THE UNIVERSITY OF MANITOBA

EFFECTS OF CYTOSINE ARABINOSIDE AND X-IRRADIATION ON THE CELL KINETICS OF DUODENAL AND MALIGNANT CELL POPULATIONS

by

WENDY JANE DAHLGREN

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WENDY JANE DAHLGREN

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Determinations of the LI, MI, and DI were employed to ascertain the effect of x-irradiation on a normal cell population and on a malignant cell population. In addition, the morphology of the duodenum and of the B16 melanoma were examined (at the LM level) as were survival patterns of treated and untreated C57Bl/6J mice bearing the tumour. Synchrony was produced by the administration of 8 i.p. injections of 12.5 mg/kg cytosine arabinoside (ara-C) given at two-hour intervals. The ara-C-induced synchrony occurred earlier and decayed more rapidly in the tumour cell population than it did in the duodenal cell population. The regimen of ara-C administration did not exert any effect on the MST of experimental animals, but was lethal to 10% of the mice. Damage to the duodenal mucosa was severe four hours following the last injection, but recovery was almost complete by two days. Dosages of x-irradiation (500R, 1000R, 1500R, and 500R x 2) caused damage to the duodenal mucosa which, assessed on a morphological basis, was dose-dependent. Changes in the duodenal indices following x-irradiation were indicative of a general damage but did not reflect a dose-dependency. All doses of x-irradiation caused some retardation and/or regression of tumour growth, as evidenced by decreased labeling and mitotic indices and augmented degenerating index, however a dose-dependent effect was not apparent. The MST of irradiated mice appeared to be dose-dependent. Doses of 500R x 2, 1000R, and 1500R all increased the MST of mice bearing the tumour. A 1000R dose of x-irradiation was administered four, 14, 16, 18 or 20 hours following the

last injection of ara-C, when it was assumed that the cohort of tumour cells would be in the late G_1, early G_2, M, mid-G_1, and late G_1 phase of the cell cycle, respectively. The cohort of duodenal cells would likely be in late G_1 at four hours and at 14 hours and in S phase at 16, 18, and 20 hours. A 1000R dose of x-irradiation four hours following the last injection of ara-C was lethal to 75% of the animals, and a 500R dose at this time, though not nearly as lethal, was relatively ineffective in retarding tumour growth. A 1000R dose 14 hours following ara-C proved to be the most effective regimen in terms of retardation and/or regression of tumour growth, although 1000R at 16 hours or at 18 hours were also effective treatments. The degree of damage to the duodenal mucosa augmented as the time between ara-C administration and irradiation was shortened. Generally, reduction or fractionation of an irradiation dose caused less duodenal damage than the identical dose administered as a single exposure, but the therapeutic gain was offset by less retardation or regression of the tumour growth.

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CHAPTER I

STATEMENT OF THE PROBLEM

Introduction

Melanoma, the malignant lesion composed of melanin-producing cells, is among the most unpredictable tumours in oncology (Allen and Spitz, 1953). This is because some are highly malignant, with very poor prognoses, while others, even though very similar histologically, may behave in a relatively benign fashion (Robbins, 1967).

As melanomas have been generally considered to be radioresistant (Desjardins, 1934; Pack, 1948; Philips, 1963), the treatment of choice is usually extensive surgical excision, combined with resection of the appropriate regional lymph nodes (Pack, 1948; McNeer and DasGupta, 1965). Despite the reluctance of many radiotherapists to irradiate pigmented tumours, several investigators reported remissions and even cures of human melanomas treated with irradiation alone or in combination with surgery (Jorgshol and Engdahl, 1955; Hellriegel, 1963; Weitzel, 1970; von Glasser, 1972).

Although various chemotherapeutic agents have been used clinically in conjunction with irradiation, the rationale in the administration of such combinations is often based solely on the status of the patient's recovery from side effects, rather than on the actual impact on the cell kinetics of the particular tumour. The growth fraction of a tumour represents a mixed population of cells, in regard to proliferation rate, and hence, the effects of irradiation may vary according to the position of a cell in the cell cycle at the time of administration. It is plausible

then, that the effect of irradiation on a tumour might be enhanced if the growth fraction of cells were more homogeneous with regard to the position in the cell cycle. If, for example, a drug could be used to synchronize the cells in a particular phase of the cell cycle, that cohort of cells could subsequently be irradiated while in the most radiosensitive phase. It would be expected that more cells would thereby be eradicated using synchrony and irradiation in combination than would be killed by mere irradiation alone of the (normally) heterogeneous asynchronous population of proliferating cells.

In any investigation dealing with the effects of therapy on malignant cell populations, the therapeutic ratio is of primary importance, that is, the effect of the treatment on the malignant cells compared to the effect of the treatment on normal proliferating cell populations. Indeed, the success or failure of any treatment schedule is dependent on the amount of damage sustained by normal tissues adjacent to the tumour, and the ability of those normal tissues to recover from any such injury.

Statement of the Problem

The main purpose of this investigation was to determine the effect of x-irradiation on the synchronized growth fraction of the B16 mouse melanoma. Subsidiary problems were as follows:

1. To determine the effect of cytosine arabinoside (ara-C) alone, x-irradiation alone, and ara-C in combination with x-irradiation on the cell kinetics of the duodenum of the C57Bl/6J mouse.

2. To determine the effect of ara-C alone, x-irradiation alone, and ara-C in combination with x-irradiation on the cell kinetics of the B16 malignant mouse melanoma.

3. To determine the effect of ara-C alone, x-irradiation alone, and ara-C in combination with x-irradiation on the survival rates of C57B1/6J mice bearing the B16 melanoma.

Definitions of Terms

Ara-C--as used in this study, is the abbreviation of 1-B-D-arabinofuranosylcytosine, or cytosine arabinoside.

Melanoma (pl. melanomas)--refers to the malignant lesion of melanin-producing cells, also melanocarcinoma or malignant melanoma (Robbins, 1967).

Synchrony--as used in this study, synchrony refers to the condition whereby a significantly greater than normal proportion of the growth fraction of cells of a population are in one particular phase of the cell cycle. A synchronization procedure is, therefore, any procedure which produces synchrony.

X-irradiation--as used in this study, refers only to that ionizing radiation emitted by an x-ray device.

CHAPTER II

REVIEW OF RELATED LITERATURE

INTRODUCTION

The Cell Cycle

The term cell cycle refers to the phases intervening between the completion of one mitosis of a cell until the completion of the subsequent mitosis of one or both daughter cells (Baserga, 1968). Howard and Pelc (1953) divided the cell cycle into four phases: M, G_1, G_2, and S. The latter three phases are referred to, collectively and morphologically, as interphase.

Mitosis approximates one hour with the majority of cells, and constitutes that part of the cell cycle when actual cell division occurs. Following its completion, the yield is two daughter cells, morphologically and genetically identical to one another and to the original parent cell. Cells in any one of the four stages of mitosis, namely prophase, metaphase, anaphase, and telophase, can be readily recognized at the light microscope level and are generally referred to as "mitotic figures" (MF's).

Between the termination of mitosis and the commencement of S-phase is the G_1 phase, or postmitotic gap. During that phase the cell is engaged in the synthesis of RNA and proteins (Baserga, 1968). The duration of the G_1 phase varies widely among different cell types. Indeed, the main variability in the length of a cell cycle is determined by the length of the G_1 phase (Mueller, 1969).

The S-phase (DNA synthesis), when DNA is replicated preparatory to division, follows G_1 and may last from 4 to 10 hours (Patt and Quastler,

1963). Thymidine, a natural precursor of thymine, is used by cells in the synthesis of DNA. In the complex process of autoradiography, thymidine, "labeled" with the radioactive isotope tritium, is administered. It can subsequently be visualized by reduced silver grains in the emulsion, overlying the nuclei of cells which were in S phase at the time the tritiated thmidine (H^3-TdR) was available. In this manner, labeled cells can be readily recognized at the light microscope level.

The G_2 phase (premitotic gap) usually lasts between one and two hours--from the termination of DNA synthesis to the morphologically discernible onset of mitosis (prophase). G_2 cells apparently synthesize RNA with the spindle fiber proteins requisite for cell division (Baserga, 1968).

In summary, of the four phases of the cell cycle, two phases, the M phase and S phase, are discernible at the light microscope level. Although nuclei of G_2 cells differ from those of G_1 inasmuch as they contain double the amount of DNA, G_2-phase cells cannot be distinguished morphologically from cells in G_1. Moreover, G_1 and G_2 cells cannot be morphologically discerned from cells which are are not in the proliferating cycle.

Cell Renewal Systems

A population of cells in which cells are lost continuously by exfoliation and replaced simultaneously by cells newly formed by mitosis is termed a cell renewal system (Leblond and Walker, 1956). This is in contrast to a system in which more cells are added by mitosis than are extruded or die. In the latter expanding cell populations, growth is the consequence. In a renewing cell population, a steady state is maintained, as cell production precisely balances cell loss.

Cell renewal systems have been divided into compartments, based on the morphology, function, or location of the constituent cells. Proliferation and functional activity of the cells are thereby separated. The proliferating compartment consists of those cells which pass through the cell cycle, and hence will eventually divide. It has also been designated the "growth fraction". The functional compartment comprises those cells which left the cell cycle, matured or differentiated, and are no longer capable of division. That compartment represents the "differentiating cell fraction". Not all cells remain in the proliferating compartment and divide indefinitely. As cells are lost, or extruded, from the functional compartment, new cells leave the proliferating compartment to differentiate and become functional cells. Such cells, in the process of differentiation, make up a third compartment--the maturation compartment (Patt and Quastler, 1963). It must be realized that, just as the entire population of cells in a cell renewal system is in a steady state, so are the cells within the individual compartments.

For example, the principal cells of the epithelium of the small intestine comprise a cell renewal system (Leblond and Stevens, 1948). The proliferating compartment is confined to the area at the bottom of the crypts. As the cells mature, they move upward along the walls of the crypts and become the functional cells by the time they have reached the upper parts.

Growing Proliferative Systems

Growing proliferative systems are defined as those cell communities in which growth occurs, that is systems in which more cells are added to the

population by mitotic division than are lost by death or exfoliation. A simple proliferative system is in an in vitro system composed of stem cells, or cells which do not differentiate but simply continue division. Initially, the growth of such a cell culture is logarithmic, as virtually all cells are proliferating and cell death is negligible. Eventually, however, such cell cultures attain a density where growth begins to slow and, finally, a "critical density" when growth ceases altogether (Clarkson and Fried, 1971).

Tumours also often comprise growing proliferative systems, although more complicated than the aforementioned in vitro system. In contrast to stem cells in culture, tumours are composed of mixed cell fractions, comparable to population compartments of the cell renewal system described previously. A proportion of tumour cells do divide, these comprising the proliferating or growth fraction of the tumour. The other nonproliferating fraction is composed of cells which rarely, if ever, divide, and of cells which no longer divide because they are either degenerating or are already dead. A nonproliferating fraction may also contain G_0 type cells; that is, cells which do not normally divide but which may be stimulated by suitable means to do so under specific circumstances. The growth characteristics of any particular tumour will vary according to the proportion of cells in these compartments. As these proportions may become modified during the course of growth of a tumour, so will the growth characteristics of the tumour.

In vivo growth curves, analagous to growth curves of in vitro systems, have been developed for various in situ tumours. Such curves are most often based on measures of tumour volume or weight relative to

time. The growth curves of most tumour cell populations resemble the growth curves of cells growing in culture, that is, an initial rapid growth rate followed by its gradual deceleration (Laird, 1964; McCredie et al, 1965). Several factors were postulated as being responsible for that pattern, either wholly, or in part, especially for the decline of growth rates in cell cultures. Clarkson and Fried (1971) suggested that a lengthening of the mitotic cycle (especially the G_1 phase), augmented death rate, or failure of daughter cells to re-enter the cycle following completion of mitosis could all contribute to the stationary phase of the growth curve. Similar factors might also be responsible for the retardation in the in vivo growth rate of in situ tumours (Dethlefsen et al, 1968).

THE DUODENUM

Introduction

The small intestine has been the object of a great many investigations, at the light microscope level (Leblond and Stevens, 1948; Thrasher and Greulich, 1966; Merzel and Leblond, 1969), at the electron microscope level (Toner, 1968; Staehlin, 1972), and in terms of cell kinetics (Leblond and Stevens, 1948; Bertalanffy, 1960). Inasmuch as morphological and cell kinetic differences occur in the 3 segments of the small intestine, as well as species variations, this review is confined to a description of the characteristics of the mouse duodenum.

Morphology of the Duodenum (Figure 1)

The duodenum, the most proximal part of the small intestine, pursues a horseshoe-shaped course around the head of the pancreas. It extends from the pyloric sphincter to the jejunum, a distance of 5 to 10mm in the mouse (Montagna and Wilson, 1955). Just beyond the pyloric sphincter, the mucous membrane is thrown into large folds, the folds of Kerckring.

The wall of the duodenum is comprised of four layers, the mucosa, lining the lumen, submucosa, muscularis externa, and the serosa. The mucous membrane, the innermost layer of the small intestine, consists of three components: the epithelial lining, the lamina propria, and the muscularis mucosae. The epithelium is simple columnar and supported by a reticulin basement membrane. Beneath lies the lamina propria, a layer of loose connective tissue, where lymphocytic elements abound. The lamina propria of the C57Bl mouse is devoid of lymphatic nodules (Montagna and Wilson, 1955). Reticular, collagenous and elastic connective tissue fibres are present in this layer, as well as thin bundles of smooth muscle. Blood and lymphatic capillaries (lacteals) are numerous.

The deep layer of the mucosa, the muscularis mucosae, generally consists of two ill-defined and interwoven layers of smooth muscle, the inner layer being circularly disposed, the outer longitudinally. Some elastic fibres may be present in this layer.

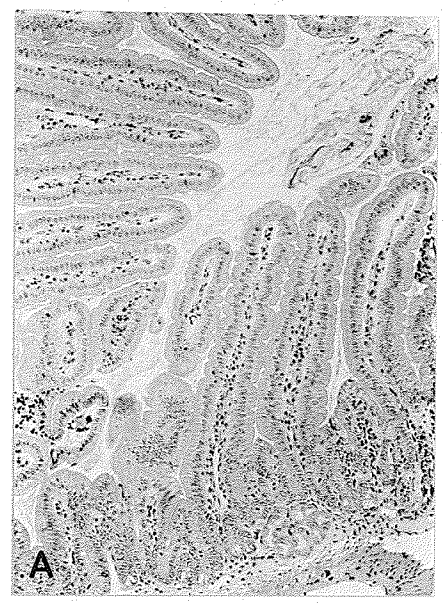
The submucosa intervenes between the mucous membrane and the muscularis externa. It is predominantly a loose connective tissue layer containing blood vessels larger than those of the mucosa, and a

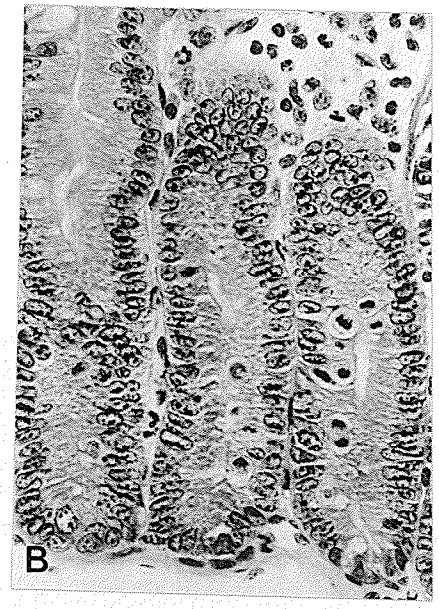
Figure 1. The Duodenum of the Untreated C57Bl/6J Mouse (H & E).

Figure 1A (x200) Low-power photomicrograph showing both villi and crypts. The duodenal lumen is discernible at the upper right corner.

Figure 1B (x800) Longitudinal sections through three crypts of Lieberkühn, such that the crypt lumina are discernible. Numerous mitotic figures (MF's) are visible in the crypts.

Figure 1





parasympathetic nervous chain and ganglia, part of the autonomic nervous system (ANS), the submucous plexus of Meissner.

The muscularis externa (propria) consists of two thick and well-defined layers of smooth muscle, an inner circular layer, and an outer longitudinal layer. Disposed in the connective tissue between these two layers is a second parasympathetic chain of the ANS, the myenteric plexus of Auerbach.

The outermost layer of the proximal portion of the duodenal wall is invested by a serosa, which is replaced distally by an adventitia.

The vascular supply to the mucosa and epithelium is by way of villus arterioles and venules running parallel to the long axis of the villus, and hence perpendicularly to the larger serosal circumferential artery and vein beneath. The capillary network intervening between the former vessels occupies the central core of the crypt and abuts the epithelium (Boyer and Conger, 1972).

The surface of the mucous membrane over and between the folds of Kerckring is characterized by finger-like projections, the intestinal villi, with a core of the lamina propria. The villi of the duodenum are broader than those of other segments of the small intestine. Because one of the functions of the duodenum is absorption, many of the tall columnar cells lining the villi bear numerous microvilli along the apical free border, forming the striated border discernible at the light microscope level. Distributed among these "striated" villus cells are goblet cells, which secrete mucus. Goblet cells are usually oblong and are placed at right angles to the surface. The amount of mucus in their cytoplasm reflects the degree of maturation and/or functional state. The proportion

of goblet cells increases toward the distal part of the duodenum (Moe, 1955).

Invaginating from the surface of the mucosa, between the villi, are the crypts of Liekerkühn. Fujii (1972) reported that the ratio of villus length to crypt depth in the mouse duodenum was $7.6 \pm 1.18/_1$. The crypts are likewise supported by the lamina propria and extend close to the muscularis mucosa. The cells lining the crypts vary in structure and function in relation to their crypt position. At the very base of the crypt are the Paneth cells, shaped like truncated cones, the apex directed toward the lumen. The nuclei of Paneth cells are disposed toward the basement membrane and the granular cytoplasm stains vividly with eosin. In the lower portion of the wall of the crypt the epithelial cells are low columnar with goblet cells, still containing little mucus, interspersed between. Other cells exhibit numerous free ribosomes, little rough endoplasmic reticulum, and as yet poorly developed microvilli, all characteristics of an immature cell. Cells in the upper section of the crypts, though still not tall columnar, resemble more closely those of the villi. Argentaffin cells have also been described as being present, in small numbers, in the duodenal crypts of mice (Carvalheira et al, 1968; Ferreira, 1971). Moreover, Andrew and Andrew (1945) observed a transformation, toward degenerative change, of lymphocytes as they migrated from the lamina propria into the lumen of the crypts. Up to 16.7% of crypt and 36.5% of villus epithelial cells of C57Bl mice contained lymphocytes within their cytoplasm. More recently (Andrew, 1965), it was shown that lymphocytes in the crypts showed marked degenerative changes, many of these cells being surrounded by vacuoles within the cytoplasm of the epithelial crypt cells.

Quastler and Sherman (1959) reported that, of all epithelial cells of the mouse small intestine, 62.9% of villus cells were absorptive columnar, while 2.2% were goblet cells; in the crypts, 32.5% were absorptive columnar, 0.7% goblet, and 1.7% Paneth cells.

In the submucosa of the duodenum lie the extensive Brunner's glands, compound tubular glands characteristic of the proximal portion of the duodenum. The excretory ducts of the Brunner's glands pierce the muscularis mucosae to empty into the bases of the crypts.

Cell Kinetics of the Duodenum

The epithelium lining the duodenum is a typical cell renewal system, with a turnover rate in the order of 1½ to 2 days, and exceeding in velocity that of any other *in vivo* tissue component (Bertalanffy and Lau, 1962). Because more than 90% of the epithelial cells are principal, or chief cells (in contrast to Paneth cells)(Quastler and Sherman, 1959), most cell kinetic studies refer to this portion of the cell population.

The proliferative compartment of the epithelium is located in the middle 2/3 of the crypts (Lamerton, 1966), and comprises approximately 55 to 60% of the total crypt population (Lesher and Bauman, 1969). The Paneth cells, which are located at the bases of the crypts, make up about 10% of crypt cells and are considered, along with the goblet cells, to be a part of the functional compartment of the crypts (Lesher and Bauman, 1969). As cells differentiate, they move from the proliferative compartment toward the mouths of the crypts, the two daughter cells arising from a division moving contiguously (Quastler and Sherman, 1959). Differentiation is likely completed over a very short distance, one or two

cell positions being the likely distance (Lesher and Bauman, 1969). The functional cells migrate subsequently as a coherent sheet from the crypts to the tips of the villi (Leblond and Stevens, 1948). Incorporation of tritiated leucine by cells in the functional compartment indicated that the cells are actively producing enzymes for molecular degradation, and subsequent transport across cell membranes. Incorporation of RNA and protein precursors becomes reduced to very low levels by cells near the villus tips, signifying that, at that level, the cells have reached the end of their functional life span. These cells are subsequently exfoliated into the intestinal lumen (Lesher and Bauman, 1969) from the extrusion zones at the villus tips.

The transit time, or time required by the cells to move up the walls of crypts and villi to be finally extruded, is shorter in the duodenum than in the jejunum or ileum (Lesher et al, 1961a; Fry et al, 1961; Fry et al, 1962). Hagemann et al (1970b), estimated the transit time of duodenal cells of the C57Bl mouse to be 41 hours, which is briefer than that reported for other mouse strains (Lesher and Bauman, 1969; Creamer et al, 1961), and significantly shorter than that estimated for the rat (Merzel and Leblond, 1969). Moreover, transit time has been claimed to increase with age (Fry et al, 1962).

The generation time of duodenal crypt epithelial cells of the C57Bl mouse is 13.1 hours with S=7.5 hours, G_2=0.5 hours, M=1 hour, and G_1=4.1 hours (Hagemann et al, 1970b). It has been reported, in another mouse strain, that the generation time of duodenal cells increased with age (Lesher et al, 1966; Lesher, 1967). Although other investigators, studying various mouse strains, reported generation times ranging from

10.3 to 18.75 hours (Lesher et al, 1966; Tsubouchi and Matsuzawa, 1974; Sherman and Quastler, 1960), the duration of the S phase was nonetheless constant at 7.5 hours. The greatest observed variation was in the length of G_1. Cameron and Greulich (1963) suggested that the S phase might be identical in all epithelial cell populations of the same mammalian species.

As the proliferative compartment is located in the crypts of the duodenum, it is logical that crypt cells became readily labeled when H^3-TdR was administered. The variances in the method of assessment of labeled cells, however, render comparisons between different studies difficult. Also, as maximal saturation of the H^3-TdR likely occurs within 10 to 20 minutes (Quastler and Sherman, 1949), some variations in labeling are to be expected when there are wide discrepancies in the time at which the animals were killed. Further, some variations are also brought about by different amounts of labeled thymidine injected (Sherman and Quastler, 1960). Several investigators (for example, Lesher, 1967; Hagemann et al, 1970a; Lesher and Lesher, 1974), ascertained by the crypt squash technique, that 60 to 77% of nuclei were labeled per crypt. This would correspond to a growth fraction of 50 to 60%, substantiated by other investigators by the autoradiographic technique (Quastler and Sherman, 1959; Edwards and Klein, 1961; Hagemann et al, 1970a). Fry et al (1961), scored labeled nuclei in twenty crypts (approximately 1000 cells) per specimen, and reported an average of 15.5 labeled cells per crypt 30 minutes after H^3-TdR administration. This corresponded to an LI of 31%. Hagemann et al (1970b) reported that 171 cells were labeled of a total of approximately 292 in the crypts, an LI of 37%. Kovacs and Potten (1973) stated that a discrepancy did not occur in the LI of DBA-2 mice jejunal cells, whether the crypt

squash technique or autoradiography of unsquashed crypts were examined. By the former method, the LI was 30.9 ± 0.7, while the latter yielded the LI of 28.1 ± 1.2. Lesher et al (1961a) observed that the LI (LI_{30}) of duodenal cells of CAF_1 mice ranged between 30 to 34%, and remained independent of age.

The duodenal crypt was described as containing only a few functional cells, approximately equally composed of goblet and Paneth cells (Montagna and Wilson, 1955; Lesher and Bauman, 1969). Cairnie (1970) reported that 2% of goblet cells and 1% of Paneth cells became labeled in the intestinal crypts of rats. Lesher (1967) observed a 10% labeling of Paneth cells in mice, while Kovacs and Potten (1973) reported 19% labeling in these cells. Conversely, Merzel and Leblond (1969) did not observe any labeling of either Paneth or argentaffin cells one hour after H^3-TdR administration, hence agreeing with Hughes et al (1958), who also failed to observe labeling of cells containing granules (meaning the Paneth cells).

Other cells of the intestinal epithelium exhibiting a slight degree of labeling were the villus epithelial cells (less than 1%, Cairnie et al, 1965) and cells of Brunner's glands (approximately 1%, Bertalanffy, 1960).

Mitotic figures (MF's) are frequently observed in the intestinal epithelium and are confined to the crypts (Quastler and Sherman, 1959). Numerous investigators reported the mitotic index (MI) of the rat duodenum (Bertalanffy, 1960; Altmann and Enesco, 1967) to range between 3.4 and 5.6. Pilgrim et al (1963) noted that the MI of the mouse jejunum was 4.8. Merzel and Leblond (1969) asserted that MF's of goblet cells or of

argentaffin cells did not occur in mouse duodenum. In contrast to Pilgrim et al (1963), who did not observe diurnal variations in the MI or LI of mouse intestinal epithelium, Grube et al (1970) reported diurnal variations in the LI of mice. Sigestad et al (1969) also suggested that the number of cells in S phase and M phase had a well-defined circadian rhythm.

Under normal conditions, there is little cell mortality in the crypts of the small intestine. Hugon and Borgers (1966a) described sporadic very dark cells in the crypts of Balb/C=+ mice; characterized by a densified cytoplasm, enlarged ergastoplasm, and inflated nuclear envelope. No explanation was offered as to the apparent death of these cells.

THE B16 MELANOMA

Introduction

The B16 melanoma initially arose spontaneously in the skin at the base of the ear of a C57Bl/6J mouse in 1954. It has been maintained by subcutaneous (s.c.) transplant (Jackson Laboratory Handbook, 1962). Demopoulos et al (1965) reported that the B16 melanoma was palpable within 5 days of s.c. transplant and measured 30 to 60 mm^3 by 8 or 9 days. At the end of 3 weeks, the tumours measured 3 by 3 by 1.5 cm, and the hosts generally succumbed thereafter. Gibson (1972) stated likewise that the tumour was palpable by 4 to 6 days after transplantation. By 10 days of tumour growth the mass averaged $80mm^3$, and weighed on the average 324 mg, by which time the mass doubling time was estimated to be 72 hours. Mean survival time (MST) of mice bearing the B16 melanoma was 29 ± 6.9 days.

CHART 1 - THE GROWTH CURVES OF THE MALIGNANT B16 MELANOMA BETWEEN DAYS FOUR TO 33 OF TUMOUR GROWTH (Gibson, 1972)

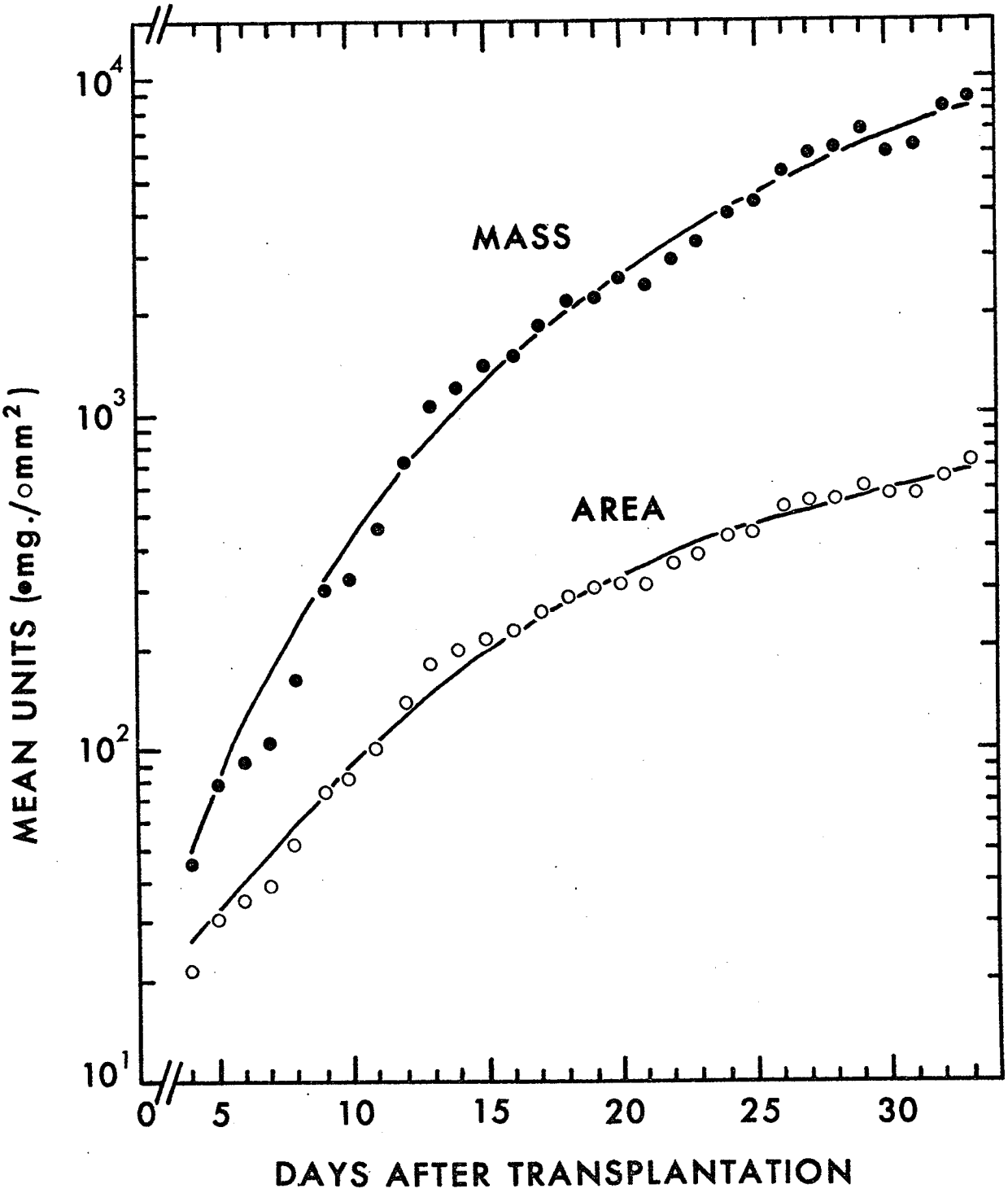


Chart I illustrates the growth curves, in terms of mass and area, of the B16 melanoma between days 4 to 33 of tumour growth (Gibson, 1972).

