sanford used 10,000 L#2 cells in 0.2 ml Ringer's solution as the tumor inoculum. The result was that 100% of the A/HeHa mice developed a tumor but only about half of the F_1 hybrid A/HeHa x C3Hf/HeHa did so. However, when the F_1 hosts were pre-irradiated (dose not specified), 100% of them developed a lethal tumor. Sanford was able to show that (A/HeHa x C3Hf/HeHa) F_1 animals which rejected the 10,000 L#2 cells could resist a subsequent inoculation of up to 2×10^7 cells.

Sanford has reported a study made with TA3 cells which may explain the hybrid effect and our observations as well (104). She found that enzymatic removal of sialic acid from the heavy sialomucin cell surface coating present on TA3 cells reduced the number of lethal takes in allogeneic C3H hosts. Removal of sialic acid was accomplished using neuraminidase. Cells so treated produced no lethal takes in C3H hosts receiving 3000 cells IP whereas a 3000 cell inoculum of untreated cells resulted in $56 \pm 5\%$ takes. When A strain mice were used as hosts, the same inoculum produced a higher percentage of takes but neuraminidase treated cells still were less effective than untreated cells. Sanford also observed that an IV injection of neuraminadase into TA3 tumor bearing hosts reduced take percentage.

Sanford felt that simple destruction of tumor cells by neuraminidase was unlikely since syngeneic A strain mice regularly became distended after being injected with treated cells although regression

often occurred later. She postulated that her results were consistent with enzymatic removal of sialic acid from the cell surface increasing tumor specificity by exposing histocompatibility antigens previously concealed by sialomucin. In view of the fact that she presents evidence that her TA3 cells had undergone some genetic drift, some immune response by A strain mice to treated cells would be expected and could be manifest in the regression of tumors in these hosts.

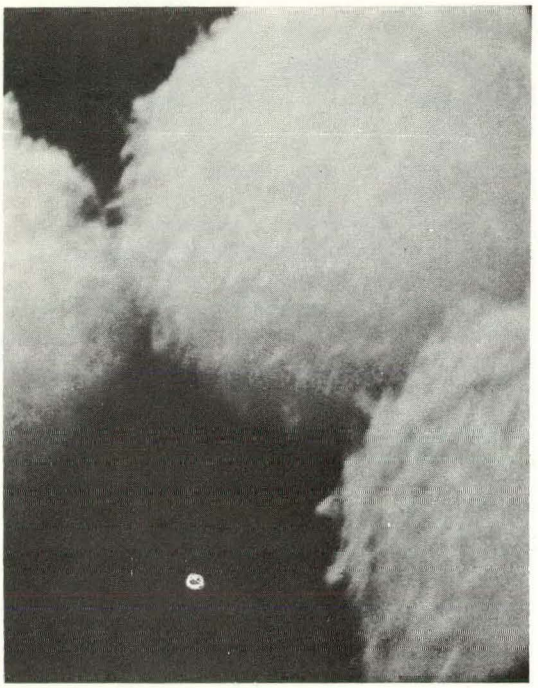
We feel that our observations would be consistent with a hypothesis like that offered by Sanford. Specifically, we postulate that dilution of cells into saline or a balanced salt solution causes the cells to lose some substance, perhaps sialic acid, from the surface thereby exposing histocompatibility antigens which were previously concealed when the cells were in the adequate medium of ascites fluid. Incubation at $0^{o}C$ somehow inhibits this loss of material. The observation that cells incubated in saline at room temperature grow quite well in irradiated hosts is due to the inability of the host to respond immunologically to histocompatibility antigens.

Electron Microscope Studies

We set about to look, in the most literal sense of the word, for changes in the cell membrane produced as a result of incubation in saline. Cells were prepared for scaning electron microscope viewing in the following way:

1. Cells were extracted from the peritoneal cavity of a donor which contained about 10^8 TA3 cells in 1 cc of fluid.
2. A 1:1000 dilution of 0.1 cc of ascites fluid was made.
3. After an incubation period of about 15 minutes at room temperature, smears were prepared of the diluted fluid and of the undiluted ascites fluid as well.
4. The smears were immediately placed in a petri dish containing a 2.5% solution of glutaraldehyde buffered with 0.1 molar Na Cacodylate and allowed to fix for 12 hours.
5. After fixation, the smears were dehydrated by exposure for 12 hours to each of the following sequence of solutions:
    A. 50% ethyl alcohol in H_2O
    B. 70% ethyl alcohol in H_2O
    C. 80% ethyl alcohol in H_2O
    D. 95% ethyl alcohol in H_2O
    E. acetone
    F. chloroform
6. When the smears were to be examined, they were removed from chloroform and air dried rapidly with a fan.
7. Gold was sputtered onto the slides just before viewing in order to improve contrast.

