Studies on the Mechanism of Acquired Radioresistance in Cancer

ALAN D. CONGER AND HELEN J. LUIPPOLD

(Biology Division, Oak Ridge National Laboratory,* Oak Ridge, Tenn.)

It is a frequent clinical, and less frequent laboratory, observation that a tumor subjected to a number of sublethal irradiations will gradually acquire radioresistance (5, 11). The usual manifestation of acquired radioresistance by a tumor is the diminished amount of regression for the same dose, or the increased dose required for an equal amount of regression or for sterilization, as the radiation treatments continue. Three possible mechanisms to explain the phenomenon are commonly proposed. Firstly, the initial sublethal irradiation kills off all but the few resistant tumor cells in a heterogeneous population. Secondly, the initial irradiations induce a mutation toward radioresistance in a tumor cell, which is then selected for by subsequent irradiations. Thirdly, the initial irradiations cause changes of one sort or another in the host ("tumor bed changes") that interact to make the tumor behave as though more resistant on subsequent irradiation. The first two hypotheses require that: (a) there must be a mutation for radioresistance in a cell (whether already existing in an originally heterogeneous population or induced by the radiation), (b) the mutation must be in a cell that survives the initial irradiations, and (c) selection pressure must be exerted so that propagation of the resistant cells is favored over propagation of the less resistant. It would seem that the likelihood of all three requirements being met, since each alone has a low probability, should be very small, at least for ordinary experiments. However, there is an important difference between the two mutational hypotheses, spontaneous vs. induced. Since the spontaneous mutation could have originated at any time in the previous history of the tumor, there is a fair likelihood if it exists at all that, by previous cell division, it will be present in considerably more than one cell at the time of irradiation, thus making the probability for its survival much greater than for the induced mutation, which at the time of irradiation will be induced in only one, or a few cells.

Given a resistant cell, the most likely means of causing formation of a resistant population is to exert selection pressure by subsequent irradiation. Theoretically, of course, selection for radioresistance could be accomplished in any way as long as it was great enough, either at once by a single drastic irradiation, or gradually by moderate selection exerted over a considerable number of cell generations. Since the resistant cells, if present, are initially present in small numbers, since radioresistance differs by a factor of only three or four and is not absolute, and since the amount of radiation killing has a probability distribution with any given dose, there is a practical limit to the amount of killing that can be caused in one cell generation without elimination of the desired resistant cells. Hence, in the practical case with tumors, many considerations favor the conclusion that the method of gradual selection by moderate doses over a large number of cell generations is the one most likely to succeed.

The present paper examines the requirements and conditions for the generation of true, or intrinsic, radioresistance in a tumor and shows, in an experiment particularly designed to maximize the likelihood of inducing it, that it has not occurred.

MATERIALS AND METHODS

A clonal line of the Ehrlich mouse ascites tumor, started from a single cell inoculum about a year previously, was used for experimental material. The tumor was transferred weekly by intraperitoneal puncture and injection of a measured ascites volume and tumor cell number into C3H × 101 F1 hybrid mice. At the beginning of the experiment, a group of animals was given injections of tumor from a single donor animal. Thenceforth, the unirradiated control line and each of the continuously irradiated lines were maintained by the injection of ascites once a week from a single animal into a pair of animals. Four irradiated tumor lines were established, receiving continuous whole-


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body Co\textsuperscript{60} gamma radiation at rates of 50, 100, 150, and 200 r/day. The desire was to obtain a tumor line surviving to the end of the experiment that had been subjected to the maximal amount of radiation selection possible. The pairs of animals were in small cages at increasing distances from a chronic Co\textsuperscript{60} gamma-ray facility in this laboratory. The animals were exposed to gamma rays 23 hours a day, 7 days a week, for the 9-week course of the experiment. Irradiation was continuous, except for the time required each day, averaging about an hour, for feeding, watering, and cleaning. The original 200 r/day line was lost at the 7th week, the 50 r/day line was dropped after the 4th week measurements showed that growth or survival in the irradiated line was only $10^{-4}$ to $10^{-10}$ as great as in the control line for the 9-week period; as desired, radiation selection had been severe. From the growth rate of the control tumor, the average doubling, or cell generation time, could be estimated as somewhere between 21 and 27 hours; doubling time would be somewhat longer in the irradiated line, because of radiation-induced mitotic delay. The 9-week irradiation therefore represents continuous radiation selection for somewhat less than 56–72 cell generations.