Morphology of the B16 Melanoma (Figure 2)

Bertalanffy and McAskill (1964) provided a detailed description of the light microscopic structure of the B16 melanoma. After s.c. injection of suspension of B16 melanoma cells, the transplant developed into a fairly well-encapsulated nodule in the s.c. fascia. Invasion of the melanoma into surrounding skeletal muscle, collagenous tissue, and adipose tissue occurred at areas where the tumour was not encapsulated. The tumour was fairly vascular, and many of the blood vessels appeared congested. Gullino and Grantham (1961) noted that various mice and rat tumours exhibited a remarkably small blood supply, being of the order of 5% of the liver vascularity. Bertalanffy and McAskill (1964) suggested that pigment-containing tumour cells, which were occasionally observed to protrude into the lumina of blood vessels, gave rise to metastatic tumours elsewhere.

The parenchyma of the B16 melanoma consisted chiefly of tumour cells, with sporadic migratory leukocytes. The parenchymal cells were often arranged in circular or oval groups around a blood vessel. Tumour tissue between such cell aggregations was rather diffuse. Hu et al (1967) observed that the cytoplasmic membranes of two abutting tumour cells did not have desmosomal connections, although interdigitations of the membranes were elaborate in some areas of contact.

Apart from blood vessels and a fine reticulin network, little other tissue or cells occurred. Demopoulos et al (1965) observed large

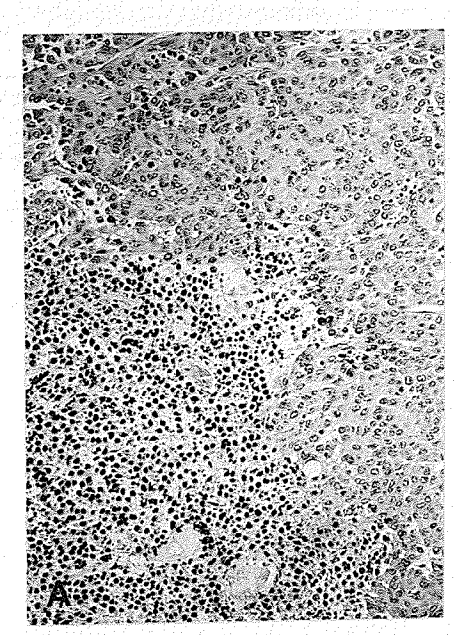
Figure 2 The Untreated Malignant B16 Melanoma on Day 10 of Tumour Growth (H & E).

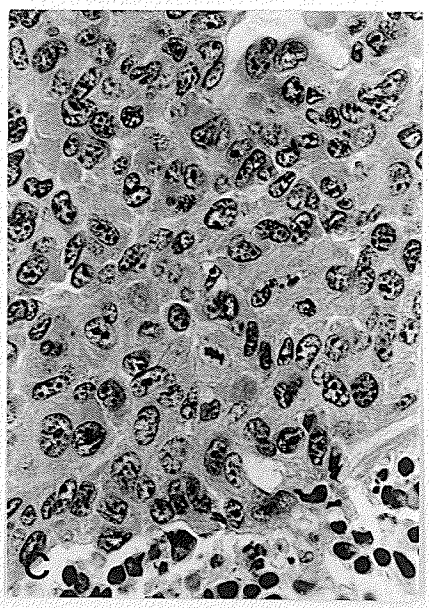
Figure 2A (x200) The centrally located necrotic area is at lower left of the photomicrograph, viable tumour tissue at upper right.

Figure 2B (x800) The subcapsular region of the tumour. Part of the tumour capsule is visible at upper left.

Figure 2C (x800) The deep (central) area of the tumour. Part of the necrotic area is visible in lower area of photomicrograph.

Figure 2





necrotic areas located in the center of the tumours at 3 weeks of tumour growth, but never as early as 8 or 9 days. Gibson and Bertalanffy (1972a) noted such a central necrotic area by day 10 of tumour growth, the area enlarging subsequently with the age of the tumour.

Characteristic vascular arrangements are often similar in histologically comparable tumour types. For example, the vascular pattern may be very similar in all carcinomas (Margulis et al, 1961). The development of a centrally located necrotic area has been attributed in many tumours to inadequate vascular supply to the area (Rubin and Casarett, 1966a; Lewis et al, 1968). Although the vascular development during early growth of tumours may be fairly constant (Margulis et al, 1961), the distribution of blood flow was noted to change eventually with the growth of the tumours (Lewis et al, 1968). Rubin and Casarett (1966a) suggested that newly formed blood vessels in tumours differed in structure from normal vessels. The rapid growth of the tumour vascular channels resulted in their imperfect architecture and the formation of many irregular channels. The endothelium was patchy in places and replaced occasionally by tumour cells. The vessel walls usually were deficient of the normal amount of elastic connective tissue and smooth muscle. In tumours with a large necrotic area, the small vessels from the viable peripheral tumour tissue attained only the margin of the necrotic area.

Bertalanffy and McAskill (1964) reported that the parenchymal cells, when round or oval, ranged in size from 12μ to 16μ. Although the majority of the tumour cells was polygonal, there were also elongated and spindle-shaped forms. Demopoulos et al, (1965) similarly described B16 melanocytes as being generally round, and endowed with abundant

cytoplasm. The cells were multipolar with extremely short, multipolar dendritic-like processes. It was noted that such cells tended to form giant cells with multiple nuclei and broad sheets of cytoplasm. The cytoplasm of the B16 melanoma cells stained slightly basophilic. It contained varying amounts of fine, light brown granules, presumably melanin (Bertalanffy and McAskill, 1964). These granules were either concentrated around the nucleus or in parallel rows in the dendritic processes (Demopoulos et al, 1965). While some cells appeared to be devoid of melanin, the cytomorphology of others was obscured by a great quantity of pigment granules (Bertalanffy and McAskill, 1964).

Hu et al (1967), with cells from a B16 melanoma, isolated pigmented and nonpigmented cell strains. By selection of cells from particular areas of tumours, they developed a pigmented tumour and a nonpigmented tumour. The black (pigmented) tumour was similar to the original melanoma from which the cells lines were isolated. The nonmelanotic tumour was completely devoid of pigment. Both tumours were composed of predominantly small, nonpigmented epithelial-like and spindle cells. The pigmented tumours contained melanocytes that varied in both size and shape. They were described as being round, oval, bipolar, and polydendritic. Some resembled epithelial cells while others were more like fibroblasts. The ultrastructure of the two types of tumours was identical except for the presence or absence of melanosomes and premelanosomes containing varying degrees of melanization. Wellings and Siegel (1963) also studied both melanotic and amelanotic tumours and observed them to have a very similar ultrastructure. The amelanotic tumour contained just pale premelanosomes. Demopoulos et al (1965)

described frequent aggregations of melanized melanosomes contained in large cytoplasmic vacuoles. There were numerous mitochondria, frequently in close proximity, if not contact, with the melanosomes. Distinct Golgi complexes frequently covered a large area and exhibited a great density of vesicles and vacuoles. The abundant rough endoplasmic reticulum formed long, parallel rows of cisternae.

The nonpigmented cells were devoid of melanosomes or melanized premelanosomes, but they did contain nonmelanized premelanosomes. In addition, nonpigmented cells contained myelin figures which were not observed in the melanocytes. These myelin figures occurred either free in the cytoplasm in close proximity to premelanosomes, or as parts of the premelanosomes.

Cell Kinetics of the B16 Melanoma

Bertalanffy and McAskill (1964), using the colchicine technique, did not observe a significant difference in the mitotic rate (MR) of the B16 melanoma on day 10 and day 14 of tumour growth. They determined that approximately 35% of new cells were added to the tumour daily, equalling a doubling time (DT) of 2.8 days. Metastatic tumours in the liver and spleen had a MR similar to that of the primary tumour.

Gibson and Bertalanffy (1972a) observed the GF of the B16 melanoma to range between .41 and .78 over a 29-day period, with a mean of .53. A significant difference in the GF was not noted on days 7 to 16 of tumour growth, signifying little, if any, loss in the proportion of proliferating cells during that period.

Observations on the generation time of B16 melanoma, both grown *in vitro* and *in vivo*, were first reported by Helpap and Maurer (1967). They

stated the length of the S phase to be 7 hours, slightly shorter than the 8.2 hours estimated by Wodinski et al (1972). None of these investigators indicated the stage of tumour growth at which these estimates were made, however. Bertalanffy and Gibson (1971) and Gibson and Bertalanffy (1972a) estimated the length of the cell cycle to be 12.5 hours, 14 hours, and 19.5 hours on days 6, 10, and 18 of tumour growth of the B16 melanoma. On day 10 of tumour growth, the following estimates were made with respect to the duration of the cell cycle phases: S=7.5 hours, G_2=1.3 hours, M=1.4 hours, and G_1=3.8 hours. The S phase was longer on both day 6 and day 18 (9.5 and 15.5 hours respectively), while $G_1+\frac{1}{2}M$ was considerably shorter (1.5 and .5 hours). $G_2+\frac{1}{2}M$ on day 6 was approximately the same as G_2 on day 10, while on day 18, G_2=M was about 3 times as long as G_2 on day 10. These investigators postulated that, particularly on day 18 of tumour growth, there were likely two distinct cell populations with differing cell cycles.

Gibson (1972) and Gibson and Bertalanffy (1972a) reported that the LI of the B16 melanoma was 48%, 46%, and 28% on days 6, 10, and 18 of tumour growth respectively. These indices were obtained following a one-hour pulse label with H^3-TdR. After a 15-minute pulse label, the LI was approximately 20% on day 10 of tumour growth. The MI declined slightly with time, being 3.4 (day 6), 2.5 (day 10), and 1.3 (day 18). The DI did not differ on any of the 3 days, remaining constant at approximately .74%. Neither the MI nor the DI differed significantly between day 7 to day 16 of tumour growth.

CYTOSINE ARABINOSIDE

Introduction

Cytosine arabinoside, or ara-C (1-β-D-arabinofuranosylcytosine, cytosar, cytarabine) is a synthetic, cell cycle specific (CCS) nucleoside analogue. It was independently synthesized by Walwick et al (1959) and Hunter (1965), and has been widely employed as both an antiviral and an antitumour agent.

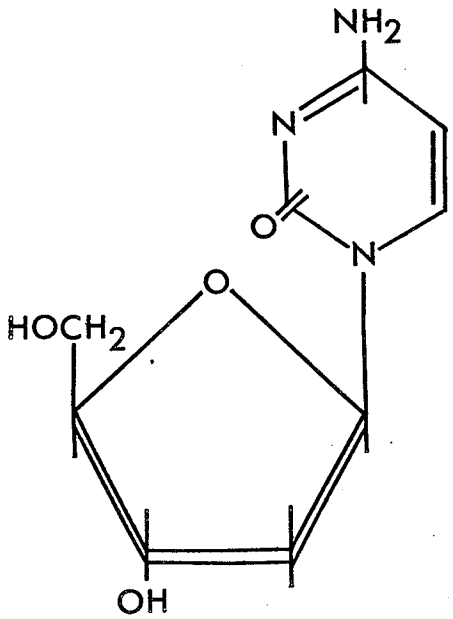
The chemical configuration of ara-C is quite similar to that of cytidine and deoxycytidine. As is evident from Chart 2, the hydroxyl group on the C-2 lies in a cis-position to the glycosol linkage rather than in the transposition. The biochemical properties of the D-arabinosyl nucleosides, with specific reference to ara-C, were reviewed by Cohen (1966) and by Smith (1966). The pharmacology of ara-C was recently discussed by Dedrick et al (1972) and by Wan et al (1974).

Mechanism and action at cellular level

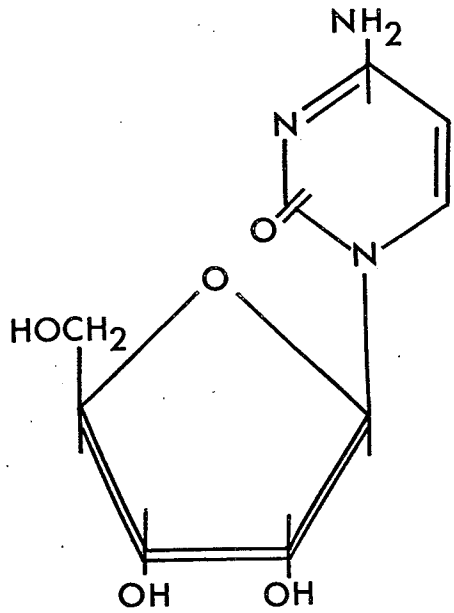
1. Metabolism of ara-C in mammals. Ara-C becomes rapidly deaminated to 1-β-D-arabinofuranosylurasil (ara-U) by cytidine deaminase (D), present primarily in the liver and kidney (Camiener, 1967a and 1967b, Camiener and Smith, 1965; Creasey, 1963). Ara-U is a noninhibitory metabolite without therapeutic action (Wilkoff et al, 1967; Stewart and Burke, 1971). Several investigators (Creasey et al, 1968; Finklestein et al, 1970; Ho and Frei, 1971) demonstrated that approximately 10 hours after the administration of radioactive ara-C, 90% of the radioactivity was excreted with the urine, indicating the rapid removal by the kidney of ara-C or its metabolites. In man, the plasma half-life of ara-C

CHART 2.

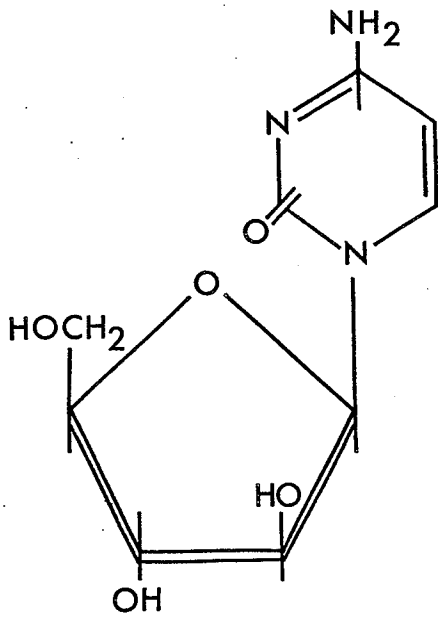
CHEMICAL STRUCTURES OF CYTOSINE NUCLEOSIDES



Deoxycytidine



Cytidine



ara-C

lasts approximately 10 minutes (Baguley et al, 1971; Momparler et al, 1972). In 5 to 6 week old mice the plasma half-life is approximately 20 minutes (Borsa et al, 1969).

Ara-C enters cells via a nucleoside transport system (Mizel and Wilson, 1972) likely by a facilitated diffusion mechanism (Kessel and Wodinski, 1967). Once within the cell, ara-C becomes phosphorylated to ara-CMP (the 5^1-monophosphate), ara-CDP (the 5^1-diphosphate), and ara-CTP (the 5^1-triphosphate), the latter being the major form of the nucleotide within the cell (Momparler, et al, 1968; Schrecker and Ursel, 1968).

It was observed that deoxycytidine kinase (K), an enzyme present chiefly in proliferating cells, catalyzes the phosphorylation of ara-C to ara-CMP (Durham and Ives, 1968; Kessel and Wodinski, 1967; Momparler, et al, 1968). Kozai and Sugino (1971) reported that ara-C becomes phosphorylated to ara-CTP by three separate actions of enzymes, namely deoxycytidine kinase, deoxycytidine monophosphate kinase, and nucleoside diphosphokinase, the intermediates being ara-CMP and ara-CDP. Chu and Fischer (1962 and 1965) noted that the inhibitory effect of ara-C on the in vitro growth of lymphoblasts was completely prevented by deoxycytidine, and further, that ara-C inhibited the conversion of uridine to deoxycytidine monophosphate (dCMP) but not to cytidine monophosphate (CMP). This suggested to these investigators that the reaction affected by ara-C was the conversion of CDP to dCDP.

It was shown that ara-C was activated by deoxycytidine and deoxycytidine kinase (K), and the activated product, ara-CTP, was a competitive inhibitor of DNA polymerase and thus inhibited DNA synthesis (Furth and Cohen, 1968; Burke et al, 1970; Ho and Frei, 1971; Owens et al, 1973).

The conversion of ara-C to the ara-C nucleotide correlated directly with the in vivo antitumour activity of the drug (Kessel et al, 1967).

Several investigators reported that either absolute values of K and of D or the K/D ratio were crucial factors in the efficacy of ara-C action. Stewart and Burke (1971) reported that leukemic cells in patients who responded to ara-C treatment contained six times less pyrimidine nucleoside deaminase than did leukemic cells of non-responders. It was postulated that the intracellular deaminase could be critical in determining whether or not ara-C would exert any antitumour effect. In contrast, Hart et al (1972) did not observe any significant difference in the levels of ara-C deaminase between "responders" and "non-responders" bearing metastatic melanoma, but they did find that the median kinase activity was significantly higher in patients who responded to ara-C treatment than in those who did not. As the K/D ratio for the responding patients was approximately five times higher than that of the non-responding patients, it was suggested that there was a positive correlation between the enzyme activity ratio (K/D) and the responsiveness to ara-C. Drewinko et al (1972) suggested kinase to be the rate limiting factor in responsiveness to ara-C. This is in agreement with Drahovsky and Morris (1972) and Kreis et al (1972) who ascertained that the sole difference between two cell lines was in their enzymatic deoxycytidine kinase activity in the ara-C sensitive line, which was reduced (almost negligible) in the ara-C resistant cells. Schrecker (1970) also found a decreased concentration of deoxycytidine kinase in L1210 cells resistant to ara-C. Ho (1973) reported that mouse L1210 cells displayed a high K activity but no D activity. Livers of one strain of mouse (C3H) had no

measurable K whereas the livers of another strain (DBA/A) did have K activity. Kidneys of both strains exhibited high D activity.

2. Effect of ara-C on the cell cycle. It is generally agreed that cells in the S phase are inhibited by ara-C, while in the other phases cells proceeded through the cell cycle to the G_1-S boundary, where they became blocked. However, Karon and Shirakawa (1970) claimed that, after ara-C administration the transit time of in vitro Don cells was greater at the S-G_2 junction than at the G_1-S boundary. In contrast, Kim and Eidnoff (1965) concluded from cell kinetic data that HeLa S-3 cells were halted before they approached S, and that cells not as yet at the end of G_1 when ara-C was administered began to accumulate at that point. Brent et al (1965) observed an augmented thymidine kinase activity in synchronized HeLa cells during the latter part of G_1. Kit et al (1966) suggested that this was indicative of cells blocked at that point. Bremerskov et al (1970) concluded that ara-C selectively killed S-phase cells, caused a major accumulation of G_1 cells, and a minor accumulation of G_2 cells.

It was further reported (Tobey, 1972) that when CHO cells were exposed during G_1 arrest (caused by an isoleucine-deficient medium) to ara-C, initiation of DNA synthesis was drastically reduced. Cells in S at the time of ara-C administration ceased DNA synthesis within an hour, and only sporadic cells passed into mitosis. Although the drug caused general accumulation of cells at the G_1-S boundary, a few cells still continued to enter S, even in the presence of high concentrations of ara-C. In a further study (Tobey and Crissman, 1972) the G_1-S block by ara-C was substantiated by the reduced time both of entry into S phase

and cell division subsequent to removal of the drug, and also by the augmented rate of entry into the S phase and the enhanced rate of mitosis in cultures recovering from the ara-C effects. It was again noted, however, that ara-C did not completely inhibit DNA synthesis in all cells, and therefore the G_1-S block was not absolute. Neil and Homan (1973) cautioned that the optimal spacing between two separate doses of ara-C, to achieve maximal killing of cells, would be longer than the predicted value (based on cell cycle kinetics) because of the slowed rate of entry of G_1 cells into S produced by the first dose of the drug. Bhuyan et al (1973) also observed that ara-C delayed the entry of L1210 cells into the S phase.

It was further ascertained that ara-C did not inhibit the progression of G_2 cells into mitosis, but it did prevent cells in S at the time of ara-C administration from progressing into G_2. Cells in G_1 at the time of administration were inhibited from entering S. These inhibitions lasted up to 28 hours and the maximal blockage was assumed to have occurred at the G_1-S junction (Wheeler et al, 1972).

More recently (Rao et al, 1974; Yataganas et al, 1974) it was shown that ara-C, even in low concentrations, could cause an accumulation of cells at the G_1-S boundary. It was suggested further that higher concentrations of ara-C, though more cytotoxic, were less favorable in achieving cell synchrony (Rao et al, 1974). Although a considerable degree of cell synchrony can be achieved with ara-C, it was suggested that progression through the cell cycle subsequent to removal of the drug (by centrifuging, washing twice in fresh medium, and then placing in fresh medium) might not be synchronous (Yataganas et al, 1974). Thus,

after removal of ara-C from in vitro cultures of leukemia cells, the LI was variable.

3. Cytotoxic activity. Ara-C is known to be cytotoxic to proliferating mammalian cells (Momparler, 1974). The cytotoxic effect of ara-C has long been blamed for causing an "unbalanced growth" of cells, or thymineless death, as demonstrated by Cohen and Barner (1954) for bacteria. The reason for the cytotoxity was attributed to synthesis of proteins and RNA continuing virtually undiminished for some time, while the rate of DNA synthesis had fallen to a very low level (Lark and Lark, 1964; Cohen, 1966). As synthesis of proteins and of RNA would continue in the absence of cell division, the unbalanced growth would result in the death of the cell. Thus, Cohen (1966) reported that the continuing RNA and protein synthesis in the absence of DNA synthesis caused a marked increase in cell volume which was morphologically reflected by an increase in cell diameter but without detectable vacuolization or other cytoplasmic damage. The chromatin was frequently condensed in the enlarged nuclei. Though Doering et al (1966) also noted that L cells exposed to ara-C doubled in size, they did not detect any clear evidence of nuclear damage. Bremerskov et al (1970) reported that ara-C added to in vitro L cells caused the appearance of pyknotic cells and a concomitant decrease in cell number which was directly related to the duration of the treatment. After 12 hours of exposure to 100 mg/ml ara-C, the nuclei became enlarged and the nuclear membrane thinned out. With prolonged treatment time, the chromatin became finer and finer, finally appearing pulverized leaving only a few remnants of heterochromatin. The cells increased in size

during the first 24 hours, after which time cytoplasmic vacuoles appeared. At 40 hours, the cell volume had increased to 2½ times normal. By 36 hours the protein content of the cells was 3 times that of normal cells.

Drewinko et al (1972) suggested that cell lethality might be the result of two consecutive mechanisms, the first being the inhibition of DNA polymerase by ara-C, resulting in unbalanced growth and subsequent death of cells. The second, that ara-C, could be incorporated into nucleic acid molecules causing strand breaks or blocking of terminal chains. Karon et al (1972) treated Don-C cells with 1, 10 or 100 mg/ml ara-C, and noted that, from 8 hours onward there was a gradual increase in cell size with a concomittant decrease in cell number. Further, cell survival correlated well with chromatid breaks, that is, cells with 5 or more chromatid breaks did not, as a rule, survive, but survival did not correlate with the inhibition of DNA synthesis. Other compounds, such as cycloheximide or tenuazonic acid, which simultaneously inhibit both protein and DNA synthesis, did not kill intestinal crypt epithelial cells. Moreover, crypt cells were protected from the cytotoxicity of ara-C by their simultaneous exposure to these latter two agents (Lieberman et al, 1970). The observations suggested that continued protein synthesis was necessary for DNA synthesis inhibition to culminate in cell death, and also, when both protein and DNA synthesis were inhibited simultaneously, the cells were protected from the lethal effects of the DNA synthesis inhibitor (Barber, 1971; Lieberman et al, 1970).

Bhuyan et al (1972) noted that the dose-survival curves of asynchronous cell populations exposed to ara-C decreased to a constant

saturation value at high doses, indicating the ara-C was cytotoxic to a specific phase or phases of the cell cycle. Observations on synchronized cells signified that, at both high and low concentrations of ara-C, the drug was specifically cytotoxic to S-phase cells. This was proved to be the case with L1210 cells (Bhuyan et al, 1973). Moreover, because ara-C blocked the entry of non-S-phase L1210 cells into the S phase, there was a greater degree of cell killing by the drug when it was administered in two doses 11 hours apart than when the cultured cells were exposed to it continuously. Both normal haematopoietic and lymphoma cells in mice, exposed to ara-C, exhibited a characteristic survival curve whereby survival decreased as the dose was increased, but only to a point, when a further increase in the drug produced no further reduction in survival. Ara-C also had a marked selective action on tumour cells when compared with normal cells, and the selectivity was most pronounced in early stages, decreasing after 12 to 24 hours (Bruce et al, 1969). Tobey (1972) reported that CHO cells in G_1 at the time of ara-C administration were spared from the cytoxic effects of the drug and Skipper et al (1970) reported likewise that ara-C was not significantly cytotoxic to resting cells which were not replicating DNA. It was recently observed (Yataganas et al, 1974) that the cytocidal effect of ara-C on in vitro leukemia cells was dose-dependent, with an initial lag phase before cells began to die. The suggestion was proposed that this lag period corresponded to the time required for cells to accumulate in S and to remain there for a sufficient time before dying. It should be noted that these investigators also found that, at all effective concentrations of ara-C, most cells continued to enter S at a near normal rate and were

blocked immediately upon entering S. Although ara-C reduced the surviving fraction in exponentially growing CHO cells to .5 from 1.0 by 1 hour after treatment with 100 mg/ml, cells exposed to the drug in the plateau phase remained unaffected by 75 mg/ml and their survival was greater than .95 following 100 mg/ml (Barranco and Novak, 1974).

According to Roberts and Loehr (1972), the addition of ara-C to cultured leukemic cells altered a number of parameters additional to DNA synthesis, hence suggesting a second locus of drug action. Higher concentrations of ara-C resulted in rapid cell lysis accompanied by changes in the synthesis of RNA and the level of thymidylate synthetase activity, suggesting that high doses of ara-C might interfere with RNA synthesis as well, and that this might contribute to cell death. Other authors (Borun et al, 1967; Chu, 1971; Nakai and Gugang, 1971) also observed that the impairment of DNA synthesis by ara-C produced an eventual inhibition of RNA synthesis.

4. Mode of action. It was ascertained that ara-C was a rapid and potent inhibitor of DNA synthesis, both in DNA viruses and in mammalian cells (Chu and Fischer, 1962 and 1965; Cohen, 1966; Robbins and Merrill, 1969; Bhuyan et al, 1972; Tobey, 1972). Although complete inhibition of DNA synthesis by ara-C was unlikely (Bremerskov et al, 1970), the inhibition was both concentration and duration-dependent (Doering et al, 1966; Bremerskov et al, 1970; Karon et al, 1972; Yataganas et al, 1974). In addition, as outlined above, ara-C was cytotoxic to cells, chiefly to those cells in the S phase of the cell cycle. The exact mechanisms whereby ara-C exerts these effects remains to be fully elucidated, but they do appear to implicate the enzyme DNA polymerase.