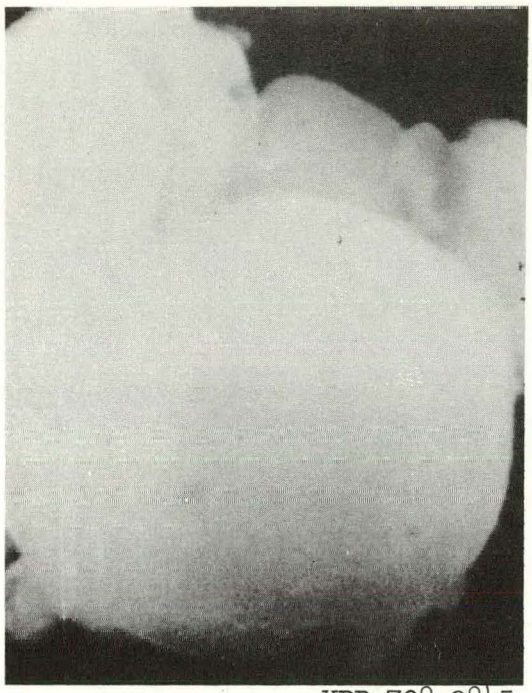
Samples of the pictures taken using a scanning electron microscope are presented in Figures 12 and 13. The cells in Figure



X 10,000

Figure 12

TA3 Cells Incubated at 23°C
in Ascites Fluid



XBB 708-3845

X 10,000

Figure 13

TA3 Cells Incubated at 23°C
in Saline

12 are typical of all cells seen in smears of undiluted ascites fluid and many cells of the 1:1000 dilution. A sizable minority of cells in the diluted fluid exhibited an appearance like that in Figure 13. No cells seen in the undiluted fluid had such an appearance.

It is not possible to directly relate the visual appearance of the TA3 cells with their fate subsequent to inoculation into a host, nor is it possible to correlate appearance with the surface antigens present and active on the cells. Nonetheless, it is interesting that a difference in appearance is evident with a difference in treatment which has influenced the fate of the cells. Such an observation is entirely consistent with our hypothesis of masked surface antigens.

Quantitation of Sialic Acid

We have looked for a difference in sialomucin coating on the surface of TA3 cells incubated at 0°C compared with that of cells incubated at 23°C in a 1:1000 diluation with saline. The result of neuraminidase treatment was identical for the two incubation temperatures. The yield of sialic acid as measured by the method of L. Warren (105) was 0.91 μmoles/10^9 cells for cells incubated at 23°C and 0.90 μmoles/10^9 cells when the temperature was 0°C.

The fact that results were identical does not necessarily indicate that the sialomucin coating was the same for both cell treatments. We observed that centrifugation of 3 liters of saline to extract

cells gave us 10 ml of fluid with an appearance much like that of the 3 ml of ascites originally added. Cell-free ascites fluid is known to contain a large concentration of sialic acid. Cook et al. (106) obtained a value of 200-300 μg/ml of Ehrlich ascites fluid which corresponds to 1 μmole/ml fluid. It then seems reasonable to assume that a major fraction of the sialic acid measured by us was from ascites fluid present with the cells following centrifugation. This assumption is supported by the data of Cook et al. (106) who obtained a value of about 0.13 μmoles/10^9 cells for washed cells. The presence of a large amount of sialic acid in the fluid could mask a significant change in that bound to cells.

The fact of the presence of such a large amount of sialic acid in ascites fluids may indicate the existence of some kind of equilibrium between sialic acid bound to the cells and acid in solution.

A recent review paper by Apffel and Peters (107) offers a postulate explaining specific tumor tolerance as being due to the formation of a complex on the cell membrane resulting in concealment of antigens and thereby preventing any immune response by the host. The authors present a large body of evidence in support of their postulate gleaned from many sources. We believe that our experience with tumor transplantation immunity provides one more piece of evidence that a condition similar to that postulated does exist in fact.

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TECHNICAL INFORMATION DIVISION
LAWRENCE RADIATION LABORATORY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA 94720