The percentage of divisions which were normal, i.e., anaphases of division free from both bridges or fragments, was also observed at the weekly transfers. A graph of the time course of the percentage of normal divisions in the various lines is shown in Chart 1. The history of the “irradiated line” later used to compare with the control line is indicated by the very heavy line on the figure. Throughout the irradiation period, only about 20–40 per cent of the divisions were normal. The irradiated line recovered to the control percentage normal divisions within about 10 days after the end of the irradiation. Subsequently, the radiosensitivity of the two lines was compared, by observation of the amount of cytological damage and amount of cell killing, as a function of x-ray dose in vitro. The methods of treatment and interpretation of results are substantially the same as those given for previous experiments (2, 3).

Ascitic fluid of control and irradiated lines was...
withdrawn from animals injected 6 days previously and diluted with 1 part Tyrode's solution to 4 parts whole ascitic fluid, plus 0.01 mg heparin/ml ascitic fluid to prevent clotting. Fluid was thoroughly mixed, subdivided into lots, then irradiated in vitro while in equilibrium with air with 250 kvP x-rays filtered through ½ mm. of Cu intervals for staining and observation of the percentage normal anaphases of division. The results from one of the cytological experiments are shown in Chart 2. Each individual point represents observation of 100 or more cells.

The percentage of normal anaphases falls rather rapidly toward a minimum value, which depends

and 3 mm. of Al, (HVL, 1.45 mm. Cu) at rates of 218 to 240 r/min for the different experiments. Doses in the range of 50–670 r were delivered. For the cytological experiments, 2 ml. of irradiated fluid from each dose was injected shortly after irradiation into one or two recipient mice, and samples were drawn thereafter at about 12 hourly

on dose, and then gradually recovers toward the normal value. If the family of dose curves for the control and for the previously irradiated lines (Chart 2A and 2B) be superimposed, the curves for the same doses are almost identical, except for the 50-r curve only, which is displaced somewhat in time but not in amount of depression. Two mice
were given injections of ascitic fluid irradiated with 400 r for each tumor line; the fairly good agreement between the values obtained from the two mice gives a notion of the reliability and accuracy of the system. The rather broad minimum of normalcy at 20–24 hours represents the degree of cytological abnormalcy induced in the initially irradiated population when it is manifested in the first division after irradiation and is a proper control lines, which are opposite in direction in the two experiments, are not significant.

The radiosensitivity of the two lines was also compared for a different kind of radiation response, namely, the effect of in vitro x-radiation on the amount of growth of the tumor occurring subsequently in a 5-day in vivo growth period. The methods of handling and in vitro irradiation were the same as for the cytological experiments, except

measurement of cytological damage as a function of dose for the two lines. These data, taken from the experiment shown in Chart 2, together with the results from another cytological experiment done in the same way as the one already described, are shown in Chart 3.

The results clearly show that there is no difference in radiosensitivity of the control and irradiated lines, as measured by the amount of cytological damage caused by x-radiation. The very slight differences that exist between the irradiated and that cell counts were made on the irradiated tumor, and a measured number of cells was injected into four recipient animals from each irradiation lot of tumor, all animals receiving the same number of tumor cells. Five days later, which is still within the period of exponential growth of the tumor, the animals were killed; the total number of cells per animal was calculated from measurements of total ascitic volume and the number of cells per milliliter determined from hemocytometer counts. The results are shown in Chart 4. The
unirradiated lots of tumor from each line had increased about 52-fold in the 5-day growth period. Total number of cells on the 5th day is roughly proportional to the number of viable cells inoculated on day zero (3). Since every animal was inoculated with the same total number of cells, the lesser amount of 5-day growth at the larger doses is believed to be a consequence of radiation killing, resulting in a smaller viable cell number in the initial inoculum. The upper pair of curves on Chart 4 shows that the amount of killing as a function of dose was approximately the same for the control and the previously irradiated tumor lines; the only point at which the curves do not overlap by one standard deviation is at the 102-r point, but this difference is not significant. The fall in the dose curve in the 50–100-r range and subsequent rise to the 200–300-r range before falling again toward the larger doses that is seen in Chart 4 is contrary to our experience in previous experiments of this sort, in which cell number declined smoothly with dose. The decline and rise may be explained by a phenomenon described by Révész (8), working with ascites tumor also, who found that, when unirradiated tumor was mixed with increasing proportions of supralethally irradiated tumor, tumor growth (increase in cell number) was at first depressed, then increased, then depressed again, as the proportion of irradiated tumor was increased. In any case, radiosensitivity of the control and previously irradiated lines, as measured by cell survival and growth, can be considered the same.