Several investigators asserted that the incorporation of ara-C (or its nucleotides) into the DNA chain caused an inhibition of DNA synthesis (Silagi, 1965; Chu and Fischer, 1969; Momparler, 1969 and 1974; Barranco, et al, 1972). Momparler (1972 and 1973), in in vitro studies with purified mammalian DNA produced a termination of polydeoxynucleotide growth. Other authors (Chu and Fischer, 1962; Graham and Whitmore, 1970) demonstrated that ara-C was incorporated into the internal nucleotides of DNA. Zahn et al (1972) added ara-C ½ hour before onset of the S phase to synchronized mouse lymphoma cells and thereby demonstrated that ara-C was incorporated into DNA. They ascertained that both the nucleoside and nucleotide fractions of arabinose were associated with labeled cytosine. It was estimated that 87% of the radioactivity incorporated into DNA was that of ara-C, whereas just 6% could be attributed to deoxycytidine. This would suggest that, of all the ara-C molecules incorporated into DNA, less than 6% were metabolically altered. Other authors also provided evidence that ara-C might become incorporated into DNA (Silagi, 1965; Creasey et al, 1968; Chu and Fischer, 1968; Chu, 1970; Graham and Whitmore, 1970).

In contrast to the aforementioned studies, Doering et al (1966), noting that DNA synthesis was accelerated when ara-C exposed cells were washed and placed in fresh medium, suggested it was unlikely that the drug had been incorporated into the DNA molecule. This assumption was further supported by Drewinko et al (1972), stating that, if ara-C did inhibit DNA polymerase, it would be difficult to understand how the analogue could be incorporated into the DNA chain by the enzyme it purportedly inhibited. He postulated rather that the inhibition of the

polymerase could be relative and dose-dependent, and that the inhibition could depend on the type of polymerase. The major effective locus of ara-C could be on the DNA template not so much by incorporation into the chain, but by the formation of complexes with the DNA which would inhibit the replication of DNA without directly affecting the polymerase.

More recently, Owens et al (1973), using proliferating mouse mammary epithelial cells as a test system, did not find any evidence for ara-C incorporation into DNA, and hence suggested that it was unlikely that ara-C could cause the formation of faulty mRNA. Graham and Whitmore (1970) stated that, in any event, the incorporation of ara-C into DNA was not the actual cause of inhibition of DNA synthesis and suggested several reasons for their contention. First, ara-C could inhibit DNA synthesis without affecting viability, suggesting that the inhibition must be reversible. Second, the incorporation of ara-C into newly synthesized strands of DNA did not protect the subsequent elongation of these chains when cells were subsequently incubated in the absence of ara-C. Third, although ara-C was incorporated into the nucleotide linkages, it was not terminally incorporated and could therefore not block further synthesis. Finally, the rate of incorporation of ara-C did not correlate with lethality. These investigators therefore concluded that the cytotoxic effect of ara-C was solely ascribable to inhibition of DNA synthesis. This is in direct contrast to the view of Momparler (1974), who concluded, after several studies, that both the incorporation of ara-C into DNA and the inhibition of DNA synthesis, as well as changes in nucleoside pools, played an important role in the production of the cytotoxic effects of the drug.

Although deoxycytidine, when added simultaneously can prevent the toxic effects of ara-C in vivo, deoxycytidine does not reverse the effects (Evans and Mengel, 1964). In addition, Moore and Cohen (1967) demonstrated that ara-CDP was no more effective in inhibiting ribonucleotide reduction in an in vitro system than was deoxycytidine diphosphate. This evidence suggests that the inhibition of the deaminase was not the major site of action of ara-C. As ara-CTP is a competitive inhibitor of deoxycytidine triphosphate for DNA polymerase, it is this latter enzyme that is now accepted as the prime target for the activity of ara-C (Kimball and Wilson, 1968; Momparler, 1972 and 1974). However, there is more than one type of DNA polymerase (Baril et al, 1973; Ove et al, 1973), and the action of ara-C may affect one or more than one type. Although Ove et al (1973) isolated 4 different DNA polymerase activities from rat livers and hepatomas, they could not assess the significance of the different activities at different cell loci. Baril et al (1973) encountered two mitochondrial forms of DNA polymerase in regenerating rat liver cells and in various hepatomas, designating them DNA polymerase I and DNA polymerase II. DNA polymerase I was the predominant form of polymerase in the normal rat liver and its quantity was not significantly augmented in regenerating liver cells, suggesting that this enzyme functioned in nonreplicative polymerization reactions, such as DNA repair. In contrast, the normally low activity of DNA polymerase II was augmented in regenerating cells and hepatomas, suggesting that this enzyme functioned as a replicative enzyme.

Studies in bacterial systems indicated that ara-C does not interfere with polymerase I (Gross and Gross, 1969), the repair enzyme, but

does inhibit DNA polymerase II, the enzyme involved in DNA replication (Rama Reddy et al, 1971). Nonetheless, although the prime enzymatic target of ara-C was a DNA polymerase, cell lethality did not bear a 1-to-1 relationship with the inhibition of DNA synthesis (Graham and Whitmore, 1970; Karon and Shirakawa, 1970).

In direct contrast to the aforementioned studies, several other investigators discovered that the activity of DNA polymerase I (6 to 8 S polymerase) increased significantly during cellular proliferation. It was therefore concluded to play a role in replicative function (Chang and Bollum, 1972a, 1972b, and 1973; Schrecker et al, 1974). DNA polymerase II (3.4 S polymerase) remained relatively constant as cells passed through the cell cycle (Chang and Bollum, 1972b and 1973).

It is generally agreed, however, that ara-C (or its nucleotides) inhibits the replicative polymerase, thereby blocking most cells in S (Fox et al, 1972; Schrecker et al, 1974; Yataganas et al, 1974; Momparler, 1974). It was suggested further that, because many other polymerases existed, one of them could well be critical in the initiation of DNA synthesis, and, when inhibited might therefore be responsible for a G_1-S block (Yataganas et al, 1974).

5. Chromosome breaks. Kihlman et al (1963) discovered after exposing human leukemia cells to a constant concentration of ara-C for varying lengths of time, that the number of chromatid aberrations increased with the length of exposure to the drug. They observed further that the addition of deoxyguanosine or cytidine reduced the indicence of aberrations, while the addition of deoxyribosides reversed completely the chromosome breakage by ara-C. On the basis of these observations, the investigators concluded that the chromosome breakage resulted from the

inhibition of DNA synthesis. In contrast, Brewen (1965) disagreed that ara-C induced chromosomal aberrations by interfering with DNA synthesis. He observed that ara-C did not induce chromosome aberrations in labeled cells, and concluded that ara-C was a very potent chromosome breaker only of G_2 cells. Although ara-C also inhibited DNA synthesis, it was thought that these two effects were not casually interrelated. The study was later confirmed by Brewen and Christie (1967), reporting that G_2 cells were uniformally sensitive to chromosome breaks by ara-C, but that such aberrations did not appear in S cells under the effect of the drug.

Benedict et al (1970) discovered that the induction of chromatid breakage in Don-C cells by ara-C occurred at essentially the same incidence whether cells were in G_1, S, or G_2 of the cell cycle. They postulated that, as chromatid breakage occurred during all phases of the cell cycle apart from M, the breakage could not have occurred during DNA synthesis alone. Because deoxycytidine prevented chromatid breakage, and it was most effective when administered to cells in the early part of S, the suggestion was proposed that ara-C may have interfered with both the DNA polymerase concerned with replication and the other involved in repair. This agreed with the theory of chromatid breakage as first postulated by Ahnstrom and Natarajan (1966). It should be noted that chromatid and chromosomal aberrations are morphologically different and may occur as the result of effects at different points in the cell cycle. Thus, some of the contradictory results in various studies could be ascribable to different definitions of breaks in chromosomes or chromatids. At the time DNA replication commences, a transition from chromosome- to chromatid-aberrations takes place. Thus, chromatid

aberrations are induced just prior to the onset of detectable DNA replication (Evans and Savage, 1963; Wolff and Luippold, 1964; Hsu et al, 1972). Accordingly, late S and early G_2 are likely the most sensitive phases of the cell cycle with respect to ara-C induced chromatid breaks (Benedict et al, 1970).

Effect of Ara-C on the Duodenal Mucosa

Inasmuch as ara-C is a potent inhibitor of DNA synthesis and is also cytotoxic to cells in the S phase of the cell cycle, it is not surprising that this analogue could exert these effects on any proliferating cell population. Ara-C has been observed to cause morphologic abnormalities and cell death in haematopoietic tissues in man (Bell et al, 1966; Dubois-Ferriere et al, 1970), in the mouse (Bruce et al, 1969), as well as in a variety of cultured cells (see cytotoxic effects). Moreover, single injections of ara-C to pregnant rats on days 10 to 12 of gestation produced malformations, including cleft palate and lip, encephalocele, and deformed appendages in foetuses which survived to gestation (Chaube et al, 1968). The lower half of the crypts of the small intestine comprises an active proliferation zone (Lamerton, 1972); therefore it is plausible that ara-C would adversely affect this normal tissue region.

1. Effect on morphology. Although severe morphological abnormalities of haematopoietic cells were observed in patients treated with ara-C, the small intestine of one of these patients did not reveal morphological abnormalities (Bell et al, 1966). This might indicate that the intestinal mucosa was less susceptible to damage by ara-C than were haematopoietic tissues.

Dethlefsen and Riley (1973) observed in mice that following a dose of 3 mg/gm of hydroxyurea (HU), recovery of the duodenal crypts was manifested by a lengthening of the crypts. Although the acute cellular responses ceased within 24 to 48 hours, the length of the crypts had not returned to normal by that time. These same investigators (1971) reported that the mean number of cells per duodenal crypt decreased initially after HU, returned to normal by 10 hours, and at 34 hours had increased. (It should be noted that the effect of HU on cells is similar to that of ara-C, that is, it is cytotoxic to cells in S and inhibits DNA synthesis.)

Leach et al (1969) administered to mice 15 mg/kg ara-C every 3 hours for 24 hours, and reported that the LD_{100} was 360 mg/kg (total dose). The most severe damage to the small intestine occurred 4 hours after the conclusion of the therapy. Severe degenerative changes in the crypts were observed, with large accumulations of karyorrhectic cells. In some cases there was vacuolization of the deep crypt cells. With sub-lethal doses, the small intestinal crypt was essentially normal again by 72 hours, while the villi remained normal throughout the course of the experiment. By 120 hours after the last injection, there was no observable damage to the mucosa. When higher doses were administered (close to the LD_{100}), a marked distortion of the intestinal mucosa occurred by 72 hours, with progressive disappearance of crypts and a shortening and widening of the villi. The villus stroma became infiltrated by inflammatory cells, mostly lymphocytic and histiocytic elements. The villus cells exhibited bizarre changes, such as a great increase in cell size and multiple nuclei, likely caused by duplication of the nuclei without accompanying division of the cell. Estensen and Baserga (1966)

reported that ara-C produced necrosis in the crypt cells of the mouse duodenum. However, the damage caused by ara-C was less severe than that by puromycin. Amionpterin, another drug blocking DNA synthesis, when administered to mice caused a denudation of the jejunal villi. Mitotic figures were absent in the crypts and the cells were flattened and contained large nuclei. The crypt lumina were dilated to approximately twice normal size, and were often filled with cellular debris (Rybak, 1962). Cohen et al (1972) studied the effect of bleomycin, another DNA synthesis inhibitor, on the mucosa of the mouse duodenum. A therapeutic dose (2 mg/kg) did not produce any changes in the mucosa during the first 4 hours following administration. Thereafter, cellular debris was observed in most crypts. Two hours after a dose of 200 mg/kg (1 to 1½ times the LD_{50} dose), a small amount of cellular debris appeared in the lower portions of the crypts, which became increasingly pronounced by 4 and 6 hours. Following a 300 mg/kg dose, the size and number of crypts diminished by 24 hours. The crypt nuclei were irregular and enlarged, containing less stainable chromatin material. The villi appeared normal, however. After three days, the number of crypts was reduced, while those remaining were hypertrophied and lined by cells with cytoplasmic hyperbasophilia. The nuclei of the crypt cells were irregular and slightly enlarged. Moderate numbers of inflammatory cells, chiefly mononuclear cells and polymorphonuclear leukocytes, infiltrated the interstitial stroma. The villi were slightly irregular, and shorter than normal.

Trier (1962a and b) noted after methotrexate administration that, though at the light microscope morphology of human jejunum appeared to be normal, marked ultrastructural changes had occurred within the first 6 hours.

However, despite these morphological ultrastructural changes, none of the patients exhibited gastrointestinal symptoms.

Although rats are less susceptible to the toxic effects of ara-C than mice (Lenaz et al, 1969), changes which might be induced in the intestinal mucosa by ara-C in the rat would likely be similar to those in the mouse. Though HU proved relatively non-toxic in rats (Philips et al, 1967), a single dose causing few outward signs of toxicity nonetheless was followed by large numbers of karyorrhectic cells in the duodenal crypt cells after 3 to 5 hours. At no time did the villus cells show signs of injury. With a sublethal dose of ara-C to rats, Verbin et al (1972) reported apparent crypt cell damage within $2\frac{1}{2}$ to $3\frac{1}{2}$ hours. The lesion manifested itself by numerous intracellular eosinophilic inclusions, occasionally containing fragments of basophilic debris which was often Feulgen positive. The epithelial cell nuclei appeared to be normal, however. By 8 hours, only a few intracellular inclusions remained. Recovery, as measured by the disappearance of intracellular debris, continued so that beyond 16 hours cytological differences could not be detected between the control and ara-C treated animals. In a further study Verbin et al (1973) noted the following changes in the intestinal mucosa of rats following treatment with ara-C: a. An increased prominence of lymphocytes in the epithelial lining of the crypts, b. pyknosis and karyorrhexis of the nuclei of intraepithelial lymphocytes, c. abundant, necrotic cells in the lamina propria, and d. development of cytolysosomes within the crypt epithelial cells.

2. Effect on cell kinetics. Dethlefsen and Riley (1970) reported that HU inhibited the LI in mouse duodenum for 4 to 5 hours. The inhibition was followed by a subsequent LI peak at 8 hours, another trough at 12 hours, and a return to control values by 16 to 20 hours. It was further noted that there was evidence of a partial synchronization of the crypt cells. The effect of this drug on the MI did not parallel its action on the LI. The MI remained inhibited for 6 hours, attained a peak at 11 hours, and by 16 hours had declined again below control values. The DI was increased for the first 4 to 6 hours, and then decreased steadily for the next 12 hours. The authors suggested that cells which were killed by the drug were rapidly eliminated from the duodenum. Cohen et al (1972) reported that a therapeutic dose of bleomycin caused a marked depression of the incorporation of H^3-TdR in the mouse small intestine. Maximal inhibition occurred 6 hours following the administration, and recovery was observed by 24 hours. The MI declined from normal 2.5 to less than 1.0, 2 hours following the injection; by 6 hours the MI had returned to the control values.

Leach et al (1969), administering sublethal doses of ara-C, noted a considerable reduction in the MI in the small intestine of mice who received 15 mg/kg ara-C every 3 hours; that reduction became less severe at 24 hours. By 72 hours the MI was normal or just slightly elevated.

Lenaz and Phillips (1970) reported that following an initial drastic reduction of the LI in the rat small intestine during the first 3 hours after an injection of ara-C, recovery was evident by 6 hours. Ara-FU, which produces a similar effect to that of ara-C (Lenaz et al, 1969) caused, one hour after a therapeutic dose, complete inhibition of the LI.

The LI was 10%, 30%, and 85% of those of control values at 2, 4, and 6 hours, respectively. Lieberman et al (1970) reported that, with a dose of 250 mg/kg ara-C, there was an almost complete inhibition of DNA synthesis in the rat small intestine within 10 to 15 minutes. With the same dose of ara-C, Verbin et al (1972) reported that DNA synthesis in rat tissues fell to 2% of control values within ½ hour and remained at this low level for the next 4½ hours. The rate of DNA synthesis returned to approximately 75% of control values by 10 hours. In contrast, the mitotic activity, markedly reduced at 1½ hours, completely abated within 2½ hours. Subsequently, mitoses began to reappear but their incidence was still below normal at 8 hours. They increased progressively in number from then on, reaching a peak at 12 hours when there was a 3-fold increase over control values. The rate of cell division subsequently declined, returning to normal levels by 16 to 24 hours. It should be noted that, in contrast to several investigators who reported increased degenerating indices or other indications of cell death in the intestinal mucosa, Verbin et al (1972) did not observe any definitive evidence of epithelial cell necrosis following ara-C. The latter authors reported that the preservation of the structural integrity of all stem cells was readily apparent, even at 3½ hours, the time at which karyorrhexis and other stigmata of cell death, according to other workers (using the light microscope) were maximal. Verbin et al (1972) concluded that the apparent necrosis of the intestinal crypts noted with conventional microscopy represented phagocytosis of degenerating lymphocytic elements by adjacent crypt epithelial cells.

In contrast, Lieberman et al (1970) suggested that cell damage induced by ara-C in the intestinal crypt epithelium of mice was likely ascribable to a metabolic reaction of the cells to some preceding biochemical lesion, rather than to the biochemical lesion per se. Because damage by ara-C could be prevented by the administration of agents which inhibited protein synthesis, it was suggested that the reaction depended upon new protein formation, perhaps akin to enzyme induction. Since DNA synthesis inhibition and/or the direct alteration of DNA appeared to be early and important biochemical lesions caused by ara-C, a special more or less direct metabolic link could occur between some phases of DNA metabolism and enzyme induction in some types of continually proliferating cells. The enzymes induced could be active in various forms of DNA repair, and an overproduction of one or more of these could cause irreparable damage to vital macromolecules in the cell.

It should be noted that several investigators reported that a definite relationship between morphological damage to the mucosa and cell kinetic disturbances did not exist, following exposure to CCS agents. Cohen et al (1972) noted that the morphological evidence of crypt damage in mice following bleomycin did not correlate with abnormalities in either the amount of H^3-TdR incorporated or in the MI, nor did the incorporation of H^3-TdR correlate with the MI itself. For example, the MI declined to approximately half of control values one hour following an injection of 20 mg/kg ara-C, but the incorporation of H^3-TdR remained unaffected at this time. Although a 2 mg/kg dose did not produce any morphologically discernible damage by 2 hours, mitotic figures were rare. However, by 4 hours, when both the MI and the incorporation of H^3-TdR had

returned to normal levels, small amounts of cellular debris appeared in the crypts. Verbin et al (1972) reported that, at the time intracellular inclusions began to disappear from epithelial cells of rats following treatment with ara-C, mitoses reappeared, although their incidence was still much below normal. The latter investigators suggested that the return of a normal MI seemed to parallel the recovery in terms of morphological damage.

3. Synchrony of the intestinal epithelial cells. Dethlefsen and Riley (1973) studied duodenal crypt cells of mice before and after a single injection of HU. They reported that this dose killed cells in S at the time of injection and inhibited DNA synthesis completely for 4 to 5 hours. This caused a partial synchronization of cells when they recovered from the inhibitory effects of the drug. Verbin et al (1974) synchronized the intestinal cells of the rat by a 250 mg/kg dose of ara-C, followed 2 hours later by an injection of colcemid. When ara-C was followed by colcemid, 33-38% of the crypts were in mitosis, compared to control values of 3 to 5%.

The Effect of Ara-C on Tumours

Ara-C was shown to be markedly inhibitory to the growth of many animal tumours (Cardeilhac and Cohen, 1964; Wodinsky et al, 1965), to various forms of human and animal leukemias (Dowling et al, 1972; Evans et al, 1965; Kline et al, 1966), and to melanoma cells in vitro (Barranco et al, 1972).

Evans et al (1961) noted that if a dosage of ara-C (5 to 50 mg/kg/day) was administered on the first day of transplant and continued

for 7 days, a high proportion of mice were free of Sarcoma 180, Ehrlich ascites carcinoma, or L1210 tumours 7 days after discontinuation of the drug. When observed over a 25 to 30 day period, many transplanted tumours which did actually develop regressed completely without ever becoming necrotic and perforating. When the daily treatment was delayed until the fifth day after transplantation, the number of tumours which developed was almost unaltered from that in untreated animals, yet there was a significantly greater number of subsequent regressions. Chen and Mealey (1972) reported a significant effect of ara-C on the growth of 5 glial tumour cell strains. Growth inhibition was found to be both concentration and duration-dependent. However, when doses reached the level of .5 mg/ml, the inhibitory response was not further accentuated. Barranco et al (1972) obtained, from a single melanoma, 4 culture lines ranging in pigment production from heavy to light. Following a one-hour treatment with 10 mg/ml ara-C, the survival of the heaviest pigmented strain rapidly decreased to 50%; higher doses did not significantly enhance cell death. It was noted that the percentage of cell kill was very close to the LI of the strain. In comparison, the lightly pigmented cell strain was not significantly affected by the identical dose of ara-C. It was suggested that, because the lightly pigmented strain contained 50% less kinase than the heavy strain, it was less susceptible to the effects of ara-C, as the reduced kinase activity would lead to a reduced rate of phosphorylation. Hart et al (1972) treated 18 patients afflicted with metastatic melanoma with a five-day regimen of ara-C every three weeks. Of the 13 patients who received adequate treatment, there was only one excellent response (as indicated by a decrease in skin tumour size),

persisting for 8 months. Two responses were transient partial responses. Bertalanffy and Gibson (1971) reported that a 50 mg/kg dose of ara-C reduced the daily mitotic rate (DMR) of the B16 melanoma from approximately 46% to 15%, whereas an 80 mg/kg dose reduced the DMR to just 25%. Hart et al (1972) observed that in 18 melanoma bearing patients, the highest LI of the melanoma occurred in those patients who responded effectively to ara-C treatment. In patients who failed to respond to the treatment, a 50% to 100% decrease occurred in the LI on day 5 of therapy, but there was a complete or nearly complete recovery by the twenty-first post-therapy day. In the one patient, who achieved maximal response, the marked decrease in the LI was maintained past the twenty-first post-therapy day.

Bertalanffy and Gibson (1971) reported that the DMR of the B16 melanoma was reduced from a control value of approximately 45% to 15% and 25% following either a 50 mg/kg or 80 mg/kg single dose of ara-C, respectively. Moreover, when 3 series (each one followed by a host recovery period) of 4 injections were administered of either 50 mg/kg, 25 mg/kg, or 12.5 mg/kg of ara-C, though the DMR was most effectively reduced following the 50 mg/kg procedure, the latter regimen was toxic to the animals. The 12.5 mg/kg dose was not toxic to the host. However, the reduced MR (to less than 2%) recovered following the second recovery period to exceed that of the controls. The 25 mg/kg regimen depressed the MR to less than 1%, but proved somewhat toxic to the host animals. Following a single 50 mg/kg dose of ara-C, the LI of the B16 melanoma, with a control value of approximately 14%, was reduced to 2% and to 0.21% 15 minutes and one hour following the injection. The effect was transitory, however, as this index had recovered by 12 hours, and the MR overshoot exceeded control values at 16 hours. Finally, these investigators estimated that, though a 50 mg/kg dose

of ara-C shortened the S phase of the B16 melanoma from 7.5 to 6 hours, the total duration of the cell cycle was lengthened by approximately 6 hours, most of which could be accounted for by an extension of the G_1 phase.

Although there are several reports of ara-C synchronizing the cell cycle in in vitro systems (Rao et al, 1974), there is a dearth of recent information with regard to in vivo synchronization. Lampkin et al (1972) noted that ara-C caused synchronization of leukemia cells in humans at 18 to 24 hours following ara-C treatment. Subsequent ara-C infusions were administered 6 to 12 hours following synchrony in an attempt to kill more cells. Though remissions of varying durations occurred in all patients treated in this manner, the therapy was accompanied by marked thrombocytopenia and severe leukopenia. Gibson and Bertalanffy (1972b) attempted to synchronize the cell cycle of the B16 melanoma, applying 6 different time and dose combinations of ara-C. In one series, the synchrony index (SI) was 40.7, following 8 i.p. injections of 12.5 mg/kg given 2 hours apart. This SI was 77%, adjusted to take into account the G.F. of the melanoma of 0.53. According to these investigators (Gibson and Beralanffy, 1972b), maximum labeling occurred 10 hours and again 24 hours following the last ara-C injection. These investigators calculated that the cell cycle time of the melanoma cells remained unaltered at 14 hours, assuming that the peaks of the LI indicated the mid-point of the S phase.

Effect of Ara-C Treatment on the Survival of Mice Bearing Tumours

Several investigators (Evans et al, 1965; Kline et al, 1966; Vadlumdi and Goldin, 1971; Grindey et al, 1972; Avery and Roberts, 1973) provided evidence that ara-C was effective in extending the MST of mice

bearing L1210 leukemia. Evans et al (1965) demonstrated ara-C to be effective against the L1210 leukemia and three types of ascites tumours in mice, as judged by the increased MST. However, ara-C did not exert any significant effect on the MST of mice bearing a transplantable mast cell or plasma cell tumour. Further to this, Kline et al (1966) noted, in terms of MST, that ara-C was more effective in the early stage of L1210 leukemia growth than in the more advanced stages. It was also reported that multiple treatments of mice with ara-C resulted in a marked increase of the MST afflicted with both early and advanced forms of leukemia. It was noted, however, that there was a very narrow therapeutic margin of safety, that is, the optimal treatment dose was very close to the toxic dose. Several smaller doses spread over a day were found to be less toxic than a larger single daily dose. This was substantiated by Skipper et al (1967). Bertalanffy and Gibson (1971) reported likewise that the toxic dose was rather close to the generally employed optimal therapeutic dose. Wodinsky et al (1967) noted that 2 administrations of 250 mg/kg ara-C doubled the MST of L1210 leukemia bearing mice.

X-IRRADIATION

Introduction

Since the discovery of x-rays by Roentgen in 1895, and the subsequent description of the injurious effects of x-irradiation on the skin by Marcuse (1896), these rays have been widely employed in both diagnostic and in therapeutic procedures.

X-rays are shorter than ultraviolet (UV) rays but longer than gamma

rays (Glasser et al, 1949). They are propagated in straight lines, can penetrate many materials, become "harder" after having penetrated several absorbers, and produce different types of scattered and secondary radiations.

The roentgen (R) is a unit of x-ray quantity based on the ionization in air. An x-ray transfers its energy to matter by interacting with an atomic electron, thereby yielding an energetic secondary electron which subsequently loses its kinetic energy by multiple interactions with other electrons. In a gas, this causes a trail of ions along the path of the secondary electron. Most of the ionization present in air resulted from such secondary electrons. A roentgen, then, is defined as the exposure of x- or gamma-radiation, so that the associated corpuscular emission per 0.001293 gram of air produces, in air, ions carrying one electrostatic unit of electricity of either sign (International Commission on Radiation Units and Measurements, 1956). The term "associated corpuscular emission" refers to the secondary electrons.

When dealing with biological materials, it is usually more expedient to refer to x-irradiation in terms of absorbed dose, or tissue dose. The absorbed dose is one rad (radiation absorbed dose), and may be defined as the energy absorption of 100 ergs per gram of any material. The rad/roentgen ratio for deep x-rays (250 kVp) is approximately 0.95. With x-rays, the maximum absorbed dose occurs at the surface of the tissue.

Mechanism of Action of X-irradiation and Its Effects on Cells

Generally, x-rays dissipate energy in matter by causing ionization of atoms, that is, ejection of an electron from its atom or molecule with

the resultant production of two ions, one being the negatively charged electron, the other the positively charged residual atom or molecule. The exact mechanism of action of ionizing radiation in tissues is undetermined. However, it is generally believed that the biologic effects of radiation are in some way ascribable to this production of ions within living cells (Glasser et al, 1949).

There are two concepts regarding the effects of ionized radiation on living systems, a direct or molecular concept, and an indirect or metabolic concept. The first concept, known variously as the "direct action", "target", "quantum hit", or "point heat" theory, states that molecular particles constitute the vital target or sensitive areas within a cell. If one (or several) ion pairs are formed within this sensitive volume, the cell will be severely damaged, or will die (Dunlap, 1961).

The indirect action theory assumes that changes of a more diffuse nature occur within the cell, releasing injurious substances, usually assumed to be protein metabolite products or peroxides. Whether cells survive or succumb depends on their individual ability to withstand these injurious agents. Water was implicated to play an important role in some of these indirect reactions (Patt, 1950; Lea, 1956; Bacq and Alexander, 1961).

Both concepts assume that chemical changes are produced by the ionizing radiation, either by the destruction of important molecular species, or by the production of agents that influence cell metabolism.

1. Cell morphology. Although a great deal of work was done on the effect of x-irradiation on chromosomes (see below), there were relatively few studies of other cytological effects. At the light

microscope level, cellular irradiation damage may become expressed as pyknosis or karyolysis of the nucleus, margination of chromatin, and either shrinking or swelling of the nucleus or cytoplasm (Prasad, 1971). Giant cells were reported by Puck and Marcus (1956) to appear, following x-irradiation, in cell culture suggesting that they formed as a consequence of the inactivation of the reproductive capacity by irradiation (Puck et al, 1957). These cells often contained a single large nucleus, many micronuclei, or a combination of both, and likely resulted from the mitotic apparatus having been so severely damaged that it was incapable of organizing the chromosomes into two equal groups in metaphase. The chromosomes became either enveloped by a single membrane, thereby forming a giant polyploid nucleus, or else they fragmented into smaller groups, causing micronuclei. Till and Whitmore (1958) observed that irradiated cells could proceed through a number of chromosome duplications following irradiation without dividing, thereby giving rise to giant cells.

The observations by Puck and Marcus (1956) on irradiated HeLa cells, suggest that mammalian cells, irradiated with high doses, can maintain at least some degree of metabolic activity even though they are no longer able to continue proliferation. Moreover, Tolmach and Marcus (1960) reported that HeLa cells increased in volume immediately after irradiation and maintained an exponential increase over at least 10 days, during which time the average volume augmented to approximately 20-fold. The volume doubling time was estimated to be approximately 34 hours and was relatively dose-independent (Tolmach, 1961). Whitmore et al (1958) observed that the cell volume of irradiated mouse L cells increased logarithmically for approximately 160 hours, with a cell volume doubling time

of 70 hours. It was asserted that all cultured cells would increase in volume following irradiation (Tolmach and Marcus, 1960).

Mouse oocytes are known to be particularly sensitive to the effects of ionizing irradiation (Parsons, 1962), and have hence been used to observe cytological effects brought about by small doses of x-irradiation. Following a dose of 7R, some cell shrinkage was evident (at the light microscope level) after 6 to 7 hours, but there was no evidence of cell necrosis at this time. At 7 hours, the nucleus exhibited clumped chromatin and irregularities in the nuclear shape, and after 8 hours some of the cells appeared swollen. Necrosis of cells was complete by 12 hours when the cells were destined to die; other cells were still essentially normal by 24 hours and survived.

Parsons (1962) reported that similar alterations were observed with higher doses as well, though the changes tended to occur earlier. Upton (1963) concurred, and reported that, within minutes after irradiation, the nucleus of the cell displayed characteristics of degeneration, including swelling, vacuolization, rupture, and fragmentation. It was noted further that intranuclear components, such as chromosomes and nucleoli, may leak out of the severely damaged nucleus (Hugon et al, 1960).

Although nuclear damage caused by x-irradiation is very readily observed, the greatest impact of irradiation damage to cells may be in the more radiosensitive cytoplasm (Berdjis, 1971). It was thus reported that all cytoplasmic organelles showed abnormalities following x-irradiation (Bloom, 1958; Errera, 1959; Errera and Forsberg, 1961). According to Parsons (1962), following a dose of 200R (250 kv) to mouse oocytes, a marked depletion of mitochondria occurred within 2 minutes, returning to

normal numbers 10 to 20 minutes later. As the number of mitochondria began to increase, they diminished strikingly in average diameter. Frederick and Chevremont (1953) noted that spherical mitochondria could readily fragment into two or more mitochondria within one minute after x-irradiation. Other workers (Noyes and Smith, 1959) found the number of mitochondria in the rat liver cells markedly decreased following doses of 1000R gamma rays. These same investigators noted further that the average unit mass of mitochondria increased as the mitochondria decreased in quantity. At the light microscope level, MacCardle and Congdon (1955) observed that doses of 500, 900, and 1200R caused mouse liver mitochondria to become globulated, fragmented, and vesiculated 6 to 8 hours following irradiation. These changes were more pronounced with increased doses, when the mitochondria tended to become concentrated at the periphery of the cytoplasm.

Parsons (1962) reported that a 200R dose in mouse oocytes caused, within a few minutes, a slight loss of ribosomes from the endoplasmic reticulum. Hampton and Quastler (1950) observed that, 3 days following a 3000R dose of x-rays, there was a marked depletion of RNA granules in mouse intestinal epithelial cells. Bacq and Alexander (1961) postulated that many of the severe effects of irradiation were caused by damage to the cell membranes, specifically, damage to the macromolecules constituting both intracellular components and cellular membranes. Alteration of cellular permeability following irradiation is well-known (Bacq and Alexander, 1961), as are marked disruptions of the cell membrane of irradiated tumour cells (Goldfeder and Miller, 1963) and of duodenal cells (Quastler and Hampton, 1962). It is unknown, however, whether the loss of

RNA from the endoplasmic reticulum is accompanied by an actual loss of RNA from the cell.

Various types of vacuolization following x-irradiation to cells were observed (Bari and Sorenson, 1961; Gerendas, 1965; Hugon et al, 1965; Leroy et al, 1966; Berdjis, 1968), some of them discernible with the light microscope. Hampton and Quastler (1960), following irradiation to the crypt intestinal cells of mice, further noted that the cells were markedly abnormal, some being binucleated, containing large and numerous lipid granules, and disorganized endoplasmic membranes. The number of mitochondria was reduced as was the amount of endoplasmic reticulum.

2. Biochemical effects of irradiation. A précis of the biochemical effects of x-irradiation follows. More complete reviews were published by Kelly (1957), Lajtha (1960), Goutier (1961), Quastler (1963b), Elkind and Whitmore (1967), and Berdjis (1971).

According to Lajtha et al (1958), and Ord and Stocken (1958) cells irradiated while in the S phase exhibited DNA synthesis inhibition that was dose-dependent. However, the response curve was a typical "two component" curve, indicative of two cell components with different sensitivities. Creasy and Stocken (1958) observed that nuclear phosphorylation of nucleotides was inhibited within 3 minutes after doses of 50 to 100R, suggesting that the sensitive component in the aforementioned curves was a suppression of nuclear phosphorylation. Both Lajtha et al (1958) and Ord and Stocken (1958) postulated that the least sensitive component was radiation-induced loss of the ability of the existing DNA to act as a template for the synthesis of new DNA. Wheeler and Okada (1961) discovered that doses much higher than those used by the previous investigators were

required to reduce the priming ability of DNA of calf thymus cells. Also, Weiss and Wheeler (1964) demonstrated that if DNA alone was irradiated, a dose of 12 krad reduced the priming ability to 2% of control values. When nucleoprotein was irradiated with the same dose, however, the priming ability was 72% of control values. This suggested that irradiation affected only the unsheathed (by histones) portions of DNA and that large doses resulted in a separation of DNA and nuclear proteins, thereby increasing the priming ability. More recently, Baver and Oliphant (1972) reported that the loss of histone from the irradiated rat thymus paralleled the DNA loss. However, a preferential loss of a specific histone was not observed. These investigators postulated that the DNA-histone relationship could play an important role in the mechanism whereby ionizing radiation ultimately led to death in cells.

Sherman and Quastler (1960) and Cattaneo et al (1960) reported that in vivo irradiation of mouse intestinal epithelial cells and hair follicles was followed almost immediately by a 50 to 70% inhibition of DNA synthesis; that inhibition was relatively independent of the dose. Small doses of irradiation, given before the onset of DNA synthesis, delayed synthesis in the regenerating rat liver, suggesting that subsequent DNA synthesis was inhibited even when irradiation was administered during the G_1 phase (Beltz et al, 1957; Bollum et al, 1960; van Lancker, 1959 and 1960a and b). Because it is likely the level of kinase activity which limits the rate of DNA synthesis rather than the level of polymerase activity (Bollum and Potter, 1959), that enzyme might also control the initiation of DNA synthesis. Bollum and Potter (1959) reported that whole body irradiation (WBI) to partially hepatectomized rats before onset of

enzyme formation or DNA synthesis, resulted in kinase and polymerase activities being reduced to 1 and 20% (respectively) of the control values. When the animals were irradiated just prior to the peak levels of both enzymes or when appreciable DNA synthesis occurred, there was no effect on either enzyme activity. This would suggest that, rather than affecting the enzymes directly, irradiation interfered with their formation, that of the kinase more so than that of the polymerase. Elkind and Whitmore (1967) agreed that irradiation late in G_1 exerted little effect on DNA synthesis, because the previously formed enzymes would be insensitive to irradiation damage. Mak and Till (1963) made the observation that though DNA synthesis, once it had commenced, could be impaired by irradiation, the impairment was likely temporary.

It was reported that RNA synthesis was less sensitive to irradiation than DNA synthesis. Thus, Holmes and Mee (1952) noted that a dose of 2000R had reduced DNA synthesis in a rat sarcoma by 75% 1.5 hours after irradiation, whereas RNA synthesis was not appreciably affected. Beltz et al (1957) similarly observed that doses up to 3000R remained without impact on RNA synthesis in regenerating rat liver 24 hours after irradiation. Whitmore et al (1961) reported that 2000R administered to mouse L cells did not alter the rate of RNA synthesis for the duration of one generation time. In contrast to these findings, Abrams (1951) noted that a dose of 800R reduced both RNA and DNA synthesis of rabbit bone marrow cells to an approximately equal degree during a 5-hour post-irradiation period. Logan et al (1959) similarly reported that doses of 50 to 9000R to calf thymus nuclei reduced RNA synthesis to 40% of the control values during the first 2 hours after irradiation, but that DNA

sythesis remained unaffected. Although RNA synthesis was relatively unchanged in a rat sarcoma soon after irradiation, 48 hours later the degree of both DNA and RNA inhibtion was approximately equal. Whitmore et al (1961) reported similar findings with mouse L cells that had passed through more than one generation time after the administration of irradiation. Harbers and Heidelberger (1959) observed that nuclear RNA synthesis was reduced by 50% in Ehrlich ascites cells, whereas cytoplasmic RNA synthesis was slightly greater than normal. More recently, Banarjee and Hornsey (1972) ascertained that with a dose of 2 krads, DNA and protein content of cells was similar to that of controls. Nevertheless, the RNA content was lower. They suggested that the synthesis of RNA was more easily impaired by high doses of irradiation than was that of either DNA or protein. Schjeide et al (1972) reported a decrease in the synthesis of new ribosomes and a slowing in the growth of endoplasmic reticulum following irradiation to the livers of young chickens. Verma and van Huystee (1971) observed that a 500 krad dose reduced protein synthesis to 45% to 55% of control values in peanut cells in suspension. At 3 to 4 days after irradiation, a recovery occurred, with an overshoot at one week. Although the quantity of protein synthesis returned to normal, the kinds of proteins that were synthesized were markedly abnormal. It was suggested that induction of such errors in protein metabolism could culminate in giant cell formation, abnormal growth, and subsequent cell death.

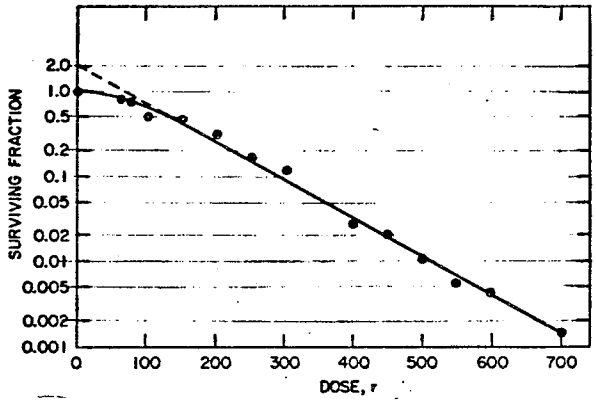
3. Radiosensitivity and its relationship to the cell cycle. In 1906 Bergonie and Tribondeau asserted that radiosensitivity of a cell was proportional to its functional and morphological differentiation. It was

pointed out, however (Berry, 1969), that there was no clear difference in the radiosensitivity of tumour cells of wide range of morphological types nor were there large differences in the radiosensitivities of normal cells in comparison to many tumour cells. Nonetheless, it is now an accepted fact that proliferating cells are much more radiosensitive than non-proliferating cell populations. Consequently, tissues with a relatively large proportion of the cell population proliferating (such as haematopoietic tissues, intestinal epithelia, or tumours with a large growth fraction) are much more radiosensitive than are tissues with minimal cell proliferation (for instance kidney or central nervous system tissue). Moreover, it is unlikely that even proliferating cells remain uniformly radiosensitive throughout the cell cycle.

Much of the reported research on irradiation effects on in vitro cell systems were reported in relation to cell survival curves. A review of the survival curve theory was presented by Elkind and Whitmore (1967).

Puck and Marcus (1956) published the first survival curve of in vitro cells following irradiation. Following irradiation with 100 to 200R per minute of HeLa cells, the log of survival was plotted as a linear function of the dose. As is evident from Chart 3, the sigmoidal curve has a threshold in the region of small doses, followed by two or three decades of exponential survival. When the straight line portion of the curve is extrapolated back to zero dose, it intersects the ordinate at a value greater than 1.0. This intersection is termed the extrapolation number (n). The dose required to reduce survival by the factor 1/e (≈ 0.37)

CHART 3 - SURVIVAL CURVE OF HeLa CELLS EXPOSED TO 250 kVp X-RAYS, 100-200R PER MINUTE (Puck and Marcus, 1956).



is referred to as Do. The width of the shoulder region can be determined by that dose at which the back extrapolate intersects the abscissa. It is known as the quasi-threshold dose (Dq). In this particular case n is greater than 1.0, indicating that damage must be accumulated before a cell is killed (Elkind and Sutton, 1960). Because a given increment of the dose becomes more effective as total dose is increased, this is a further indication that damage must be accumulated before a cell could be killed. Subsequent to this initial work, survival curves were published for a variety of cell lines, including those of normal human liver, conjunctiva, appendix, spleen, skin, and ovary (Puck et al, 1957), of two sublines of the original HeLa S3 line (Lockart et al, 1961), and mouse L-cells (Whitfield and Rixon, 1960). Although the shape of all these curves was sigmoidal, it was noted that there was some variation in the radioresponse, even within a particular cell line. It was recognized early that these variations (particularly in the $\tilde{n}$ and Do) could be representative of mixed cell populations, specifically of subpopulations of cells with varying radiosensitivities. The dependence of the survival

response on cell age is referred to as the age-response function and was investigated in a number of in vitro systems.

In a series of experiments, Terasima and Tolmach (1961, 1963a and b) examined several properties of partially synchronized HeLa S3 cells. They concluded that mitotic cells were likely radiosensitive, that cells became more responsive during G_1, less responsive during S, and exhibited a near minimum constant response in G_2. They suggested that DNA was the sensitive molecule in x-ray killing (Terasima and Tolmach, 1963b). Other investigators studying the same cell line, concurred that the M phase was the most radiosensitive, the S phase the least radiosensitive phase, and that radiosensitivity varied within G_1 and G_2. Thus, Terasima and Tolmach (1963a) reported a near minimal response of G_2 cells, although sensitivity increased as cells progressed through this phase (Djordjevic and Tolmach, 1967). Other workers ascertained that the radiosensitivity of cells throughout G_1 was not constant, cells becoming more sensitive as they progressed to reach a maximal sensitivity late in the phase (Tolmach et al, 1965; Phillips and Tolmach, 1966; Kollmorgen, 1969). It was further reported that, though cells were eventually killed when irradiated in G_1, they progressed through one and one-half cycles before dying (Hopwood, 1972).

Sinclair and Morton (1964, 1965a and b) examined the age-response dependence of a subline of CH cells following partial synchronization by mitotic selection. Survival during the first G_1, G_2, and M periods following irradiation was approximately equal and amounted to about one-third that of S-phase cells. Analysis of the survival curves of the various subpopulations revealed that the shoulder of the curve of S cells was

broader than that of the curve of cells not in S at the time of irradiation. Subsequently, Sinclair (1967) reported that CH cells were equally radiosensitive in M and G_2, followed by G_1, early S, and late S. This conclusion was substantiated by several other investigators (Dewey et al, 1970; Hall et al, 1972; Kellerer et al, 1972; Nagle and Humphrey, 1973). Dewey et al (1970) noted that to kill synchronized CH cells in G_1 required doses approximately 1.5 times greater than that required to kill cells in metaphase. Cells in late S required 2.8 to 4.2 times the dose necessary for killing in late metaphase. In contrast, Hsu et al (1962) reported that cells in G_2 were three times as radiosensitive as S-phase cells, but the radioresponse of G_1 cells did not differ from that of S-phase cells.

With regard to other cell lines, Drewinko and Humphrey (1973) observed that the radioresponse of synchronized human lymphoid cells was similar to that of CH cells as previously reported by Sinclair (1967). Ludovici et al (1961) claimed the M phase to be the most radiosensitive in three of four human cancer cell strains. Evans (1967) found that, although the G_1 phase was the most radiosensitive of a plant cell line and a human leukocyte cell line, that phase did not appear to be uniformly radiosensitive. Concurring with this is a more recent study in which the cells of a mammary tumour exhibited two radiosensitive periods, namely M-G_1 (or very early G_1) and G_1-S and lasting well into S. The two radioresistant phases were mid-G_1 and S-G_2 (Rockwell and Kallman, 1974).

Dewey and Humphrey (1963 and 1965) reported that mouse L cells were most radiosensitive in S, most radioresistant in G_1, with G_2 cells being intermediate. It was proposed that the different radioresponse of

these cells (when compared to many others) might be ascribable to the lower number of chromosomes in this line. According to Erikson and Szybalski (1963), the survival of human, marrow-derived cells increased when the cells were irradiated in the S phase as compared to irradiation of non-S-phase cells.

Elkind and Whitmore (1967) cautioned that comparison between studies of this type must take into account the fact that different cell lines were employed, both of normal and malignant cells. In addition, variations in the synchronization procedure could conceivably account for some of the variation. Despite these obstacles, the principal features of the survival of in vitro cells exposed to irradiation at various times in the cell cycle can be summarized as follows:

1. Generally, cells are most sensitive to irradiation during the M phase.

2. If G_1 occupies an appreciable length of the cycle, a resistant period may be evident early in this phase while radiosensitivity late in this phase is much more pronounced, approaching that of M-phase cells.

3. Radiosensitivity increases sharply from early G_2 to a level approaching that of mitosis.

4. The S phase is least radiosensitive, and less so in the later part of the phase.

Hewitt and Wilson (1961) were the first workers to plot suvival curves of CBA leukemia cells irradiated and assayed in vivo. The survival curves were very similar to the in vitro curves described previously. Hornsey and Silini (1962) and Silini and Hornsey (1961 and 1962) irradiated Ehrlich ascites cells in vitro and assayed for survival in vivo. Again,

the survival curves were similar to in vitro survival curves, though the $\tilde{n}$ value was larger.

Dendy and Cleaver (1965) observed that ultraviolet (UV) radiation depressed DNA synthesis, the degree of which depended on the time a cell spent in the S phase. Further, the sensitivity was greater with increasing DNA content of cells. Dewey et al (1970 and 1972b) noted a linear correlation between survival of irradiated cells and incidence of chromosome aberrations, suggesting that as cells moved from M to S, there was a dispersion of chromatin which correlated well with increased radioresistance. Condensation of the chromatin could lead to enhanced radiosensitivity and inhibition of repair of chromosomal damage. Finally, Langguth and Beam (1973) ascertained that diploid strains of cells were more radioresistant than hexaploids. Haploid strains exhibited the longest radioresistant phase, while hexaploids showed the shortest.

Other factors contributing to radiosensitivity include: (a) cells are more radioresistant under hypoxic conditions than when well-oxygenated (Berry et al, 1960 and 1970; Littbrand and Revesz, 1969; Malone et al, 1971; McNally, 1972; Koch et al, 1973b) yet they may be unable to repair sublethal damage (SLD) under severe hypoxic or anoxic conditions (Koch et al, 1973b), (b) cells may become more radioresistant once they have been exposed to repeated x-irradiation, and (c) in vitro cells while in the density inhibition plateau phase (DIPP) may be more radiosensitive than cells in exponential growth (Little, 1969; Little et al, 1973; Zinninger and Little, 1973). This phenomenon was referred to as repair of potentially lethal damage (PLD).

4. The effect of x-irradiation on the cell cycle. Following irradiation, the incidence of mitotic cells is suppressed for variable periods, and the extent of this delay period is dose-dependent. Elkind et al (1963) and Witcofski et al (1972) measured the division delay in CH cells following x-irradiation and noted that it increased linearly with dose at a rate of approximately one hour per 100R (up to 1000R). Whitmore et al (1961) reported that division delay in mouse L cells following irradiation had an initial slope of about two hours per 100R. Conceivably, irradiation damaged the division mechanism, thereby affecting the rate at which cells could perform the metabolic processes required for mitosis. The division delay for HeLa cells was reported to be 1.6 hours per 100R (Tolmach, 1961). However, HeLa cells irradiated midway in G_1 had a relatively short division delay per unit dose, but the delay increased as cells progressed through S and G_2. This suggested that division delay was dependent on DNA synthesis. Whitmore et al (1961) reported similar observations with mouse L cells and surmised that mitotic delay was age-independent for cells in G_1, but increased with age for cells in S, and was again age-independent for cells in G_2. When cells were irradiated in M or in G_1, there was a relatively short division delay which augmented in duration as cells passed through the cell cycle. Thus, cells irradiated while in S or in G_2 are prone to a longer division delay than when they are irradiated in M or G_1 (Lajtha et al, 1955; Sinclair, 1967; Leeper et al, 1970 and 1972a; Muller et al, 1973). Cells already in M at the time of irradiation completed division with little or no delay, however, new cells were prevented from entering M (Carlson, 1972; Dewey et al, 1972a). This indicates that cells are likely blocked in G_2 by

irradiation (Carlson, 1972; Dewey et al, 1972a; Bedford and Mitchell, 1973). Cells that eventually die following irradiation can remain capable of dividing a limted number of times before death. Elkind et al (1963) noted that even after a dose as high as 1000R, potentially "killed" CH cells divided at least once. Tolmach (1961) reported that HeLa cells exhibited different degrees of abilities to reach their first mitosis after irradiation, and that 5 to 10% of these cells appeared to be completely resistant to radiation.

5. Chromosome damage produced by x-irradiation. Many physical factors are known to result in the breakage of entire chromosomes (chromosome breaks) or chromosome subunits (chromatid breaks). X-irradiation is one of these factors. Chromatid breaks are generally believed to occur after DNA replication, while chromosome breaks occur before replication (Elkind and Whitmore, 1967). According to Chu et al (1961), various doses of x-irradiation administered to a variety of human cell lines caused chromatid aberrations 25 hours, and chromosome aberrations 42 hours after irradiation. Also, the incidence of both types of aberrations increased linearly with the dose, with a particular dose producing approximately three times as many chromatid as chromosome deletions. Sax (1958 and 1960) reported that more aberrations were produced during prophase than metaphase, and the total number of observed breaks per cell decreased with time after irradiation. Sinclair (1967) observed chromosome aberrations in CH cells following irradiation while in G_1, whereas chromatid aberrations occurred chiefly when cells were irradiated in G_2, followed by early S, late S, and G_1. Dewey and Humphrey (1962) reported that L

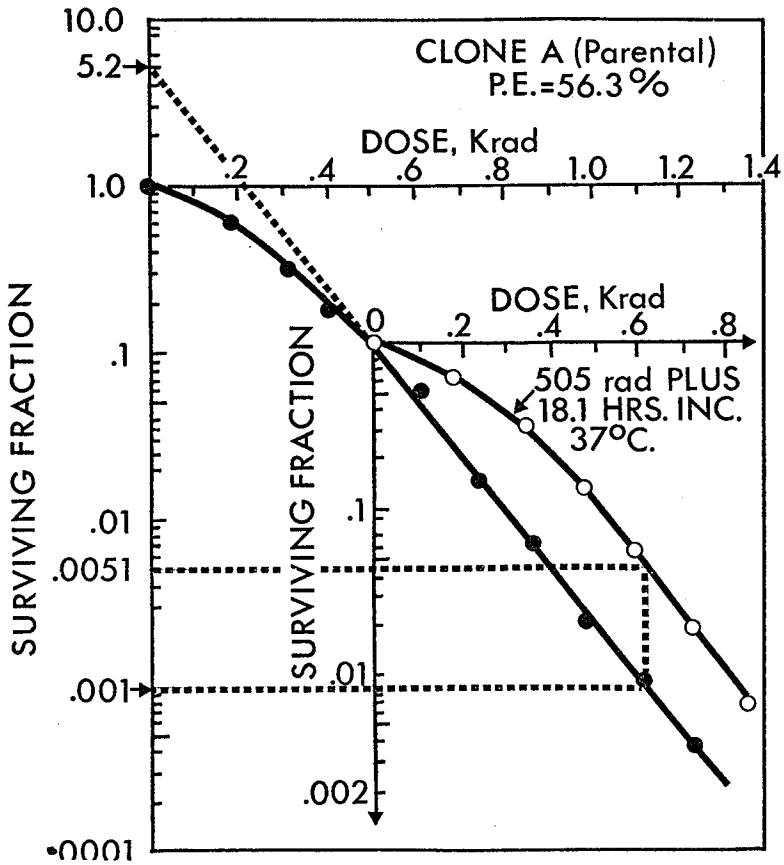
cells and Lettré ascites cells irradiated in S exhibited a higher number of aberrations than cells irradiated while in G_2 or G_1; G_2 cells showed as many or more aberrations as cells irradiated while in G_1.

It was postulated that the chromosome aberrations may be responsible for the death of cells following exposure to irradiation. Thus, Dewey et al (1972a) ascertained that the frequency of chromosomal aberrations induced by x-rays in synchronized CH cells correlated well with cell mortality. They consequently hypothesized that x-ray induced cell mortality was caused primarily by this lethal chromosomal aberration. Carrano (1972) agreed with this view, adding that survival of CH cells coincided with that predicted from visible chromosome aberrations. Billen (1972) postulated that cell death could be caused by failure to maintain the minimal integrity of a replicative system required for the normal duplication of DNA. Abnormal DNA replication would also explain the delayed death of progeny of irradiated cells. In contrast, Sinclair (1964) asserted that the incidence of chromosome aberrations in CH lung cells exposed to irradiation, did not correlate with the survival of cells. Alexander et al (1965) could neither find a convincing reason for DNA damage to be the primary lesions that determined radiosensitivity. According to Fox and Fox (1973), there was no significant difference between cell lines with different radiosensitivities and the number of single-strand breaks or the rates of rejoining following irradiation. It was concluded that single strand breaks were not, in themselves, lethal events. Belli and Brown (1972) concluded that injury registered by a second x-ray dose was not additive to that produced by a first dose. Instead, DNA, having repaired its damage following the

initial irradiation, became less susceptible to subsequent irradiation. Whether or not damage to chromosomes may directly lead to cell death, it is known that the nuclear portion of the cell is a radiosensitive component with regard to cell lethality following x-irradiation.

6. Recovery from irradiation damage and the effect of fractionation of dose. When a particular dose of irradiation is to be fractionated (that is, divided into smaller doses administered at intervals with variable recovery periods between doses), it is important to ascertain what, if any, recovery takes place between dose administrations. Elkind and Sutton (1959 and 1960) demonstrated this recovery from sublethal damage (SLD) with mammalian cells. Chart 4 presents a typical survival curve of mammalian cells following fractionated doses of irradiation. The shoulder of the survival curve indicates that damage must be accumulated for cell killing to occur. The survivors of the conditioning dose, in terms of the second survival curve, could not be distinguished from the single dose population, indicating (at least some) repair of SLD. It is obvious that, for a particular dose, the net survival of cells is larger if the dose is fractionated. The survival kinetics observed in this type of curve are partially ascribable to repair of SLD and partially to the progression of cells through the cell cycle. Studies with HeLa cells (Lockart et al, 1961), Ehrlich ascites cells (Hornsey and Silini, 1962), mouse marrow cells (Till and McCulloch, 1961) and mouse leukemia cells (Berry and Andrews, 1961a and b) all indicated that repair of SLD was a general phenomenon.

CHART 4 - INITIAL SURVIVAL CURVE (CLOSED CIRCLES) AND SURVIVAL CURVE (OPEN CIRCLES) AFTER THE SAME DOSE FRACTIONATED FOR "CLONE A" CULTURED CHINESE HAMSTER CELLS (Elkind and Sutton, 1959).



Effect of X-irradiation on the Survival of Mice

Deaths due to the effects of irradiation are generally categorized according to one of three syndromes. These syndromes are established mainly on the basis of survival time differences. At very high doses, the exposed animal dies, often in a matter of minutes, surviving at most a couple of days, and symptoms are consistent with damage to the central nervous system (CNS syndrome). A remarkably constant mean survival time occurs in the dose range from 1000R to several thousand R, and death in

this range is chiefly associated with damage to the gastrointestinal tract (GI syndrome). Below 1000R, animals succumb mostly to the bone-marrow syndrome.

While it is convenient to establish these syndromes on the basis of survival time differences, little information is revealed with respect to the actual mechanism involved. Moreover, in the transitional zones between the three syndromes, it is often difficult to determine from which syndrome the animal was primarily dying. Further, classic descriptions of the syndromes are really encountered only under laboratory conditions in which uniform whole body radiation had been accomplished. With lead shielding, for example, the classical syndromes are much less defined. Despite these difficulties, estimates for the mean lethal dose (LD) can be obtained by determining the time limits for each of the syndromes, and by calculating the LD_{50} (dosage which causes death to 50% of the animals) based on that period of observation.