DISCUSSION

Consideration of the requirements and conditions for either mutation induction and selection, or selection of mutation, given briefly in the introduction, indicated that the probability was low that acquired radioresistance was to be explained on the basis of mutation induction and selection in tumors; the possibility was not excluded, of course, but was shown to be unlikely. The present experiment on ascites tumor, designed to maximize the likelihood of induction of intrinsic radioresistance in a tumor by subjecting it to continuous maximal radiation selection (killing) for a very large number of cell generations, showed that no radiation resistance was induced. It is important to notice that the complication of some sort of irradiated host interaction's giving the appearance of tumor radioresistance, or "effective radioresistance," was eliminated by testing the irradiated line separately from the animals in which it was irradiated. The negative results, of course, cannot be absolute proof that intrinsic radioresistance of a tumor does not occur. There are unquestionably great differences between newly originated tumors and an ancient one such as the Ehrlich ascites tumor used for this experiment, so that arguments and results from an old tumor may not be generally applicable to new tumors. It would not seem unreasonable, however, that an ancient tumor, having had a longer time to do so, might well bear a greater accumulation of spontaneous mutants than a new one, and among these could be a mutant for radioresistance.

Montgomery and Warren (6) also failed to cause acquired radioresistance in a mouse lymphosarcoma irradiated in vitro at each transfer for six successive transfers; radiation selection pressure was not so great as in the present experiments, however, nor was selection exerted continuously. Rubin (9) irradiated bacteria continuously at high

![Chart 4](chart4.png)
dose rates with x-rays and P\textsuperscript{32} beta rays and failed to detect radioresistance, in spite of the facts that in his experiments radiation selection was very severe and was exerted continuously for a very large number of cell generations, and that mutation for factors other than radioresistance was found. Radioresistance to both ultraviolet and x-rays can be obtained, however, as Witkin (12) has shown for bacteria, in an excellent paper that presents many of the considerations involved in the kinetics of selection in a cell population. She enjoyed, however, the advantage of selection by single-cell isolation, a technic of bacterial experiments that, in the practical sense, is denied in cancer selection experiments of the kind under consideration. What appears to be true genetic radioresistance of a tumor has been reported once (4); radioresistance of a carcinoma was increased, we estimate about 24-fold, by a total dose of 22 kr administered 2 kr at a time over eleven successive transplant generations.

Turning away, for the moment, from the negative evidence against mutation being the cause of radioresistance, there is positive evidence that radioresistance can be altered by host responses, independent of the tumor. For example, Nice (7) was able, by repeated \textit{in vivo} sublethal irradiations, to make a lymphosarcoma behave as though radioresistant. The acquired radioresistance was not a property of the tumor itself, however, for it was immediately lost upon transfer of the tumor to a new host; the "effective resistance" was thus an effect of the irradiated host upon the tumor. Russ and Scott (10) made a clear demonstration of an effect of irradiated host upon tumor response. They found that irradiation of the area around a tumor, the tumor itself being shielded, would cause regression of the tumor in about a third of the cases. Similar evidence is the common observation that a tumor, after what otherwise would be a lethal dose, if transplanted to a new unirradiated host will survive. Another example is the observation that an unirradiated tumor that would normally take, if transplanted to an irradiated area, will fail to take. Cohen and Cohen (1) found a mutant C3H carcinoma strain that was twice as radiosensitive as normal when carried in C3H hosts, but had the same radiosensitivity as normal when in factor-free F\textsubscript{1} hybrid hosts. The cases just cited show clearly that tumor bed host response can account for a change in radioresistance in some solid tumors; whether this also applies to ascites tumors is not proved, however.

For a cell or cell population to develop intrinsic acquired radioresistance, strict point mutation is not required—any kind of heritable change of the desired sort is sufficient. There are a number of cellular mechanisms already known that confer inherited radioresistance upon a cell. Polyploidy is such a heritable change, and other indirect cellular changes that cause radioresistance may be cited. For example, cells in division are generally more radiosensitive than those that are not; a mutation that diminished the proportion of time spent in the sensitive division stages could cause radioresistance. Cellular radiosensitivity is profoundly affected by oxygen concentration; a mutation for lower intracellular oxygen concentration could cause it also. It is pertinent to notice here that the oft-noted changes in the tumor bed as a result of irradiation are mostly in the direction of reduced oxygen tension for the tumor therein. No investigation was made of the intracellular oxygen concentration of our irradiated line, but it was examined for both polyploidization and a change in the percentage of cells in division and found to be the same as the control.

SUMMARY

The problem of the mechanism by which radioresistance of a tumor can be acquired is examined. Three hypotheses to explain the phenomenon are commonly proposed; namely, (a) radiation selection of spontaneous mutant resistant cells in the tumor population, (b) radiation-induced mutation in a tumor cell to resistance, followed by selection, and (c) a complex of radiation effects on the host that interact with the tumor to give the appearance of resistance to the tumor.

An experiment with mouse ascites tumor, specifically designed to maximize the likelihood of producing a true, or genetically, radioresistant tumor line is described; radioresistance was not produced.

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