For mice, a time-limit of 30 days was set to calculate the LD_{50} in the bone-marrow syndrome. It is based on the assumption that animals which are destined to die as a result of these low exposures will have succumbed within that period. The $LD_{50/30}$ is approximately 650R, the MST of decedents being 13 days. Although manifestations of disturbances in the GI tract often begin within a few hours after irradiation, they again disappear within hours or a very few days. Following this initial episode, from which the animal readily recovers, there is a latent, virtually symptom-free period. However, destruction of the bone marrow, with subsequent depletion of the peripheral blood elements, nonetheless occurs. Once these symptoms become marked, functional consequences begin

to become manifest, such as infections (associated with neutropenia), hemorrhage and anemia (associated with thrombocytopenia), and fluid imbalance (likely owing to GI malfunction secondary to hemorrhage and agranulocytic ulcerations in the small intestine). Death of the animal results from the sequelae of infection and hemorrhage, affecting virtually every organ system in the body. While the bone-marrow syndrome is attributed primarily to bone marrow damage, it is evident that this is not the only organ affected. Nonetheless, it is the detrimental effect on the bone marrow that leads directly to the changes resulting in hemorrhage and infection, whereby damage to other organs plays only a secondary role in lethality.

In the GI syndrome, the LD_{50} is usually assessed as five days, to distinguish between those animals dying from the GI syndrome and those that will succumb later to the bone-marrow syndrome. For mice, the $LD_{50/5}$ is approximately 1200R WBI and it is likely that the entire intestine, or at least a large proportion of it, must be irradiated to induce the syndrome (Quastler et al, 1951). Quastler (1963a) stated that a C5F mouse exposed to a dose of 1200R or more would develop the GI syndrome, ending in death about three and one-half days after the irradiation. The mouse would appear normal up to the time when onset of the classic symptoms of the GI syndrome would occur, including hunched back, ruffled fur, and severe diarrhea. The three and one-half day process was referred to as "acute intestinal irradiation death" (Quastler et al, 1951). Generalized symptoms, in all species, include anorexia, increasing lethargy, diarrhea, evidence of infection, and dehydration. They are accompanied by diminished food and water intake, weight loss, gastric

retention, and decreasing intestinal absorption. The leukocyte count virtually disappears. A rather abrupt terminal phase (of less than one day duration) is characterized by severe diarrhea and prostration. With the onset of diarrhea, dehydration becomes marked and weight loss and emaciation are accentuated. Kallman (1962) observed that lethal injury to the intestinal crypt cells resulted in a failure of the functional integrity of the mucosa, causing a drastically altered fluid balance, disturbed assimilatory function, and increased passage of intestinal microorganisms into the blood stream. With the accompanying leukopenia, the animal was unable to cope with the rapidly progressing bacteremia. Taketa (1962) reported that dehydration resulted chiefly from the impaired intestinal absorptive function and the consequential net flow of water and electrolytes from the blood into the intestinal lumen. Yet, attempts to treat the rats with electrolytes alone had no effect on survival, while treatment with antibiotics alone, or else in combination with electrolytes, increased the proportion of survivors. This suggested to Taketa that microorganisms might participate to a greater extent in the GI syndrome than mere body dehydration. Recently, Gits and Gerber (1973) ascertained that the loss of sodium through the GI tract and feces amounted to less than 10% of the sodium body pool, and it occurred during the entire post-irradiation period. Sodium diminished in the blood, however, only during the last hours immediately prior to death. This signified that death was due not so much to electrolyte loss into the intestinal lumen, but rather to changes in the permeability of all organs leading to a state of irreversible shock.

Boyer and Conger (1972) suggested that some of the damage caused by low doses of irradiation might arise secondarily from capillary damage. Twenty-four hours following 500R WBI, there appeared to be fewer capillaries present in the mouse small intestine. Those visualized were ragged, that is, the entire capillary bed was not as patent as normally. Such changes were not observed as early as four to five hours, nor were they present at five days after irradiation, implying that they were transitory. Further, these changes were not observed following a lower dose (250R).

It must be noted that severe depression of bone marrow activity, with the resultant neutropenia and infection, may have a major role in the GI syndrome. With partial body irradiation (PBI) of the intestine alone, though some bone marrow depression might also occur depending on how much marrow was inadvertedly irradiated, the severe levels of neutropenia seen following WBI are not observed. It would therefore be expected that the infection component of the GI syndrome was eliminated. This is substantiated by two observations. First, the dose necessary to kill by the GI syndrome is higher with PBI of the intestine than with WBI. Second, the survival time with irradiation of the intestine alone is longer than with WBI. The complete GI syndrome evident with WBI is therefore not observed with PBI.

For the CNS syndrome, an $LD_{50/2}$ is used to distinguish between those animals dying with this syndrome and those destined to die later from the GI syndrome. This is explained by the observation that animals dying earlier than two days exhibit few, if any, GI signs and symptoms. The $LD_{50/2}$ for mice is between 18,000R and 40,000R.

The Effect of X-irradiation on the Intestinal Epithelium

Because of the high rate of proliferation in the small intestine, specifically in the crypts, and inasmuch as proliferating cells are particularly susceptible to the damaging effects of irradiation, this segment of the digestive system is extremely radiosensitive. Desjardins (1931) estimated that epithelial cells were the third most radiosensitive in the body, less so only than lymphoid cells and white blood cells. As early as 1912, Regand, Nogier, and Lacussagne described severe intestinal lesions following x-irradiation, relating them to systemic effects. A more detailed description of the nature of such lesions followed in 1922 by Warren and Whipple. Numerous investigators studied and reviewed the effects of various doses of x-irradiation on the intestinal epithelium in a variety of mammalian species. The mucosa was shown to be the radiosensitive portion, while the muscularis externa was much more radioresistant (Maisin et al, 1971). The duodenum was more radiosensitive than other segments of the gastrointestinal (GI) tract.

Several investigators (for example, Quastler, 1963a; Bond et al, 1965) reviewed the time sequence of the effects in the mouse intestinal epithelium of radiation damage on the basis of the GI syndrome (employing irradiation doses up to 3000R). Initially, the crypts were rapidly depleted of epithelial cells, with a virtual depletion by one or two days. Villus cells were not as rapidly depleted, with complete depletion seen at three to three and one-half days. Giant epithelial cells appeared in the crypts as early as six to 12 hours, remaining approximately two days. These abnormal cells increased in size with time after irradiation, and were also encountered in the villi, beginning at about 18

hours. By two or three days the entire cell population of the villi could be composed of such cells. The cells usually desquamated from the villi after four days. This degenerative phase of the GI syndrome was generally followed by a rapid regenerative phase, the two phases occurring within six days (Davids, 1973).

Early destructive changes were observed following doses as low as 100R WBI (Knowlton and Hempelman, 1949), far below the dose level required to produce the GI syndrome. The changes were minimal however, and a more distinct degenerative phase was created by doses of 600R to 1200R (Montagna and Wilson, 1955; McGrath, 1960). Destructive changes were extremely minimal during the regenerative phase.

Montagna and Wilson (1955) traced the chronology of intestinal changes following administration of 1000R WBI to mice. They noted that the cells of the villi began to show progressive degenerative changes only once the peak of damage had been passed in the crypts. Brunner's glands did not show detectable damage during the duration of the experiments. Within 30 minutes of irradiation, nearly all of the crypt cell nuclei were slightly enlarged, and the nuclear membrane was thinner and less basophilic. Each nucleus contained greatly enlarged, basophilic nucleoli and fragments of basophilic material. After one and one-half hours most of the crypt nuclei had undergone karyolysis. Quastler (1963) stated the critical sequence of events in the GI syndrome as beginning one to two hours after irradiation, with pyknosis of the crypt cells, and Hugon et al (1965), noted mitochondrial enlargement and membrane ruptures, multiplication of dense bodies, formation of numerous cytolysomes, and nuclear anomalies occurring within four hours of 1350r WBI to mice.

Hugon and Borgers (1966) reported that by three hours post-irradiation, the most striking observation in the crypt epithelium was the appearance of round, regular structures (karyolytic bodies) frequently surrounded by a clear halo. These bodies, with the appearance of nuclear fragments, were located in all crypts, either above a slightly swollen nucleus of a cell (often depressing its nuclear membrane), or at the base of the cells, pushing against neighboring cell nuclei. The largest bodies (5 to 7μ) were irregular and vacuolated, and at the EM level were observed to lie within crypt cells. The bodies were shown to consist partly of autophagic vacuoles (developed in the cells by seclusion of a necrotic area from the rest of the cytoplasm) and partly of phagosomes (formed by engulfment of degenerated leukocytes and dying cells). Ghidoni and Campbell (1969) described similar findings in the jejunum of the Rhesus monkey, six to 24 hours following irradiation. The karyolytic bodies resembled degenerating cells, some being up to 17μ in diameter and located next to the basement membrane within the crypt epithelium. The lamina propria also contained many dense bodies, morphologically similar to those in the crypts. During the remainder of the first day post-irradiation period, there was a continued reduction in the number of mitoses in the crypts, as well as progressive morphological changes of the cells. These alterations increased with increasing irradiation dose (Becciolini et al, 1973). By the end of one day, the crypt cell population was vastly reduced (Quastler, 1963; Hagemann et al, 1972), surviving cells displaying atrophy of microvilli and enlarged and multilobated nuclei (Hugon et al, 1965). During this same time period, goblet,

Paneth, and enterochromaffin cells underwent only slight morphological changes (Hugon et al, 1965). During the initial phase (four to 24 hours post-irradiation) enzyme activities in the rat small intestine increased (Becciolini et al, 1973).

Patt and Quastler (1963) stated that one day following irradiation and later, pyknotic cells were infrequent in the crypts, though the crypt cell population, unlike the villus cell population, became vastly reduced. Although some enlargement of villus cells was observed as early as 18 hours following irradiation (McGrath and Congdon, 1959) it was generally agreed that few, if any, changes occurred in the cells of the villi during the first post-irradiation day (Montagna and Wilson, 1955; McGrath, 1959 and 1960).

During the second and/or third post-irradiation day, the few remaining crypt cells were enlarged and poorly organized (Patt and Quastler, 1963), and by three days, the crypts (depending on the x-ray dose) could be denuded (Montagna and Wilson, 1955). By that time (two to three days) villi might shrink and lose cells (Quastler, 1956; Quastler and Hampton, 1962), their nuclei often having enlarged. If the villi were depleted of cells, they collapsed into small mounds, and the submucosal connective tissue became very edaematous (Quastler, 1963). According to Becciolini et al (1973), the 48 to 72 hour post-irradiation period coincided with the period of the most marked morphological changes in the epithelial cells of both crypts and villi in the rat small intestine. There was a marked reduction in the number of epithelial cells. McGrath (1960) reported that the most pronounced enlargement of crypt cells occurred 48 to 60 hours following irradiation. Montagna and

Wilson (1955) noted that by two days post-irradiation, the degenerative process in most crypt cells passed its peak, yet the nuclei of Paneth cells were just beginning to swell. Villus cells, which seemed normal till this time, exhibited loss of cytoplasmic basophilia and swollen nuclei. By 72 hours, the Paneth cells were fragmented and had been extruded into the lumen, while the crypts commenced to show evidence of repair, containing some normal cells. The villus cells still exhibited severe degenerative changes, and often appeared as multinucleated giant cell masses. By four days many of the crypts appeared normal while most villi showed a maximal amount of damage. Goblet cells in both crypts and villi became progressively larger until about two days, at which time they degenerated. Poulakos and Osborne (1973) noted that, three days following 1000R to the exteriorized rat ileum, there was complete denudation of the epithelial lining. Vestiges of damaged crypts contained abscesses filled with polymorphonuclear leukocytes and cellular debris. Withers and Elkind (1970) observed that, three and one-half days following 1000R WBI or whole abdomen irradiation to mice, the number of crypts per jejunal cross-section had been reduced to 120, from the normal 160.

Although McGrath and Congdon (1959) reported that the greatest enlargement of crypt cells occurred only four to five days following irradiation, the period from three and one-half to five days onward is generally considered to be one of recovery or regeneration (Quastler, 1963; Patt and Quastler, 1963; Becciolini et al, 1973; Poulakos and Osborne, 1973). Enzyme activities and the morphology of the epithelium returned to normal (Becciolini et al, 1973), though the number of cells per crypt may continue to increase between one and two and one-half weeks

following irradiation (Poulakos and Osborne, 1973). One week after a dose of 1000R to the exteriorized rat jejunum, Wiernik et al (1966) observed no morphological differences between the irradiated and normal epithlium.

Effect of X-irradiation on the Cell Kinetics of the Small Intestine

Almost immediately following irradiation, cells become blocked in the transit from G_2 into mitosis, as well as from G_1 into S (Patt and Quastler, 1963). A wave of death of proliferative cells follows which is concomitant with a maturation of some previously proliferating cells. According to Sherman and Quastler (1960), maturing cells followed approximately normal kinetics, that is, the migration of cells along the villi was not markedly affected by irradiation. In contrast, Brandt et al (1972) reported that, following irradiation of the exteriorized rat duodenum, cell migration rates were increased for one to three days and had returned to normal rates only by seven days post-irradiation. Friedman (1945) noted numerous rat duodenal crypt cells laden with mucous 24 hours following irradiation, attributing that phenomenon to a transitory arrest in the migration of newly formed cells away from the proliferation zone. More recently, Rijke and Galjaard (1973) reported that the cell migration rate along the villus was considerably slowed following a dose of 700R, and this decreased rate was independent of the concurrent shortening of the villi. Cell migration following irradiation was observed to be one cell position per hour as compared to the control rate of two to three positions per hour. Also, reduced enzyme activity observed during the recovery after irradiation was attributed not to a shortening of the cell

cycle but to an insufficient cell maturation brought about by increased proliferative activity of the crypt cells. Lesher et al (1967), Lesher and Bauman (1969), and Lesher and Lesher (1970), employing various doses of WBI to mice noted that the intestinal epithelial cells were blocked in G_2 with all doses, concomitant with a mitotic lag ranging from 1.5 to 10 hours, depending on the dose. For example, following a dose of 300R WBI, cells remained arrested in G_2 for approximately four and one-half hours, whereas with 1000R WBI, cells were blocked in G_2 for approximately 18 hours (Lesher et al, 1967). Cells in S at the time of irradiation were able to complete DNA synthesis, though at a slower rate, and moved into G_2. Cells also became blocked near the end of G_1, the duration of that block being dose-dependent (Bauman, 1970). Lesher et al (1961b), and Lesher and Lesher (1970), suggested that a return to normal of the intestinal epithelial cell population was brought about by two compensatory mechanisms, namely, an acceleration of the generation cycle, and an increase in the number of cells in the proliferative compartment. Fry et al (1963) also noted that, following continuous irradiation of the intestine, the cell cycle became reduced by one hour following one day exposure, and two hours following 14 days exposure. Lesher and Lesher (1974) observed that, 24 hours following 300R or 600R partial body irradiation of C57Bl/6J mice, the cell cycle of jejunal crypt cells was accelerated by approximately three hours. However, following 900R, the cell cycle was not significantly accelerated. Twenty-four hours after the same doses, the cell cycle was shortened by approximately four hours following 300R or 600R, but only by two hours following 900R. Lamerton (1972) reported a shortening of the intermitotic time following

irradiation but believed that the temporary extension of the zone of proliferation up the crypt was more significant in repair of the epithelium as it allowed more cell divisions to occur.

1. Intestinal Degenerating Index (DI). Though the phenomenon of extensive cell death in the intestinal epithelium following irradiation is well known, few quantitative studies are available. Withers (1972) reported that a 660R dose to the entire mouse abdomen reduced cellular survival in the intestinal crypts to approximately 5% of normal. According to Sherman and Quastler (1960) a cell depletion of mouse intestinal crypts, following massive irradiation, occurred chiefly on the first and third days after irradiation, that is, at times when DNA synthesis was minimal. Sigdestad et al (1972) reported that the crypt survival curve of mouse jejunal cells displayed a Do of 377R and that the survival curve was similar to those of other mammalian cells. Lesher and Lesher (1970) observed that approximately 60% of mouse jejunal epithelial cells became destroyed by a single 1000R dose, and further that at the $LD_{50/5}$ (1413R) level, mouse jejunal crypt cell survival ranged between 18 and 29% (Sigdestad et al, 1973).

Tsubouchi and Matsuzawa (1974) reported that the maximum number of pyknotic cells in duodenal crypts appeared four hours following 1000R WBI to mice. Because the incidence of pyknotic cells declined by 50% by six hours, the half-life of the cells was estimated to be two hours. It was suggested that, as mitotic figures were not observed during the first four hours after irradiation, at the time of a sharp peak in the number of pyknotic cells, a considerable proportion of premitotic cells had died during this period. Eight hours after irradiation, many abnormal

mitoses were recorded, but the sharp peak of pyknotic cell frequency observed at four hours was not repeated, suggesting that there was post-mitotic cell death after the reappearance of normal mitotic figures. The relationship between rapid cell death and the cell cycle was investigated by obtaining the labeling indices of pyknotic cells for S phase cells alone and for S + G_2 phase cells. From this, the population size of each cell phase was calculated on the assumption of a steady-state model and the frequency of pyknotic cell formation in each phase was calculated. It was shown that the production of pyknotic cells was high in the G_2 phase and substantially lower in the S and G_1 phases, with the S phase being intermediary to the G_1 and G_2 phases. The greater resistance of G_1 phase cells, in terms of cell death by pyknosis, suggested that these resistant cells were important for the recovery of the cell population during the two to four day post-irradiation period.

2. Intestinal Mitotic Index (MI). Absence of mitotic figures (MF's) in intestinal crypts occurs immediately following x-irradiation (Patt and Quastler, 1963; Lesher and Lesher, 1974; Tsubouchi and Matsuzawa, 1974) but the time of subsequent reappearance of MF's may vary with the mode of x-irradiation. Poulokos and Osborne (1973) ascertained that MF's were absent in the ileal crypts of rats three days after 1000R to the exteriorized gut, but their number steadily increased by five days post-irradiation. Between seven and 35 days the numbers of MF's per crypt was not significantly different from control values. Tsubouchi and Matsuzawa (1974) observed that the MI of the mouse intestine declined to zero by about one hour following 1000R WBI and remained well below normal for eight hours. It had returned to the normal level by 14 hours,

then decreased again, remaining depressed for two more days. Between the second and third days after irradiation, the MI increased rapidly, overshooting the control values by approximately 100%. By the fourth day, the MI began to fall, reaching normal values by the fifth day. Lesher and Lesher (1974) observed that, following 300R, 600R, or 900R of partial body irradiation to C57Bl/6J mice, MF's were absent in the crypts by one hour. The MF's per crypt (MF/crypt) returned to near normal levels by 24, 30, or 60 hours following each of the three doses, respectively, and then rose rapidly to a peak at 60, 66, or 80 hours. The MI's decreased subsequently, attaining the normal level by 96 hours following the two lower doses. Although the MF/crypt was also declining 120 hours following the 900R dose, it was then still well above control values. The maximal number of MF's/crypt was approximately 11 divisions following any of the three doses. This compared to a control value of approximately 4 mitoses per crypt.

3. Intestinal Labeling Index (LI). Sherman and Quastler (1960) noted that irradiation caused a reduction in the LI of the mouse intestinal epithelium, both the rapidity of development and the ultimate severity being dose-dependent. They suggested that the initial phase of this effect was caused by a block of DNA synthesis of those cells irradiated while in the S phase. After moderate to heavy doses, DNA synthesis resumed long before cell division, and even in massively irradiated mice, cells could pass through one or two cycles of DNA synthesis which were not followed by division. Lesher and Lesher (1970 and 1974) observed that the number of labeled nuclei per jejunal crypt (LN/crypt) dropped rapidly from a control value of about 70 to a low of 40 by six

hours following 300R partial body irradiation, to 25 by 20 hours after 600R, and to 12 labeled nuclei by 20 hours following 900R. The numbers then augmented rapidly, attaining control values by 12 hours after 300R, and 24 hours after 600R and 900R. An overshoot peak (approximately 200 LN/crypt, 260 LN/crypt, and 290 LN/crypt, following 300R, 600R, and 900R, respectively) occurred at approximately 84 hours following any of the three doses. The LN/crypt subsequently decreased rapidly, approximating control values 100 hours following the two lower doses. By 110 hours, the LN/crypt of animals that received 900R was still well above control values. Similar observations were reported following the administration of different doses of WBI. In addition, a repeating pattern of highs and lows of LN/crypt was noted following the overshoot, which was independent of the regular fluctuations of the circadian rhythm (Lesher and Lesher, 1970). According to Hagemann and Lesher (1971), the time of onset of the overshoot was three days following low doses (200R to 500R), and four days following 1000R WBI. At that time, cell proliferation per crypt was 2.6 times that of control values. Sigdestad et al (1973) found that, three days after either 1000R or 1400R WBI, the LN of mouse crypts had increased from approximately 75 to 260 and 163, respectively.

Cooper and Hagemann (1973), administering two 500R doses 24 hours apart, ascertained that the amount of labeling in the mouse jejunum was less than 50% that of the control values one day following the last exposure. It increased to a maximum (175% above control) by about two days. The maximum labeling (250% of control) following a single 1000R dose occurred four days following the exposure. There was a gradual decrease to control values by eight days, with an ensuing six-day period of

slightly elevated levels of labeling. Although Poulokos and Osborne (1972) observed a higher number of labeled cells per crypt between seven and 35 days following 1000R to the exteriorized rat ileum, there was also an increase in the total number of cells per crypt, hence the normal percentage of labeled cells was not attained.

Toal et al (1958) noted that DNA synthesis declined by 50% concomitant with a five-fold increase in the mitotic rate, and suggested that a significant rate of DNA synthesis could occur while mitosis was blocked. Moreover, DNA synthesis remained elevated well past the time the MI had recovered normal values.

The Effect of Fractionated Irradiation Doses on the Intestine

According to Corril et al (1970), biologic systems can survive even daily fractionated irradiation as long as cellular repair is greater than the rate of cell death. The cellular replicating capabilities of the small intestine are largely responsible for its (relative) radioresistance. Hagemann and Lesher (1971) observed that, following 200R, 500R, or 1000R, there was a dose-dependent post-irradiation overshoot in proliferative cellularity in the jejunum, signifying the ability of increased cell proliferation as a response to radiation injury. The peak of proliferation occurred between three and four days. It was suggested that large doses given three to four days apart could enhance the therapeutic ratio. Hornsey and Vatistas (1963) noted that the shoulder of the survival curve for crypt cells was large, indicating an ability of crypt cells to accumulate sublethal damage. The percentage of surviving mice varied with the duration between doses. The sum of the two doses,

separated in time, was greater than that of a single dose required to effect death of the animals after three to four days. Withers and Elkind (1968 and 1969) observed the broad shoulder in the curve of stem cells, and also noted that repair of sublethal damage was unusually great.

The Effect of X-irradiation on Tumours

1. Radiosensitivity. Just as radiosensitivity differs with the age of a cell and also from one normal cell population to another, radiosensitivity also varies within a tumour cell population and from one neoplastic cell population to another. Some of the factors affecting radiosensitivity were elucidated, whereas in other instances the reasons for the radioresponse of a particular tumour are unknown. Factors that may be significant in the radioresponse of tumour cell populations are:

a. Amount and composition of chromatin. Neoplastic cells exhibit, as a rule, polyploidy and/or aneuploidy (Robbins, 1967). Sparrow (1965) observed an inverse relationship between the amount of radiation required to produce lethality of tumour cells and the volume of interphase chromosomes. Berry (1962 and 1963) reported that the $\tilde{n}$ for a tetraploid line of murine lymphocytic leukemia cells exceeded that of a diploid line. Further, following 50 irradiations to the diploid leukemic line, there was an increased radioresistance concomitant with an increase of nucleic acid content per cell. Both tetraploid and aneuploid cells appeared following irradiation in this population.

Dewey et al (1972b) noted a decreased radiosensitivity (as measured by chromosomal aberrations) as cells passed from M into S, and correlated this decline with a dispersion of chromatin. Mitotic cells were most sensitive because their tightly packed chromatin fibers would provide the highest probability for interactions between lesions.

b. Oxygenation. Oxygenated cells proved more radiosensitive than hypoxic cells (Hahn et al, 1972; McNally, 1972). The radioresponse of tumours also varies with the degree of oxygenation (Thomlinson, 1960 and 1968; Powers and Tolmack, 1965; Thomlinson and Craddock, 1967. Enhanced cell killing occurred in the tumours of animals breathing hyperbaric oxygen while irradiated as compared to control animals breathing ordinary air (O'Brien and Sturbaum, 1962: Powers and Tolmack, 1964; Mori-Chavez et al, 1970; McNally, 1972; Milne et al, 1973).

c. Location of cells within the tumour. It is well-known that many solid tumours develop central necrotic, and possibly hypoxic foci, consequently these areas of the tumour would be relatively radioresistant (Bond et al, 1967). Durand and Sutherland (1973) irradiated multicellular "spheroids" of CH cells grown in vitro and which were morphologically similar to different areas of some solid tumours. They reported that three specific states developed in the spheroids. Following irradiation, the peripheral cells continued to pass through the cell cycle in a relatively normal manner, surviving irradiation equally well as when irradiated after only one day growth as spheroids. Deeper cells tended to collect

in a G_1-like population, were hypoxic and thus highly radioresistant. It was postulated that intercellular contact could stimulate a "repair mechanism" with different functional capacities at various stages of the cell cycle. Urtasun and Merz (1969) suggested two possibilities regarding the hypoxic fraction of cells. On the one hand, the peripheral cells would be expected to be more radiosensitive as they were better oxygenated; on the other, if some tumour cells were unable to produce ATP in the absence of oxygen, they would suffer less damage, but would also be unable to repair any damage, suggesting that the "core" cells would be more radiosensitive. In contrast to current radiobiologic principles indicating that cells at the periphery of large tumours are more radiosensitive than those in the centre, Urtasun and Merz (1969), and Urtasun et al (1973), reported that the central cells of both a mammary carcinoma and human osteogenic sarcomas were more radiosensitive than peripheral cells.

d. Growth rate and/or tumour size. Mendelsohn (1967) suggested that, for any particular tumour, larger tumours required higher irradiation doses for cure than did smaller tumours. Breur (1966) and van Peperzeel (1972) found a high correlation between radiosensitivity and growth rate, as expressed by doubling time; with increasing doubling time radiosensitivity decreased.

2. Tumour Growth. Although the gross response of a tumour during the course of a treatment may be a poor indicator of the success or failure in the regimen (Suit, 1973), it is evident that, in instances where direct measurements of the tumour are infeasible, they may be the only indicator available. Tumours may regress for a period of time

following irradiation and then resume growth at a normal or near normal rate. Curtis et al (1973) described the model of the irradiated tumour as consisting of a cell population with two components--a "doomed" cell fraction and the "surviving" fraction. The former component was composed of those cells which sustained damage that caused them to die, although they may still have passed through several cell divisions. The second component consisted of those cells that recovered following irradiation and remained capable of unlimited proliferation, bringing about regrowth of the tumour. It was assumed that tumour regrowth was due to the proliferation of oxygenated cells, either present before irradiation or reoxygenated following irradiation. Hollcroft et al (1950) reported that regression of a mouse lymphosarcoma following irradiation was dose-dependent, but to achieve significant tumour regression it was necessary to use WBI. Garnic et al (1972) stated that WBI did not significantly affect regression of tumours which were also locally irradiated. According to Pearlman (1973), doses ranging from 3000R (given over ten days) to 3500R (given over 40 days) were necessary to destroy a Ewing's sarcoma. Several investigators reported that irradiation caused a delay in the growth of tumours. Field et al (1967) observed that the delay was dose-dependent. Thomlinson and Craddock (1967) found that the decrease in tumour volume of a rat sarcoma irradiated with from 500R to 6000R was not strictly dose-dependent. Following an initial enlargement in tumour volume after irradiation, there was a volume reduction lasting up to five days, followed by resumed growth at near control rates. The time for a tumour to grow from 9 mm to 25 mm in diameter was approximately 20 days after exposure to 2000R, compared to just six days normally.

Van Peperzeel (1972) noted that, after irradiation with 300R to 700R, human lung metastases decreased in volume from 100% to 22%, attaining the lowest volume three to five days after the exposure. Further, although the maximal volume reduction occurred shortly after irradiation (as early as 24 hours in mice), tumour volume subsequently increased, with a growth rate exceeding that prior to irradiation. In mice, the volume doubling time (VDT) returned to control values 15 hours after the irradiation. It was suggested that the elimination of necrotic material from a tumour was very rapid, beginning immediately after irradiation. Eddy and Casarett (1972) calculated tumour volumes of three transplantable hamster sarcomas following single irradiation doses up to 6000R. Although 1000R caused a significant retardation in tumour growth, higher doses caused longer periods of growth inhibition or reversal of volume. Denekamp and Thomlinson (1971) and Denekamp (1972) studied several rat and mice carcinomas and sarcomas and noted that all carcinomas exhibited high cell loss factors and that these tumours shrank rapidly following irradiation, whereas the sarcomas, with relatively low cell loss factors, did not shrink. Although the carcinomas continued to shrink for several days, they soon commenced regrowth at approximately the same rates as non-irradiated tumours. It was postulated that the initial shrinkage in the carcinomas was not a direct result of radiation-induced cell death, but was rather an unmasking of the normal rate of cell loss when cell proliferation was halted by radiation-induced mitotic delay. Tannock and Howes (1973) reported that 600R to 2000R doses to a mouse mammary tumour produced delays in growth but, contrary to other findings, significant reduction in tumour volume did not occur. Histologically, tumour

cell cords shrank to about 50% of the control thickness by 12 hours after all doses. The investigators suggested therefore that gross changes in tumour volume brought about by irradiation were not a reliable indicator of microscopic changes in cell number, or in the thickness of tumour cords, which occurred more rapidly and were more pronounced than changes in tumour volume would indicate. The workers postulated a mechanism of cell loss similar to that of Denekamp (1972). It was suggested that tumour cord thickness was maintained by a balance between cell production in the tumour cords and cell death at the necrotic interface. Following irradiation, two effects were expected to cause the cords to shrink. First, some of the severely hypoxic cells at the necrotic interface would die, as they also would in an untreated tumour. The lost cells would not be replaced by newly formed cells from the normally proliferating region of the cord. Shrinkage by this method would therefore be characterized initially by a halving-time equal to the cell turnover time of unirradiated tumour cords. Second, irradiation-killed cells would lyse and be absorbed into the capillary, while some of the previously hypoxic cells might migrate to the blood vessel, where they would now be better oxygenated.

3. Tumour cell kinetics. Although radiotherapy, ideally, should be based on the tumour cell kinetics of the tumour to be treated (Fletcher, 1973), obstacles arise in regard to ascertaining such data, in human or in animal tumours. Furthermore, some cells may go through several divisions following irradiation before dying, thus altering the proliferative parameters of the regrowing tumour (Curtis et al, 1972).

a. Cell cycle effects. Brown (1970) and Brown and Berry (1969) reported that irradiation to a chemically induced squamous cell carcinoma caused slight prolongation of the cell cycle. In contrast, irradiation of the normal epithelium of the cheek led to a significant shortening of the cell cycle. According to Evans et al (1971), in vivo irradiation of ascites tumour cells delayed the progression of cells through S, and blocked completely the further progression of cells at the end of G_2, suggesting that, for a period after an initial exposure, cells would accumulate in S. In contrast, Frindel et al (1972) noted that, although continuous in vivo irradiation caused ascites tumour cells to accumulate in G_2, acute irradiation (up to 1200R) of cells led to their accumulation in G_1. The time interval over which this accumulation was observed was dose-dependent.

b. Mitotic Index (MI). Several investigators reported an absence of mitotic figures following irradiation, the length of time of the mitotic delay being dose-dependent. Brown and Berry (1969) did not observe any mitoses in a hamster carcinoma for nine hours following a single dose of 500R, and for 20 hours following 1000R. A mitotic delay of four hours was observed in ascites tumour cells even after a dose of as low as 200R (Evans et al, 1971). With a dose of 500R or more, the MI dropped to almost zero within one hour (Kim and Evans, 1962; Klouwen, 1965).

c. Labeling Index (LI). Frindel et al (1972) reported that, following continuous irradiation of ascites tumour cells, the LI declined with increasing time of irradiation. Similarly, Goldfeder and Ghosh

(1973) noted that doses between 500R and 3000R administered to a mouse mammary adenocarcinoma brought about a dose-dependent decrease in the LI, the largest decline being from the normal level of 13% to one percent. The period over which these decreases occurred was not stated. According to Lindgren and Riley (1973), testing the wound response of the rat lens epithelium following 1000R given just before or after wounding, there was suppression of both DNA synthesis and mitosis. However, when irradiation was administered 12 hours after wounding (and therefore two hours before onset of DNA synthesis), DNA synthesis remained unaffected, while mitotic activity was decreased and delayed.

d. Degenerating index (DI) or cell death. Brown (1970) reported that when potential doubling time of a carcinoma was measured against the actual doubling time following irradiation, the cell loss factor was 0.75. Klouwen (1965) observed that, even after a 10,000R dose, destruction did not occur of the cells in a transplantable lymphosarcoma up to 12 hours after the exposure; thereafter both MF's and pyknotic cells appeared. Kallman and Rockwell (1973) observed a cyclic fluctuation in the survival of irradiated solid mouse tumour cells, with a 10 to 12 hour period between subsequent peaks and a three to four-fold amplitude of highs over lows. Hofer (1970) ascertained that during the first two days following <u>in vivo</u> ^{60}Co-irradiation (200R, 500R, or 1000R) of mouse leukemia cells, cell death was not significantly enhanced initially. This was followed, however, by a period of extensive and rapid cell death, the "killing phase" lasting from two to six days. The death rate then returned to normal levels. It was further noted

that radiation damage was most severe when cells were irradiated during the early phases of tumour growth. Goldfeder and Ghosh (1973) described a mouse mammary adenocarcinoma as being composed of large islands of parenchymal cells surrounded by a thin connective tissue stroma interwoven by blood capillaries. Following doses of 500R to 3000R, infiltrating cells and stromal proliferation were noted to cause distortions in the morphology of the tumour, the islands of parenchyma cells diminishing in size and being replaced by stroma. Pyknotic nuclei, hemorrhages, and amorphous eosinophilic material and debris were observed with all doses, the severity of the changes being dose-dependent. Following doses of 600R, 2000R, or 3000R to a mouse mammary carcinoma, Tannock and Howes (1973) observed extensive cell degeneration in the tumour cords six to 12 hours after all of these dose exposures. Twenty-four hours after 600R, most of the pyknotic cells had disappeared and the tumour cords were very thin. The mean cell concentration decreased after all irradiation doses employed, and this was attributed to increases in both cell size and intercellular distances. Twelve hours following 600R, the mean cell concentration had fallen to about 75% of the control value, but by four days gradually returned to normal. Following 2000R, the mean cell concentration decreased steadily to about 50% of normal at two to three days, remaining low for at least one week.

4. Fractionated irradiation doses. Marcial (1967) stated that, although a greater cytocidal effect was obtained by a single dose of irradiation than when the same dose was fractionated, it was often necessary to fractionate the dose because of an intolerance of damage to

normal tissues. Because fractionation allows for larger doses to be given and tolerated, this method of treatment permits a greater likelihood of tumour cure compared to a single-dose treatment (Moulder and Fischer, 1973).

Several factors may play an important role in determining radiosensitivity with fractionated doses, and are generally termed "repair", "repopulation", "reoxygenation", and "redistribution" (Howes and Suit, 1974). Each factor is operationally independent, yet it is, nonetheless, critically related to the amount of time between fractionated doses, that is, the relative importance of each factor varying with the type of fractionation scheme employed.

Suit and Wette (1966) reported that the TCD_{50} (dose necessary to control the tumour in 50% of the cases) of irradiated mammary carcinomas increased significantly when the dose was fractionated and given as two treatments six hours apart. The TCD_{50} for 10 dose fractions was 8400R, compared to 4575R for a single dose. This suggested that there was extensive repair of damage between each exposure. Further experiments with tumours which were rendered hypoxic indicated that hypoxic cells could repair sublethal radiation injury. Hahn et al (1972) reported similar findings, in that repair of potentially lethal damage could not be demonstrated in cells from young ascites tumours, where the Do was 145R, while extensive repair was noted of hypoxic cells, with a Do of 450R. The extent of repair was dose-dependent, and it occurred more slowly in solid tumours than in ascites tumours. Hornsey and Silini (1962) observed a shorter recovery period of Ehrlich ascites carcinoma

cells irradiated in vivo, than of mammalian cells treated in vitro. Consequently, repair appears to attain a greater significance as the number of irradiation fractions is increased, while cell repopulation is more decisive in the increase of fraction spacing time (Howes and Suit, 1974). Reoxygenation can be a most potent modifier of the radioresponses of tumours, enhancing radiosensitivity. The tumour response to irradiation can be determined by the number of viable hypoxic cells that exist in the tumour during treatment. Rockwell and Kallman (1972) reported that the hypoxic fraction of an untreated mouse mammary tumour was .35, while after a single dose of 1000R the hypoxic fraction increased close to 1.0, from which it returned thereafter toward the preirradiation value. An earlier study (van Putten and Kallman, 1968) also demonstrated that under fractionated radiotherapy, which was expected to preferentially eliminate well-oxygenated cells from the tumour, a continuous movement of previously anoxic cells occurred into the compartment of well-oxygenated cells. According to Howes (1969) and Howes et al (1972), following either single or fractionated doses to mouse mammary tumours, a dose-dependent reoxygenation of originally hypoxic cells took place. For example, one hour following a dose of 1500R, the hypoxic proportion of the tumour became increased from 12% to approximately 100%. By six hours, the estimated hypoxic proportion had declined and continued to fall, reaching minimum levels by approximately four days that were significantly lower than the preirradiation value. This indicated that a spacing of 48 hours between doses was likely optimal in attaining a maximal response in this tumour. Howes and Suit (1974) studied the effect of

various fractionation schemes on the C3H mouse mammary tumour. The TCD_{50} increased progressively as the fraction spacing time (overall treatment time) was prolonged. It was suggested that the greater sensitivity was ascribable to reoxygenation of the tumour. In contrast, other investigators reported that reoxygenation was not an important factor in fractionated doses of irradiation (Kallman et al, 1970; Hewitt and Sakamoto, 1971). Hill and Bush (1973) claimed that although some reoxygenation likely occurred in a sarcoma between x-ray fractions, the sensitizing effects of this reoxygenation were balanced by a tissue repair of the radiation damage.

5. Tumour vasculature. Once the vascular plexus of a tumour becomes unable to maintain an adequate blood supply to particular areas of the tumour, these areas become hypoxic and, eventually necrotic (Gosalvez et al, 1972; Wong et al, 1973). Consequently, vascularization of tumours may decrease in rapidly growing neoplasms with increased tumour volume (Hilmas and Gillette, 1972; Kallman et al, 1972). Thomlinson and Gray (1955), in a histopathological study of bronchogenic carcinoma, observed that oxygen diffusion and consumption was balanced at a distance of $\geq$150μ from the capillary. Tannock and Steel (1969) calculated the oxygen diffusion distance of tumours to range between 85 and 210μ. That study further demonstrated a correlation between circulatory stasis and cell death in tumours. Because the hypoxic fraction of cells in a tumour is of significance in regard to the radioresponse of the particular tumour (see page 98), changes in the vascularization brought about during or following irradiation may affect the radioresponse of a tumour. Reinhold and Buisman (1973) stated that the radiosensitivity of the endothelial cells of capillaries is of the same

magnitude as that of epithelial parenchymal cells and the cells of supporting tissues. Observations regarding vascularization of irradiated tumours varied in different studies. Some of this variance may be ascribable to the different time period over which changes were observed, while others may have been dependent on the size of the irradiation dose, and whether or not it was administered as a single or fractionated exposure. Rubin and Casarett (1966) reported that a Walker carcinoma regressed after three administrations of 500R each, and that a concomitant increased vascularity was not brought about by neovascularization but rather to tumour regression. When a single dose of 1500R was given, however, there was a notable reduced vascular filling due to fragmentation of vessels. Reinhold (1971) reported enhanced microcirculation in a mammary carcinoma 24 to 48 hours following a course of fractionated irradiation. Kallman et al (1972) studied the effect of irradiation on mouse sarcomas by ascertaining the clearance rate of xenon-133 (^{133}Xe). Following doses greater than 1000R, blood flow decreased (in a dose-dependent manner) three hours post-irradiation. At later times, however, there was an increase in the rate of blood flow, for example at three to five days following 1000R, and seven days following 2000 to 4000R. The increases did not correlate simply with tumour shrinkage.

Several investigators reported decreased vascularization of tumours following irradiation. Thus Wong et al (1972 and 1973) found, following a dose of 2000R, that the vascular permeability of a sarcoma began to increase at 12 hours, and that it was 70% above control values

at 18 to 24 hours. On the other hand, vascular volume, unchanged at 18 hours, was significantly decreased at 24 hours. Similarly, Song and Levitt (1972) noted that the functional vasculature of that same sarcoma was 75% of control values six days following eight fractions of 250R, and 50% of control values six days after a single 2000R dose. Song et al (1972) observed a marked decline in the vascularity of a rat carcinoma on the first day following a 2000R dose, and thereafter for 11 days. There was a remarkably increased extravasation of plasma protein during the first 24 hours after irradiation, followed by a brief decrease on the second day. A contradictory finding was the significant increase in the clearance of ^{133}Xe six days following irradiation. The rate of blood flow, calculated from this clearance, augmented in the irradiated tumours. However, the functional vascularity, as measured by newly-injected ^{51}Cr-labeled RBC's, had undoubtedly decreased (compare Kallman et al, 1972). These investigators could not offer any explanation for this phenomenon. The decline in blood flow following irradiation would correlate with observations of a narrowing of vessel caliber, decrease in number of vessels, and occlusions following irradiation of tumours (Merwin et al, 1950; McAlister and Margulis, 1963; Potchen et al, 1972).

The Effect of X-irradiation on Melanomas

Melanomas are generally considered to be radioresistant (Desjardins, 1934; Pack, 1948; Philips, 1963; Sugiura, 1963), and several investigators reported that clinical treatment of melanomas with x-irradiation has not been very successful (Asbury et al, 1972; Castro et al, 1973). The usual

therapy of choice, therefore, is extensive surgical excision combined with a resection of the particular regional lymph nodes (Pack, 1948; Philips, 1963; McNeer and Dasgupta, 1965; McBride, 1973). It was suggested that the free radical nature of melanin (that is, with one or more unpaired electrons) may provide a radioprotective activity of this substance as an "electron acceptor" (Commoner et al, 1954; Cobb, 1955 and 1956; Potts, 1964; Seisi and Itakura, 1966). By means of its stable free state, this pigment could protect a melanin-containing tissue against ionizing conditions which might otherwise set free, within living cells, reactive free radicals capable of disrupting the metabolism. Chlorpromazine (CPZ), shown to bind with melanin in vivo (Blois and Kallman, 1964; Blois et al, 1964; Blois, 1965), caused a reduction in the transplantability of Fortrer's melanotic melanoma when it preceded irradiation, as demonstrated by Cooper and Mishima (1972). These same investigators (1971) reported that the tumours that received irradiation alone (even with in vivo doses as high as 5000R) or CPZ alone did not exhibit a significant volume decrease. In contrast, after treatment of the animals with CPZ prior to irradiation, a 61% reduction in tumour volume occurred. Furthermore, there may be a direct relationship between the degree of pigmentation and the free radical content (Mason et al, 1960). Hence, the more heavily pigmented a tissue the less radiosensitive it would presumably be. X-irradiation of pigmented and of pale slices of Cloudman S91 melanoma revealed that the pigmented tissue was relatively more radioresistant (Cobb, 1955 and 1956), although irradiation did reduce the incidence of successful transplantability of both types of tissues.

The classical description of the melanoma as being uniformly radioresistant may not be true in all cases (Levitt, 1952; Murphy, 1967; Leven, 1973). Several investigators reported remissions and even cures of human melanomas by radiotherapy alone or the latter combined with surgery (Jorgshol and Engdahl, 1955; Hellriegel, 1963; Wildermuth, 1969; Weitzel, 1970; Malaise et al, 1972; von Glasser, 1972; Ghamrawi and Glennie, 1974). Biran et al (1973) asserted that, although some types of malignant melanoma were definitely radiosensitive, there was no expedient means of predicting which types would actually be. These investigators suggested, therefore, that more patients afflicted with malignant melanoma should undergo a regime of trial radiotherapy. Stallard (1964) reported better results with melanomas irradiated while eight millimeters or less in diameter.

Barranco et al (1971) ascertained the effect of zero to 1000R on three melanoma strains with varying degrees of pigmentation. All three strains exhibited a similar radiation sensitivity (n=40, Do=125R). It was noted that, although the amount of pigmentation influenced the response of cells to UV radiation, the amount of pigment did not affect the response to x-rays. Moreover, melanoma cells responded similarly to radiation as CHO cells, suggesting that the radioresponse of the melanoma cells did not correlate necessarily with the model of a classically radio-resistant tumour cell. In reference to these observations, Hornsey (1972a) cautioned that extrapolations from experiments in vitro may considerably underestimate the sterilizing doses required for tumours in radiotherapy. The Dq (D_2-D_1) of approximately 460R ascertained by Barranco et al (1971)

would be a crucial factor in the necessary fractionation of irradiation to a melanoma.

CYTOSINE ARABINOSIDE (ARA-C) COMBINED WITH IRRADIATION

Introduction

Chemotherapy and radiotherapy may be combined to produce enhanced cell killing, utilizing a number of conceivable mechanisms, such as rendering DNA strands more liable to ruptures, by inhibiting repair of sublethal damage, by inducing cell cycling, or by synchronizing cycling cell populations. Although any, some or all of these mechanisms may play a role in combinations of various drugs with irradiation, the particular concern in the present investigation was the effect of ara-C, when used as a synchronizing agent, on the irradiation response of normal and malignant tissues. Moreover, though various chemotherapeutic agents have been employed clinically in combination with x-irradiation, the rationale in the administration of such combinations was rarely based on the known cell kinetics of the tumour, either before or after the treatment with the drug. Inasmuch as the radioresponse of a cell may vary with the age of a cell (or its position in the cell cycle), enhanced cell killing would be expected if a cohort of cells, synchronized by a drug, was irradiated while in the most sensitive phase of the cell cycle.

The Effect of Ara-C Combined with Irradiation on the Intestinal Epithelium

It is obvious that, because both ara-C and irradiation alone damage the intestinal epithelium, the combined use of these two agents could result in an enhanced detrimental effect. Indeed, the success of any combination of chemotherapy with radiotherapy will ultimately depend on the differential response of actively proliferating normal tissues, such as the intestinal epithelium, and that of the malignant cell population present in the same individual. Toxicity was reported to occur in patients irradiated following treatment with various chemotherapeutic agents, and it has often been the cause of cessation of the particular treatment schedule. In instances, it even culminated in the death of the patient (Crews, 1961; von Essen et al, 1963; Ottoman et al, 1963; Ewing et al, 1965). It was observed that actinomycin D, with a similar effect to ara-C in that it causes reversible inhibition of DNA synthesis, exerted an enhanced destructive effect on the intestinal epithelium of dogs when administered during or after irradiation (Howard, 1965; Concannon et al, 1969 and 1970; Hagemann and Concannon, 1973). That combination therapy caused almost a complete cessation of mitosis in the crypts; histologically the Peyers patches were almost completely destroyed, the mucosa was very thin with a marked denudation and atrophy, and the number of intestinal crypts was drastically reduced. Cells remaining in the crypts were large and bizarre. Hydroxurea (HU), again with a similar effect to ara-C, was employed to synchronize mouse intestinal cells in S prior to irradiation four to 28 hours later (Gillette et al, 1970). An initial 0.5 mg/gm dose, followed by 0.2 mg/gm doses at 45 minute intervals depressed the LI to

zero during the first hour and to 15% at two hours. At four hours the LI was 33% (normal), at six hours it augmented to 45% from which it declined and then increased again, reaching a peak of 55% at 13 hours. When an 1150R dose was administered four to 12 hours following the last injection of HU, there were five to seven regenerating crypts per cross-section as compared to 30 present when irradiation followed 16 hours later. It was proposed that HU exerted a sensitizing effect likely just during the first passage of cells through the S phase following the last injection of the drug.

The Effect of Ara-C Combined with Irradiation on Tumours

Thomlinson (1971) treated mice bearing intracranial gliomas with ara-C alone, and followed by irradiation, noting that neither approach altered significantly the survival of the animals. In contrast, Weiss and Tolmach (1967) reported that treatment with ara-C eight hours following a 940R dose of HeLa S3 cells reduced cell survival from five to 20 fold. When cells were exposed to ara-C immediately before irradiation, there was also enhanced cell killing. It was noted that HeLa cells in G_1 were killed more rapidly than while in S by the combination therapy.

Sasaki and Toda (1972) attempted to synchronize cells by DL-isoproterenol-HCl (IPR), observed to stimulate cell proliferation in rodent salivary glands. Following a single injection of IPR, DNA synthesis began 18 hours later, reached a peak at 24 hours, and returned to normal levels by 48 hours. When a second injection was administered at the latter point, DNA synthesis recommenced at 16 hours, peaked at

20 hours, and returned to control values by about 30 hours. Because the cells were stimulated to enter the S phase in a more synchronous manner following the second injection of IPR, the animals were irradiated 1, 16 or 20 hours later, corresponding to the early prereplicative, late prereplicative, and mid-S phase, respectively. Irradiation in early G_1 (following IPR) inhibited DNA synthesis in a dose-dependent manner. But it was less effective when given in late G_1 or in the mid-S phase. Further experiments indicated that irradiation in early G_1 simply decreased the fraction of cells stimulated to enter S, and also slightly delayed the passage of these cells into S. Although a few degenerative changes were observed in the parotid glands irradiated with 1000R at one hour, degenerative changes were not noted in the cells irradiated at the other times.

CHAPTER III

MATERIALS AND METHODS

Experimental Animals

Male, C57Bl/6J mice, four to six weeks of age, were obtained from the Jackson Laboratory, Bar Harbor, Maine. At the time of experimentation, the mice were between five and seven weeks of age, with a mean body weight of 22 grams (range = 17 to 25 grams).

The animals were housed six to eight animals per cage and fed Purina Chow and tap water *ad libitum*. The temperature in the animal room was maintained at 22°C, and the light-dark cycle was alternated on a 12-hour schedule.

Four mice served as the experimental animals for each group of indices. A minimum of eight animals per experimental group were used to determine survival parameters. Factors of a technical nature caused attrition of some groups to three (from four) or six (from eight) animals.

B16 Melanoma Tumour

The tumour employed throughout this investigation was the B16 melanoma, originally obtained in two donor C57Bl/6J mice from the Jackson Laboratory, Bar Harbor, Maine in June 1973. It was henceforth perpetuated in this laboratory.

Tumours were grown in donor mice for 12 to 14 days, at which time the donors were killed by cervical dislocation. Tumour specimens were

removed and minced into approximately 1 mm^3 pieces in a Petri dish containing Hank's balanced salt solution (pH 7.0 ± 0.02). For transplantation, the experimental animals were lightly anaesthesized with ether. An incision, approximately 4 mm in length, was made in the upper left abdominal skin. Two or three pieces of tumour tissue were loaded into a Trocar with a rounded plunger end. The Trocar was inserted subcutaneously into the incision, so that the end was approximately 5 mm inferior to the lateral aspect of the lower left ribs. The plunger then deposited the tumour tissues in the desired location, the fascia, overlying the duodenum, (Figure 3). The incision was not usually closed but healed spontaneously in approximately four to six days. The incidence of successful transplantation was 96%.

The day of implantation was designated as day zero of tumour growth, and the tumours were implanted 11 days, to the hour, prior to commencing with any experimental procedure.

Cytosine Arabinoside (ara-C)

The ara-C (U-19, 920: Lot numbers 8240-BDA-120 and 9580-BDA-50) was donated by the Cancer Research Section, Upjohn Company, Kalamazoo, Michigan. The dosage of ara-C employed throughout this investigation was 12.5 mg/kg body weight. The white crystalline (ara-C) powder was dissolved in distilled water immediately before the commencement of any particular experiment, so that the correct dosage amounted to the body weight in grams times one milliliter.

The solution of ara-C was kept refrigerated at $4^{o}C$ throughout the particular experiment, the required amount only being withdrawn and

Figure 3 The *in situ* B16 Melanoma. The tumour, on day 10 of tumour growth, has been exposed and reflected superiorly.

Figure 4 The *in situ* B16 melanoma exposed through an aperture in a lead shield.

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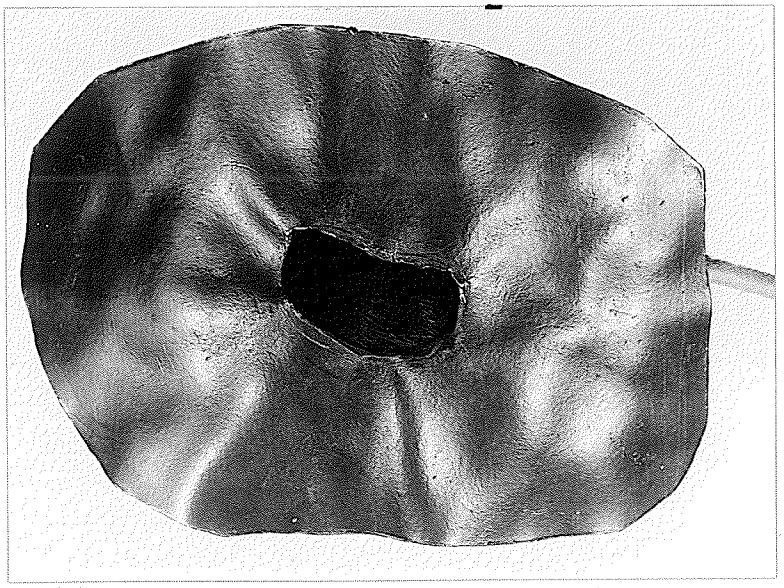

kept at room temperature not longer than one-half hour prior to the injection. All injections were given intraperitoneally (i.p.) in the lower right abdominal quadrant. Control animals received the vehicle only.

Synchronization Procedure

The synchronization procedure in this investigation was that devised by Gibson and Bertalanffy (1972). Mice received 8 i.p. injections of 12.5 mg/kg ara-C, with the injections being two hours apart.

Irradiation

The mice were anaesthesized with an i.p. injection of sodium pentobarbital (Veterinary Nembutal, 60 mg per ml, Abbott Laboratories Limited, Montreal, Quebec) according to the following formula (Hoshino, 1973): one part Nembutal to 12 parts distilled water, dosage (in cc) = $\frac{1.5 \times \text{body weight (grams)}}{100}$. The anaesthesized mice were placed under a 1 mm thick lead shield so that the tumour only was exposed through a 2.5 by 1.5 cm window cut into the shield (Figure 4). A Siemans Stabilipan unit with a Thoraeus filter (H.V.L. 2.8 mmCu) served for irradiation throughout the investigation. The dosage rate was 150 roentgens per minute (143.5 Rads) with a 30 cm focal skin distance (F.S.D.) and a 2.5 cm in diameter field size. The mice were irradiated individually (Figures 5 and 6). Control animals were similarly anaesthesized and placed under the lead shield and x-ray tube for comparable time periods to irradiated animals. The x-ray machine was not operating.

Figure 5 Control Panel of Siemans Stabilipan Radiation Unit.

Figure 6 Procedure for irradiation.

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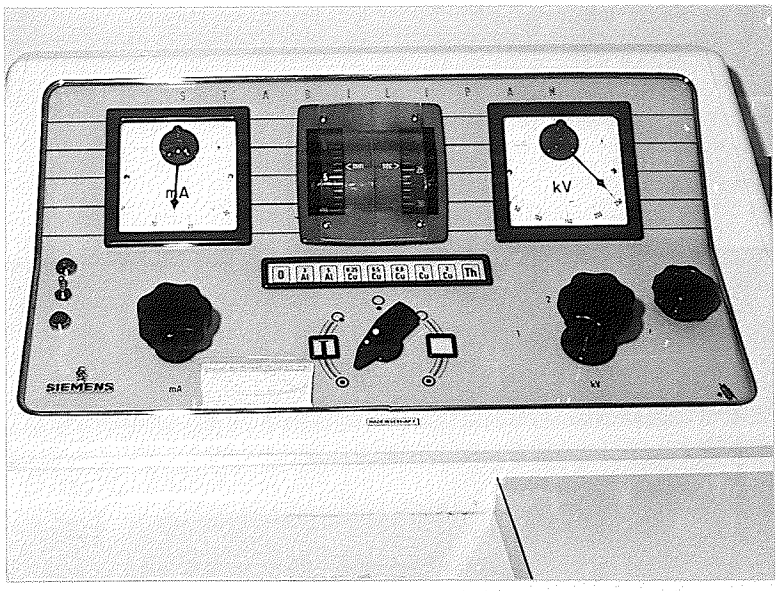
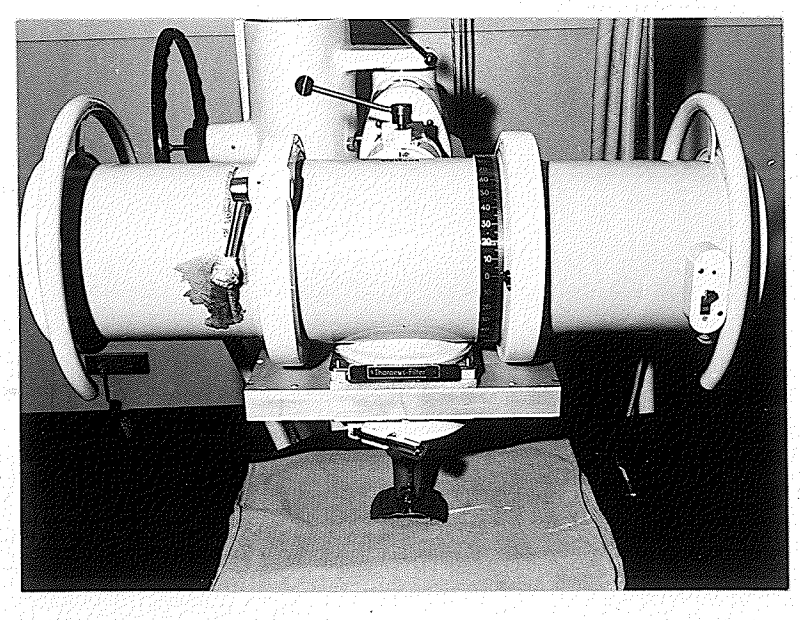


Figure



Tissue Preparation

Fifteen minutes prior to killing the mice received a 25 μCi (in .25 ml of distilled water) i.p. injection of tritiated thymidine (specific activity 5 Ci/mM, Amersham/Searle, Don Mills, Ontario). The mice were killed by cervical dislocation and the tumours removed, bisected, and placed in Davidson's fixative. A one to two cm long section of duodenum was removed, incised along the longitudinal axis, and placed with the tumour from the same animal into the fixative. Each tissue was then blocked in paraffin and routinely processed for light microscopy. Sections were cut at 3-4 μ, two to four sections being placed on each microscope slide.

For autoradiography, tumour and duodenal sections were dipped into Kodak NTB^2 type liquid nuclear tract emulsion and exposed for 10 days in black boxes at one to four degrees centigrade. These preparations were then developed in Kodak D-19 developer, fixed, and stained with haematoxylin (Gibson, 1969)(Figures X and XI).

For the determination of all other parameters, tumour and duodenal tissues were stained with haematoxylin and eosin.

Scoring of Data

Enumeration of nuclei was with a binocular light microscope under oil immersion (X1000). Tumour sections were scanned under low magnification to locate an area of viable tumour tissue. Nuclei were enumerated in an area delimited by a micrometer disc (Bausch and Lomb), and the slide was then moved to a different field. This procedure was continued

until at least 1000 cells were scored. Duodenal sections were scanned under low magnification to locate the crypts of Lieberkühn. Under oil immersion (X1000), a minimum of 1000 crypt cells were scored. The scoring was performed by the same investigator in a double-blind procedure.

The mitotic index (MI) was determined by enumerating the number of mitotic figures present among 1000 cells. Criteria used to identify the mitotic phases were those described by Leblond and Stevens (1948) as follows: (1) Prophase - appearance of definite chromatin filaments in the nucleus and adjacent to the nuclear membrane. In instances the filaments were thickened and distributed throughout the cytoplasm; the nucleolus and nuclear membrane having disappeared. (2) Metaphase - the chromosomes were arranged in the central area of the cells or accumulated at the equator. (3) Anaphase - the chromosomes had separated to opposite poles of the cell. (4) Telophase - a mid-plate divided the cytoplasm, and both nuclei (at opposite poles) were condensed. In instances two daughter cells in close proximity could be recognized.

The degenerating index (DI) was ascertained by enumerating the degenerating cells present among 1000 cells. Degenerating cells were recognized by dense, shrunken (pyknotic) nuclei and scanty, very eosinophilic cytoplasm.

The labeling index (LI) was ascertained by scoring those cells with a minimum of two grains overlying the nucleus, and their proportion among 1000 cells was recorded. Tritiated thymidine (H^3-TdR), a tracer specific for DNA, was administered 15 minutes prior to killing in a manner previously noted.

Treatment Schedules

Ten different treatment schedules were employed. In each, four mice were killed 24 hours, 48 hours, and 72 hours following each treatment. At least eight animals were maintained for the determination of survival parameters. When irradiation followed the administration of ara-C, it was applied at a specific point following the synchrony procedure, that is, following the last of eight i.p. injections of 12.5 mg/kg administered every two hours. The treatment schedules were the following:

(1) Irradiation on day 10 of tumour growth with dosages of 500R, 1000R, 1500R, and 1000R fractionated to two equal doses of 500R applied 24 hours apart.

(2) 1000R four hours following the synchrony procedure.

(3) 500R four hours following the synchrony procedure.

(4) 1000R 14 hours following the synchrony procedure.

(5) 500R 14 hours following the synchrony procedure.

(6) 1000R 16 hours following the synchrony procedure.

(7) 1000R 18 hours following the synchrony procedure.

(8) 1000R 20 hours following the synchrony procedure.

(9) 500R 14 hours and 500R 42 hours following the synchrony procedure.

(10) 500R 14 hours and 28 hours following the synchrony procedure.

In addition to the untreated control group of animals, each experimental group was also matched with another group of mice which received ara-C but without irradiation.

Analysis of Data

Statistical procedures were factorial analysis of variance and two multiple-range tests, namely Duncan's multiple-range test and Tukey's test (Rollwagen, 1973).

NOTE: In Chapters III, IV, V, and VI the letter "R" was used to denote "rads", not roentgens.

CHAPTER IV

RESULTS

CYTOSINE ARABINOSIDE

The purpose of this segment of the investigation was to ascertain achievement of cell synchrony of the B16 melanoma cell population by the method devised by Gibson and Bertalanffy (1972), namely, by eight i.p. injections of 12.5 mg/kg ara-C given at two-hour intervals. The mice, four in each group, were killed by cervical dislocation 15 minutes after a 25 μCi i.p. injection of H^3-TdR, and four, 10, 12, 14 16, 18, 20, 28, 38, 40, 42, 44, 62, 64, 66, 68, 86, 88, 90, 100, and 116 hours following the last injection of ara-C. Specimens of B16 tumour and duodenum were removed, and 3 to 4 μ sections prepared for morphological study (H & E) or for autoradiography. Groups of mice were not always killed at regular two- or four-hour intervals following the last injection of ara-C, but rather at the exact times when Gibson and Bertalanffy (1972) reported that synchrony of the B16 melanoma cell population had occurred with the ara-C regimen. Moreover, in subsequent experiments of this investigation, x-irradiation was administered at specific times following the last ara-C injection. Groups of mice were thereby killed 24, 48, and 72 hours following the x-irradiation. Other groups of animals, which had received ara-C alone but not x-irradiation, were killed at comparable times to those animals receiving both, and also at the times of the x-irradiation of the combination treated animals. Therefore, specimens of the tumour

and duodenum were not examined during the time periods between 28 to 38 hours, 44 to 62 hours, 68 to 86 hours, 92 to 100 hours, or 100 to 116 hours, and estimates of indices for these points (in the charts to follow) represent extrapolations of available data.

The Effect of Ara-C on the Duodenal Mucosa

1. Morphology (Figure 7). The normal mucosa of the duodenum of the C57Bl/6J mouse was characterized by long, narrow villi, approximately six to seven times as high as the crypts were deep. Nuclei of the crypt cells were larger and more roundish and vesicular than the nuclei of the villus cells. An exact longitudinal section through a crypt, with the lumen discernible, contained between 65 and 85 nuclei, relatively regular in size and shape. Degenerating cells were observed infrequently in the crypts; when present they were not usually in contact with the basement membrane. They were represented by round cells with very eosinophilic cytoplasm and pyknotic nuclei. Mitotic figures were abundant in the crypts. They were readily discernible, the chromosome sets being located in the apical parts of the cells.

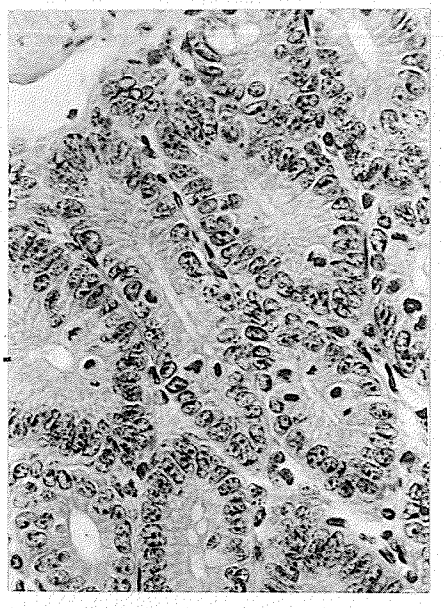
Four hours following the last ara-C injection, there were obvious morphological changes in the crypts, whereas the villi appeared morphologically normal. The crypts were noticeably shortened, much less cellular than normally, and the nuclei of many cells appeared to be swollen, with dispersed chromatin. The nuclei varied in general shape and size. Degenerating cells were numerous. By 14 hours, crypt cellularity was further reduced, and degenerating cells were fewer. Nuclei of crypt cells

Figure 7 (Pages 119 and 120)

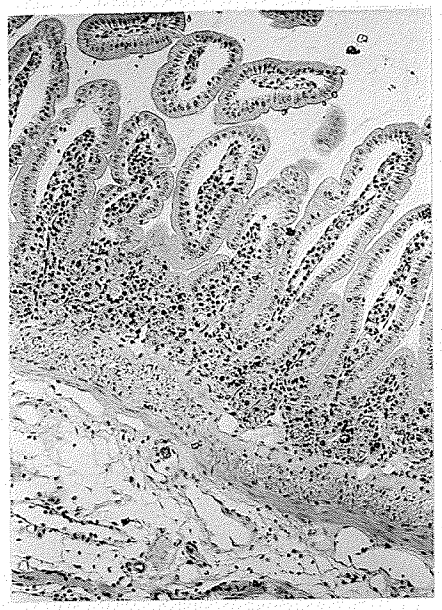
The duodenum of the C57 Bl/6J mouse at various times following 8 i.p. injections of 12.5 mg/kg ara-C administered at two-hour intervals.

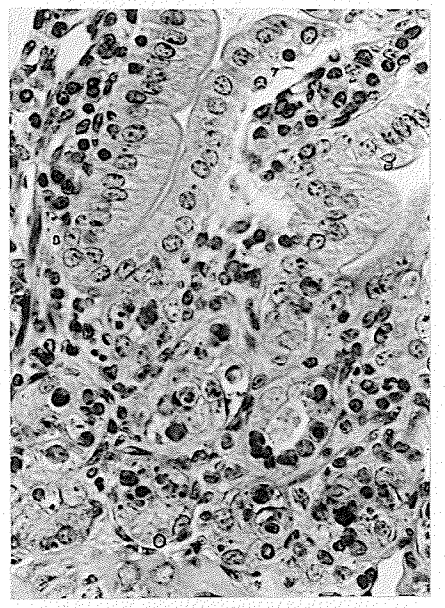
x 200 x 800

Control



4 Hrs



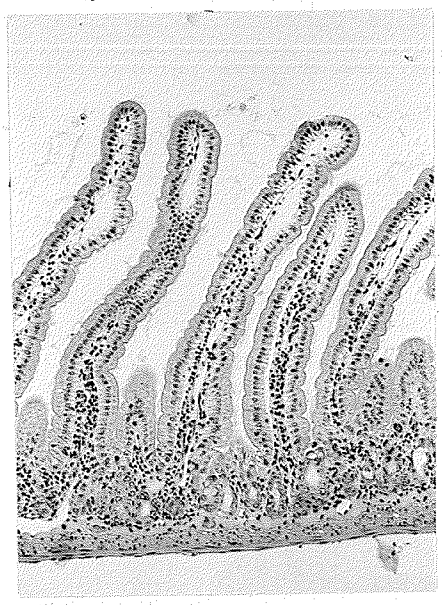


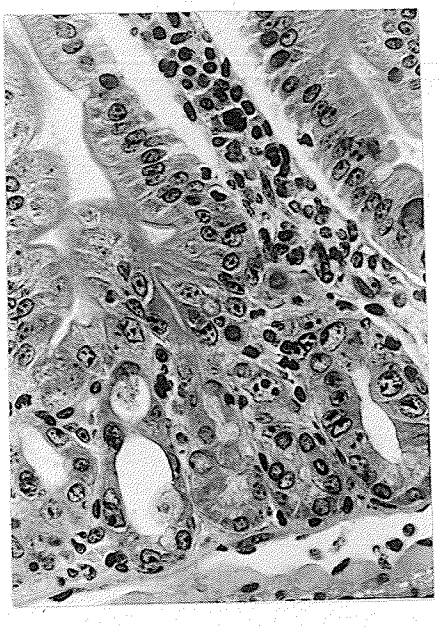
18 Hrs

re 7

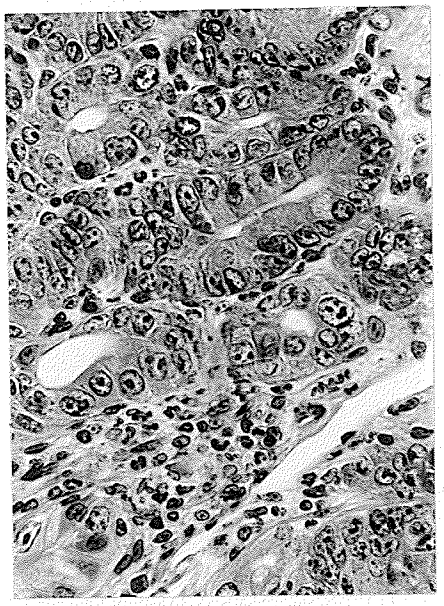
2 0

x 800

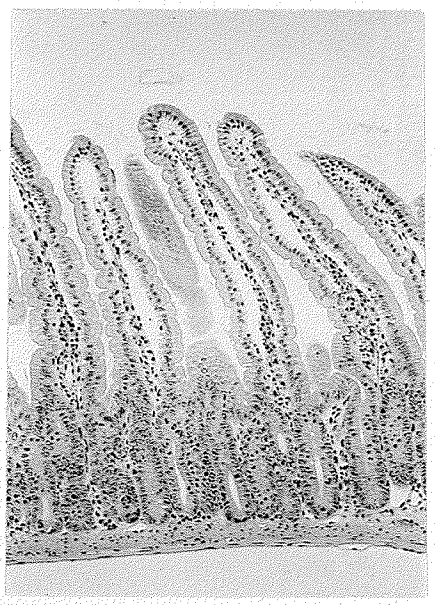


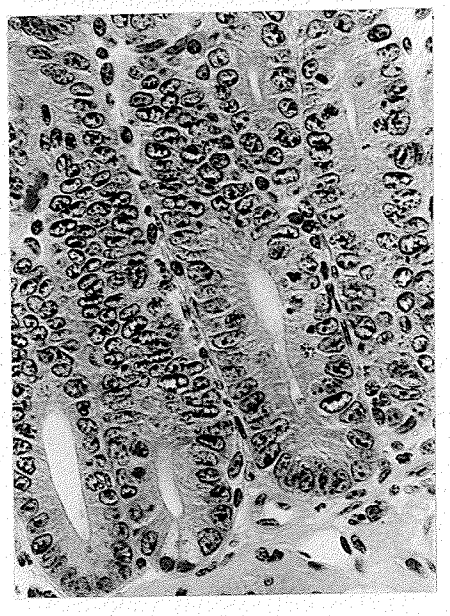


28 Hrs



52 Hrs



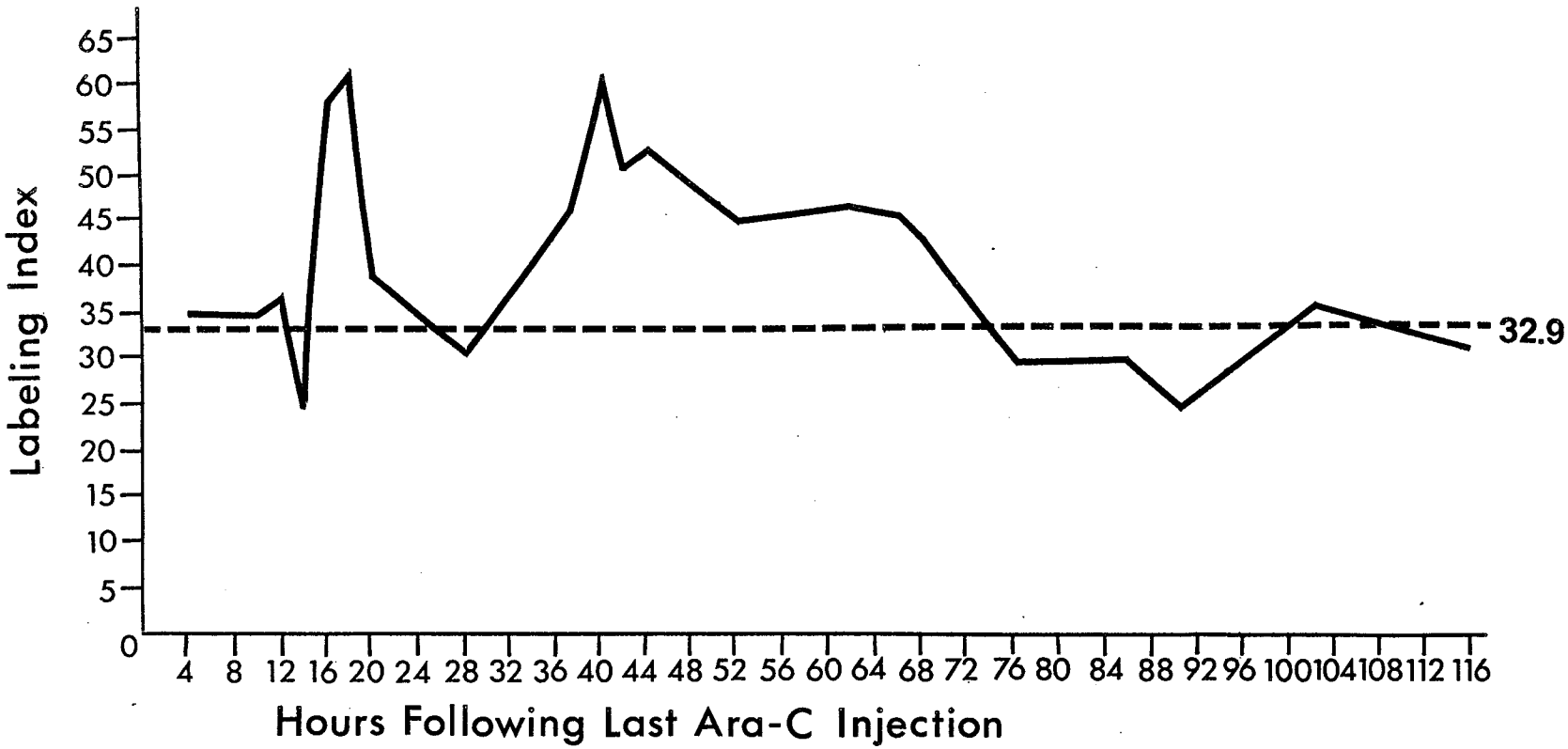


were still swollen and variable in both size and shape. Blood vessels in the lamina propria were very much distended. Any further changes of epithelial cells were not noted at 28 hours, but the lamina propria now appeared sparse and infiltrated with cells, most of which were leukocytes. Degenerating cells were observed for the first time at that stage throughout the entire length of the villi. An increase in crypt cellularity occurred subsequently, with a reduction of swollen crypt cell nuclei and disappearing irregularity of cell size and shape. By 52 hours, the crypts appeared to be close to normal. The lamina propria, however, still seemed sparse. By 76 hours, and thereafter, the epithelial cells assumed a more uniform appearance, and the nuclear irregularity in size and shape diminished. The lamina propria was still less dense than normally, and remained infiltrated by leukocytes.

2. Duodenal Indices

a. Labeling Index (LI). The labeling index, determined by the proportion of nuclei of crypt cells overlayed by a minimum of two silver grains among all crypt cells, ranged normally between 31.2 and 35.1%, with an average of 33.6% in the C57Bl/6J mouse, following a 15-minute pulse label with H^3-TdR (Chart 5). The apparent increases in the LI four and 12 hours following the last of eight ara-C injections were followed by a decrease at 14 hours; they were not significant from the control levels. The number of labeled cells then increased sharply, reaching a peak at 16 to 18 hours following the last injection, which was significantly higher than normally ($p<0.01$). It was followed by a sharp decline, until, by 28 hours, the LI was just slightly below control values

CHART 5 - THE EFFECT OF EIGHT i.p. INJECTIONS OF 12.5 mg/kg ARA-C GIVEN AT TWO-HOUR INTERVALS ON THE LABELING INDEX OF THE C57B1/6J MOUSE DUODENUM OVER A 116-HOUR PERIOD



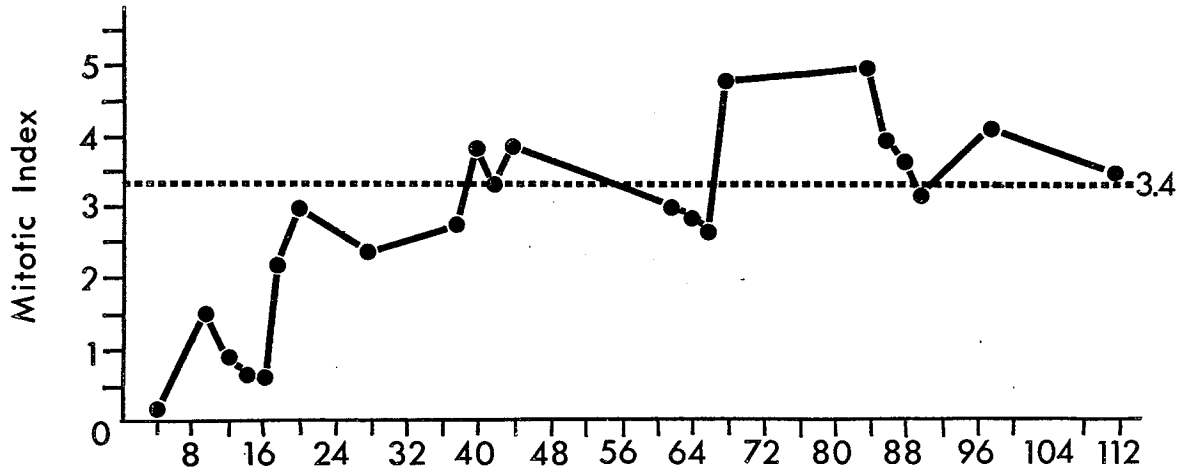
but within the normal range. A second increase, more gradual than the first, resulted in a second peak of the LI at 40 hours. The LI's at the two peaks (approximately 60%) were not significantly different from one another. Following the second peak, the LI gradually declined, so that by 68 hours, and thereafter, it was not significantly different from control values.

b. Mitotic Index (MI). There was a significant decline from control values of the duodenal MI four hours following the last ara-C injection; the index remained significantly depressed for the next 12 hours (Chart 6). Between 16 and 20 hours, the MI augmented significantly, so that by 20 hours, and thereafter, it was no longer significantly different from that of control animals. It should be noted that, at 66 hours, the MI was slightly below control values, however. It then increased significantly by 68 hours, still remaining, nonetheless, within the normal range. An ensuing significant decline between 86 and 114 hours was also within the normal range. The pronounced fluctuations were significant at the 5% level of confidence.

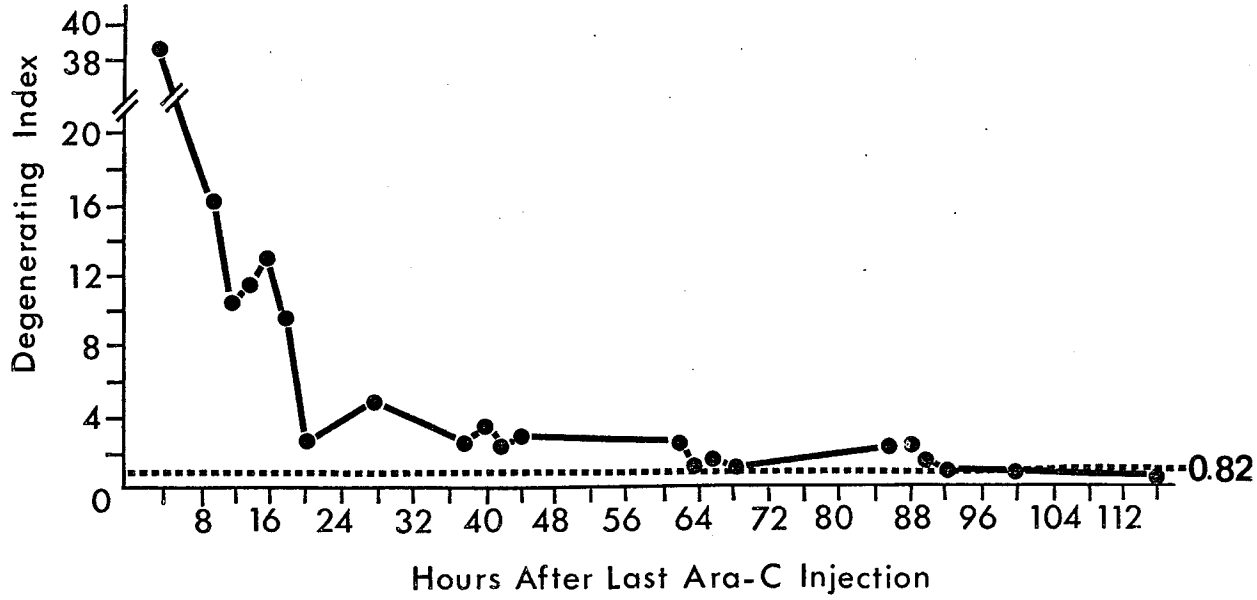
c. Degenerating Index (DI). As evident from Chart 7, the DI of the duodenum was 38.7% four hours following the last ara-C injection. It augmented hence significantly from the control value of 0.82% ($p<0.01$). Although significant decreases in the DI occurred between four and 10 hours, 10 and 12 hours, and 16 and 20 hours ($p<0.05$), the decrease during the last interval alone brought this index again within the normal range, where it remained throughout the later experimental period, despite some minor fluctuations.

THE EFFECT OF EIGHT i.p. INJECTIONS OF 12.5 mg/kg ARA-C GIVEN AT TWO-HOUR INTERVALS ON (A) THE MITOTIC INDEX AND (B) THE DEGENERATING INDEX OF THE C57B1/6J MOUSE DUODENUM OVER A 116-HOUR PERIOD

(A) CHART 6 - THE MITOTIC INDEX



(B) CHART 7 - THE DEGENERATING INDEX



Summary. Although there were slight fluctuations of the LI soon after the last injection of ara-C, none of them differed significantly from the control values during the first 16 hours. In contrast, during that same period, the MI was severely depressed, whereas the DI became greatly elevated. When the LI attained its first peak, between 16 and 18 hours, overshooting the control value by approximately 90%, the MI also augmented, yet it did not reach the control level until 20 hours. At that time also, the LI declined again to the normal level. During that same period, the DI steadily declined to control levels, where it remained throughout the duration of the experimental period. The second peak of the LI, which occurred at 40 hours, was neither accompanied nor followed by a comparable peak in the MI, which continued to remain within the normal range.

The Effect of Ara-C on the B16 Melanoma

1. Morphology (Figures 2A to 2C, page 20). The microscopic morphology of the untreated B16 melanoma on the tenth day of growth revealed a large necrotic area in the center of the tumour, surrounded by a peripheral area of viable cells. The necrotic core contained tumour cells with pyknotic nuclei and scanty eosinophilic cytoplasm, some leukocytes and cell debris. The nuclei of the cells in the viable peripheral growth zone were large, ranging in shape from ovoid to round to rather irregularly polygonal. Usually one or two nucleoli were discernible. The cells were most often arranged in irregular cords or as "swirls" around a blood vessel. Brown granules, likely of melanin, were abundant in the cells of some areas, absent in those of others.

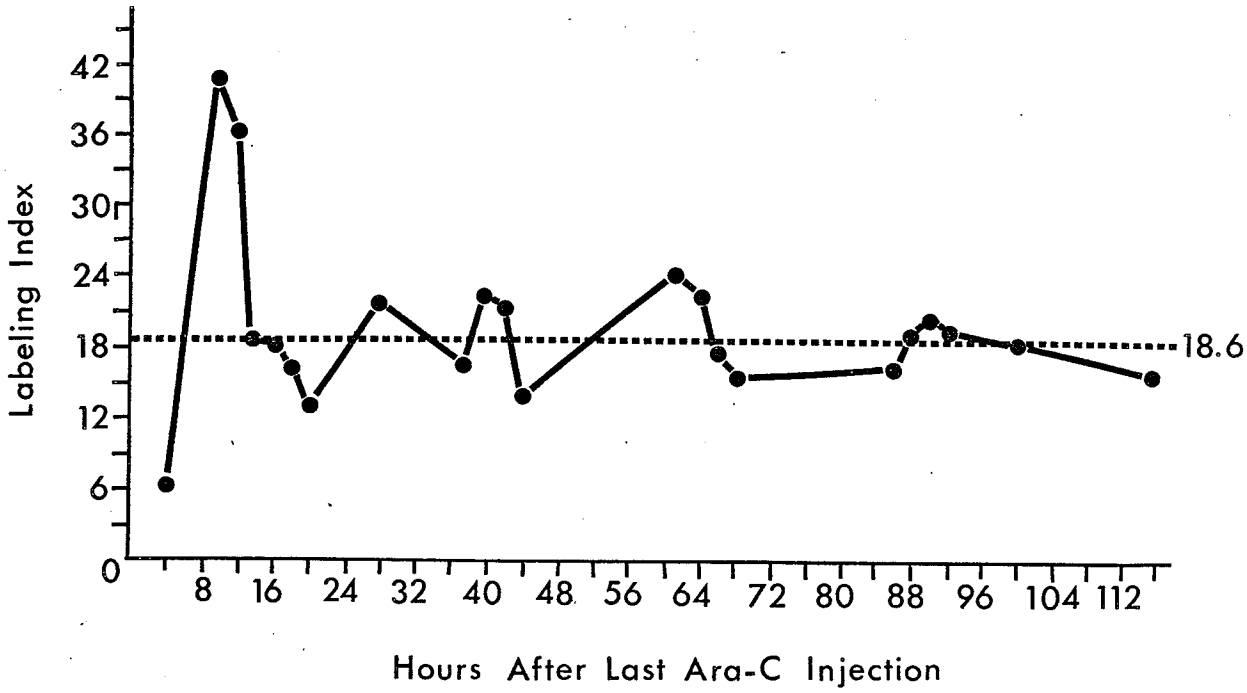
Morphological differences could not be discerned between tumours of the 10 to 15 day growth period, except for a proportionate increase of necrotic areas with the age of the tumour. Morphological differences were neither apparent between untreated and ara-C treated B16 melanoma tumours.

2. Indices of the B16 Melanoma

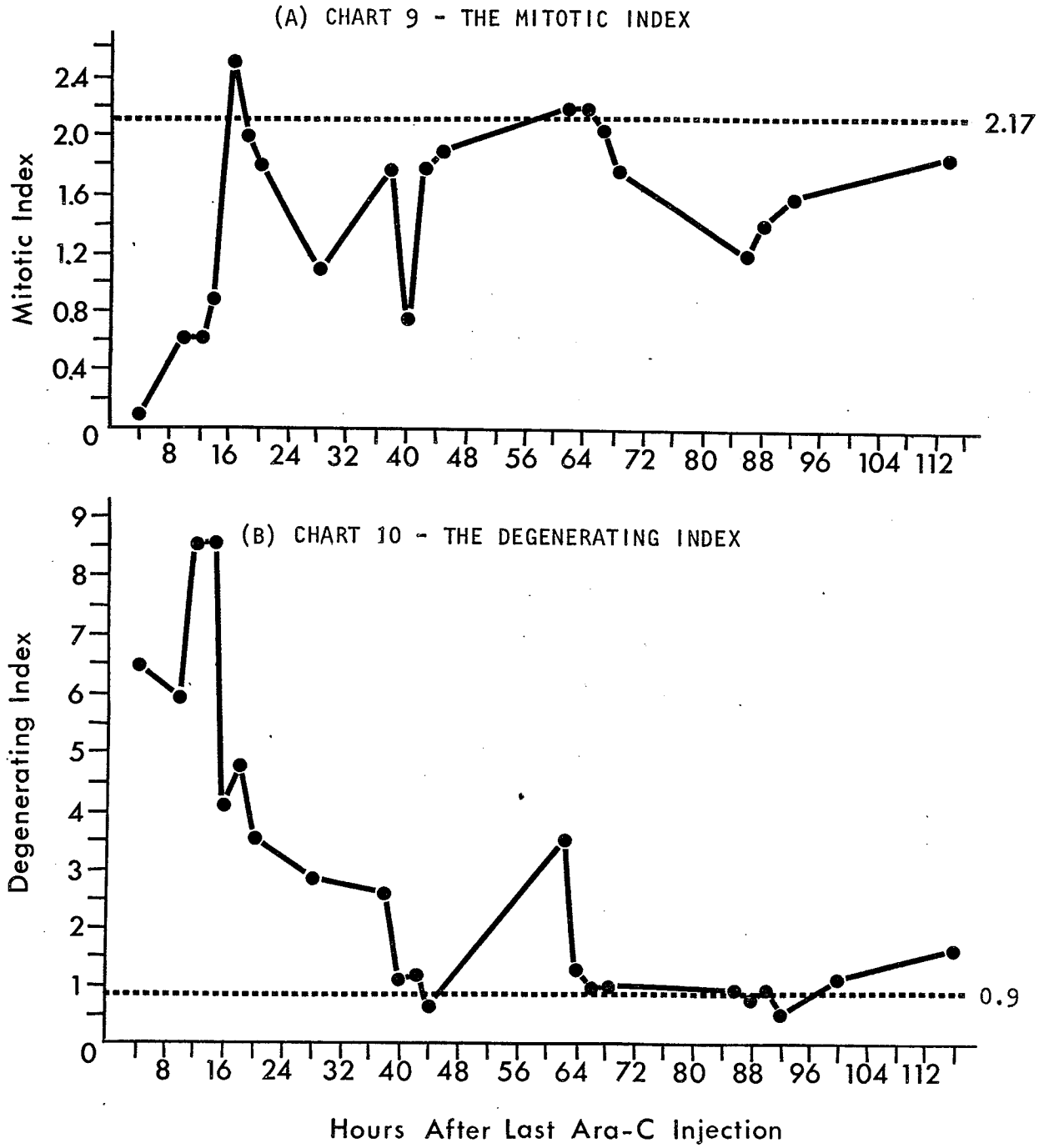
a. Labeling Index (LI), Chart 8. The LI of the untreated B16 melanoma, ascertained over a five-day period, that is, between day 10 and day 15 of tumour growth, was 18.6%. Differences were not observed on a day-to-day basis. Four hours following the last injection of ara-C, the number of labeled cells declined significantly. This was followed, in turn, by a significant increase in labeled cells, both at 10 and 12 hours, when the LI was 41.1 and 37%, respectively, that is, at both points significantly higher than control values. Both differences were significant at the 1% level of confidence. By 14 hours, the LI was again within the normal range, oscillating on either side of the control mean for the duration of the remaining experimental period, that is, for the next 102 hours.

b. Mitotic Index (MI), Chart 9. The MI of the untreated B16 melanoma, determined over a five-day period, was 2.4%. It did not differ from day to day. Four hours after the last ara-C injection, the MI was significantly depressed from control values, and it remained lower at 10, 12, and 14 hours. These differences were significant at the 1% level of confidence. Between 14 and 16 hours, there was a significant increase in the MI ($p<0.01$), so that by 16 hours the index was within the

CHART 8 - THE EFFECT OF EIGHT i.p. INJECTIONS OF 12.5 mg/kg ARA-C GIVEN AT TWO-HOUR INTERVALS ON THE LABELING INDEX OF THE B16 MELANOMA



THE EFFECT OF EIGHT i.p. INJECTIONS OF 12.5 mg/kg ARA-C GIVEN AT TWO-HOUR INTERVALS ON (A) THE MITOTIC INDEX AND (B) THE DEGENERATING INDEX OF THE B16 MELANOMA



normal range. It remained there until 28 hours, when it began to again decline ($p<0.05$). By 38 hours, at which time the MI had increased slightly, there was a sharp decrease until 40 hours, which was immediately followed by an increase at 42 hours. These latter two changes were significant at the 5% level of confidence. The MI was, therefore, within the normal range at 38 and 42 hours, but was significantly depressed at 40 hours. Although there were some fluctuations in the MI subsequently, these changes were insignificant, implying that, from 42 hours onward, the MI was not different from the normal value.

c. Degenerating Index (DI), Chart 10. The DI of the untreated B16 melanoma was 0.9% over a five-day period without significant changes between days. Four hours following the last ara-C injection, the DI was 6.5%, hence, significantly higher than the control value. The DI was still significantly elevated at 10 hours, despite some apparent decrease, and between 10 and 12 hours and 12 and 14 hours became again significantly augmented, reaching its second peak of 8.7% at 14 hours. Although significant changes did not occur between 16 and 36 hours, there was a definite downward trend in the DI. The number of degenerating cells was still above the control level at 38 hours ($p<0.05$), but by 40 hours it had declined to be within the normal range. From that time throughout the remainder of the experimental period, the DI remained within the normal range, with the exception of one instance, namely at 62 hours, when it was once more significantly increased ($p<0.01$).

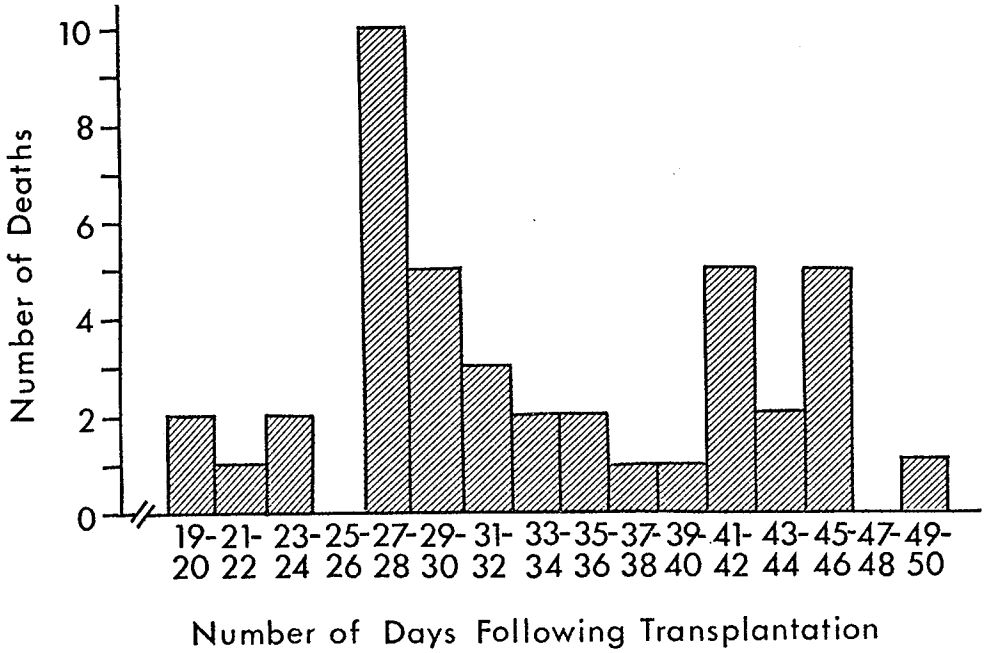
Summary. Both the LI and the MI were significantly lower than control values for the first four hours following the last ara-C injection,

when the DI was significantly higher. The LI then sharply increased, reaching peak values at 10 and 12 hours, and returning to control levels by 14 hours. Concomitantly, the MI, though still depressed, displayed an upward trend, reaching the control level at 16 hours. The DI remained elevated, far above control levels, though displaying a downward trend, reaching the control level at 40 hours. From 16 hours onward, the MI was depressed in only two instances, namely at 28 hours and 40 hours; at all other times, it was within the normal range.

The Effect of Ara-C on the Survival of Mice Bearing the B16 Melanoma

The survival pattern of untreated C57Bl/6J mice bearing the B16 melanoma is evident from Chart 11. The range in the number of days of survival following tumour implantation was between 19 and 50 days, with a mean duration of 33.4 ± 8.2 days. The TL_{50} (day by which 50% of the animals had succumbed) was 30.5 days.

CHART 11 - THE SURVIVAL PATTERN OF 42 MICE BEARING THE B16 MELANOMA



The range of survival of 10 tumour bearing animals that received eight i.p. injections of 12.5 mg/kg ara-C at two-hour intervals on day 10 of tumour growth, was from 14 to 48 days, the MST was 36.1 ± 11.9 days, and the TL_{50} was 38 days. When the animal which died earlier than the first death incident of the untreated group was eliminated from the calculations (at 14 days versus 19 days), the range of survival of ara-C treated animals was between 25 and 48 days, the MST 38.6 ± 9.6 days, and the TL_{50} 41.5 days. The mice that received the ara-C treatment did not survive any longer than the untreated tumour bearing animals, irrespective of whether any "early deaths" were included.

X-IRRADIATION

It was the purpose of this aspect of the investigation to establish that dose of x-irradiation which could subsequently be employed in experiments where x-irradiation would be administered at various time intervals following the last injection of ara-C regimens. For x-irradiation, the mice were anaesthetized. The tumour was exposed to the ionizing rays through a 2.5 by 1.5 cm aperture in a lead shield. As the tumour had been implanted s.c. in the left abdominal area, it was likely that the underlying duodenum was also irradiated. Dosages administered were 500R, 1000R, or 1500R, as single exposures, or 1000R in two equal fractionated exposures of 500R, 24 hours apart. The mice, four in each group, were killed 15 minutes following a 25 μCi i.p. injection of H^3-TdR, either 24 hours (one day), 48 hours (two days), or 72 hours (three

days) following irradiation. Histologic sections, of both tumour and duodenum, were prepared for morphological study (H & E) and for autoradiography.

The Effect of X-irradiation on the Duodenum

1. Morphology (Figure 8). Obvious damage to the duodenal crypts was evident as early as one day following any of the irradiation doses. The degree of damage appeared to be dose-dependent, however. The crypts were shorter than normally, less cellular, and the crypt cell nuclei were swollen and irregular. MF's were observed infrequently. Duodenal epithelia of mice that received the two fractionated doses totalling 1000R (500R x 2), did not seem to be as severely damaged as those exposed to the 1000R dose by a single dosage. By the second day the crypts of both groups appeared to be only slightly less cellular than normally. In contrast, on the second day the crypts of animals that received a single dose of 1000R or 1500R were very similar, perhaps even less cellular, to those of animals killed one day following irradiation.

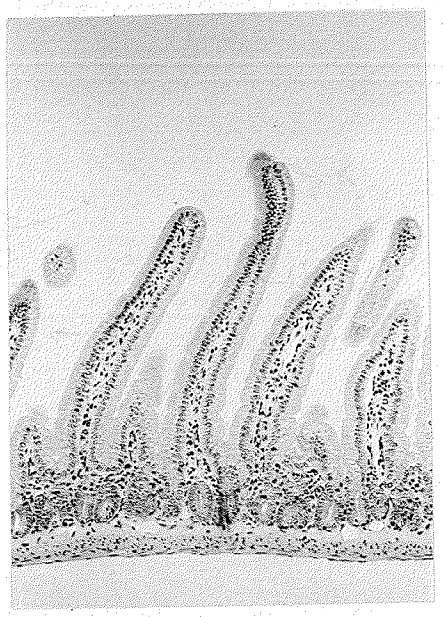
By the third day, the crypts of the 500R and 500R x 2 groups of mice appeared to be almost completely recovered, at least morphologically, and the crypts of the 1000R and 1500R groups of animals displayed some but much less damage than those of the animals on the previous day. Although the crypts of the latter groups had lengthened between the second and the third day, they were, nonetheless, still shorter than those of unirradiated animals, and also less cellular. The crypt cell nuclei were still swollen and irregular in both size and shape. Moreover, following 1000R and 1500R, the villi were shortened, particularly on the second

Figure 8 (pages 133 and 134)

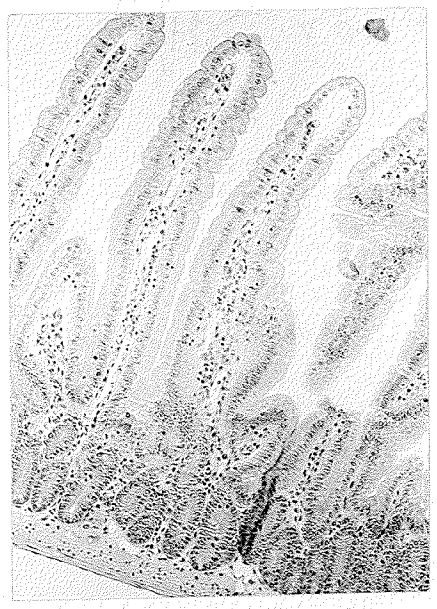
The duodenum of the C57Bl/6J mouse on each of three days following 500R, 1000R, 1500R, and 500R x 2 X-irradiation (H & E). Magnification x200.

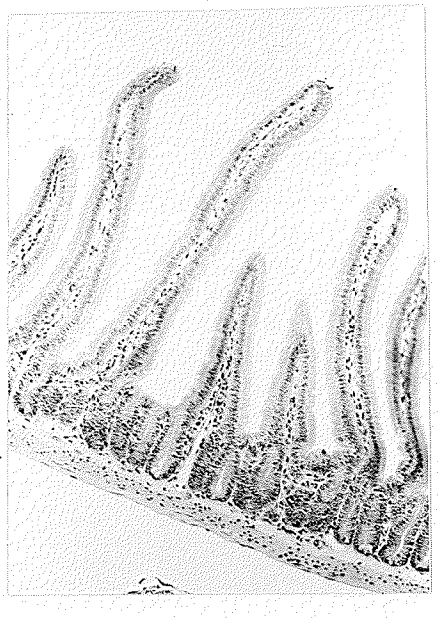
5 g

500 R x

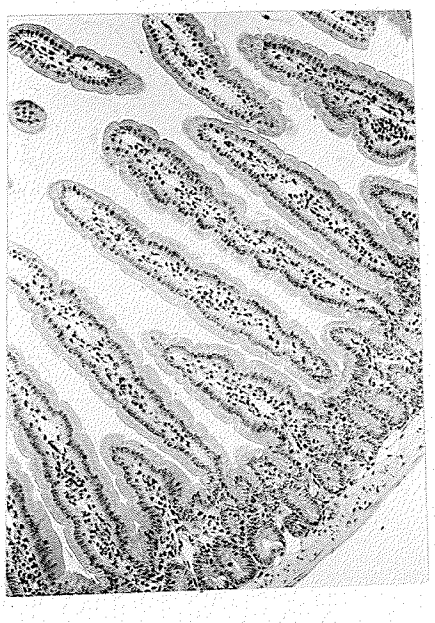


Day 1





Day 2

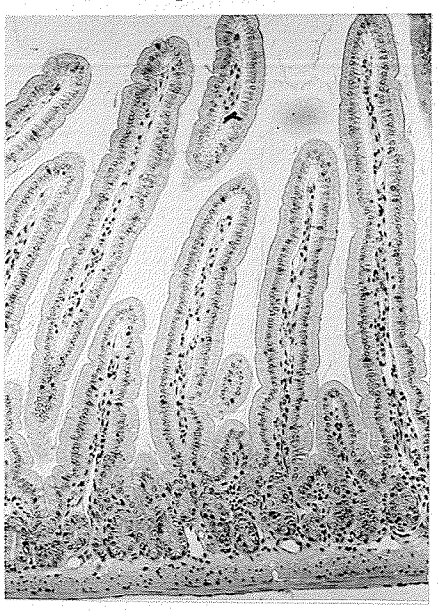


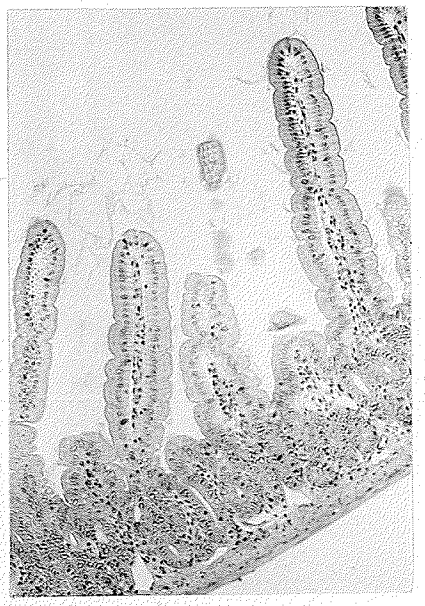
Dav 3

Fi e 8

1000 R

1500 R





post-irradiation day, and the lamina propria was rather sparse. With all doses, the blood vessels in the latter were distended.

2. Duodenal Indices (Table 1 and Chart 12).

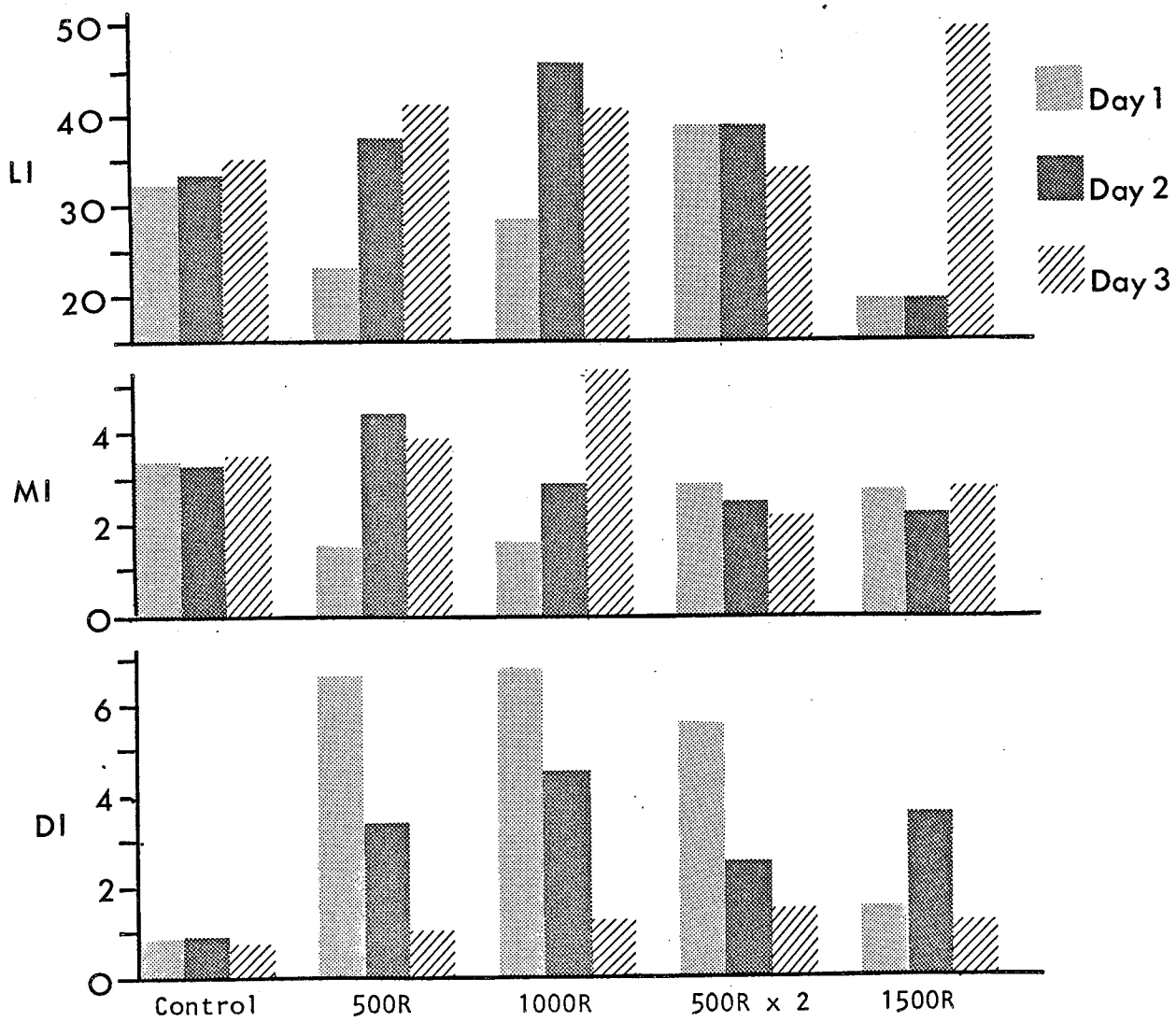
a. Labeling Index (LI). The LI seemingly decreased by 24 hours following irradiation doses of 500R, 1000R, or 1500R. However, the difference was actually significant just at the 1500R dose level ($p<0.05$). The specious increase in the LI of the 500R x 2 group was not significant from control levels, however, the LI of this treated group was significantly greater than those of either the 500R or the 1500R groups ($p<0.05$). On the second post-irradiation day, the LI's of the 500R and 1000R groups had significantly increased compared to those of the previous day ($p<0.05$), but these indices still remained within the normal range. The LI of the 1500R group remained significantly lower than those of all other groups, whether treated or untreated ($p<0.01$). There were slight variations in the LI's from the second to the third day. However, the change was significant only in the 1500R group, when the LI was more than doubled ($p<0.01$). In addition, the LI of this group was now significantly higher than those of the control animals and the 500R x 2 animals ($p<0.05$).

b. Mitotic Index (MI). Although the MI appeared reduced from control values on the first day following irradiation, it remained within the normal range. Following a dose of 500R, the MI became significantly augmented from the first post-irradiation day to the second ($p<0.01$), but did not markedly change following any other dose. Further, the MI of the 500R group was significantly higher than those of the 500R x 2 and 1500R groups ($p<0.05$), but was still not higher than that of the

TABLE 1 - THE EFFECT OF VARIOUS DOSAGES OF X-IRRADIATION ON THE CELL KINETICS OF THE C57B1/6J MOUSE DUODENUM OVER A THREE-DAY PERIOD

	DAYS AFTER IRRADIATION	CONTROL	500R	1000R	500R x 2	1500R
		Mean S.D.	Mean S.D.	Mean S.D.	Mean S.D.	Mean S.D.
LABELING INDEX	1	33.6 ± 4.9	24.2 ± 9.6	29.3 ± 5.8	39.0 ± 4.0	19.9 ± 2.5
	2	34.3 ± 6.6	38.0 ± 4.0	45.1 ± 4.3	39.1 ± 5.0	19.5 ± 4.0
	3	35.1 ± 9.7	41.4 ± 7.3	41.4 ± 7.3	34.8 ± 2.0	49.1 ± 9.2
MITOTIC INDEX	1	3.3 ± 0.5	1.6 ± 0.5	1.7 ± 0.8	2.9 ± 1.0	2.7 ± 0.6
	2	3.3 ± 0.5	4.5 ± 1.9	2.9 ± 1.6	2.5 ± 0.4	2.2 ± 1.9
	3	3.5 ± 0.4	3.9 ± 1.5	5.3 ± 1.3	2.2 ± 0.4	2.8 ± 1.8
DEGENERATING INDEX	1	0.9 ± 0.4	6.7 ± 0.9	6.8 ± 1.7	5.6 ± 4.1	1.6 ± 0.8
	2	0.9 ± 0.3	3.3 ± 2.6	4.6 ± 3.7	2.4 ± 1.4	3.6 ± 2.3
	3	0.7 ± 0.3	1.0 ± 0.4	1.2 ± 0.5	1.5 ± 0.3	1.3 ± 0.5

CHART 12 - THE EFFECTS OF VARIOUS DOSAGES OF X-IRRADIATION ON THE CELL KINETICS OF THE C57B1/6J MOUSE DUODENUM OVER A THREE-DAY PERIOD



control animals. On the third post-irradiation day, the MI of the mice which received a 1000R dose had augmented significantly from the previous day ($p<0.01$), but it was still not different from that of the non-irradiated animals. It should be noted that the MI of the mice receiving the fractionated dose of 1000R declined steadily, though not significantly, over the three-day period. By the third day it was significantly lower than that of animals receiving the 1000R as a single exposure.

c. Degenerating Index (DI). On the first day following irradiation, the duodenal DI's were significantly augmented in those animals which received doses of 500R, 1000R, or 1000R in two fractions. Further, the indices of these animals were significantly greater than that of animals which had received a dose of 1500R ($p<0.01$), in which case the DI was not different from normal. By the second post-irradiation day, the DI's of the 500R, 1000R, and 500R x 2 groups had all seemingly decreased. Although none of these changes were significant, only the DI of the 1000R group was not now within the normal range ($p<0.01$). By the third day, the DI's of all irradiated groups were again within the normal range. It is of interest to note that, although the DI's of four of the irradiated groups of animals became steadily lower over the three-day period of observation, the DI was normal on the first day, it was two-fold on the second day, and then decreased on the third day in that group receiving the 1500R exposure.

Summary. On the first post-irradiation day, the LI was decreased only in those animals which were exposed to a dose of 1500R. The fractionated group exhibited a higher LI than either the 500R or 1500R groups,

yet all of these LI's were within the normal range. The MI's were not significantly changed in any of the groups on the first day, while the DI's were augmented in all irradiated groups except in that receiving a dose of 1500R. There were increases of the LI's from the first to the second day in two groups (500R and 1000R), however the LI's of both these groups remained within the normal range. The LI of the 1500R group remained decreased on the second day, but doubled on the third day, thereby surpassing control levels. The MI changed from the first day to the second in only the 500R group, and from the second day to the third just in the 1000R group; both indices increased ($p<0.01$). The DI's, significantly augmented on the first day, declined steadily from the first day to the second and the second day to the third, reaching control levels on the latter day. Only the 1500R group did not conform with this pattern.

The Effect of X-irradiation on the B16 Melanoma

1. Morphology (Figures 9A to 9D). Following all doses of x-irradiation, some giant nuclei and nuclei displaying margination of chromatin were noted. However, a dose-dependent effect of that phenomenon was not evident. Further, it was often necessary to scan more fields of irradiated tumours than normally in order to score at least 1000 cells. It could not be determined if this was because of an enlargement of nuclei (thereby occupying more space) or to greater intercellular spaces.

2. Indices of the B16 Melanoma (Table 2 and Chart 13).

a. Labeling Index (LI). In three of the experimental groups of animals, namely 500R, 1000R, and 1500R (administered as single exposures),

Figure 9 The B16 melanoma, untreated and on each of three days following 1500R x-irradiation (H & E, X800).

The untreated B16 melanoma on day 10 of tumour growth (Figure 9A) and one day (Figure 9B), two days (Figure 9C) and three days (Figure 9D) following 1500R X-irradiation.

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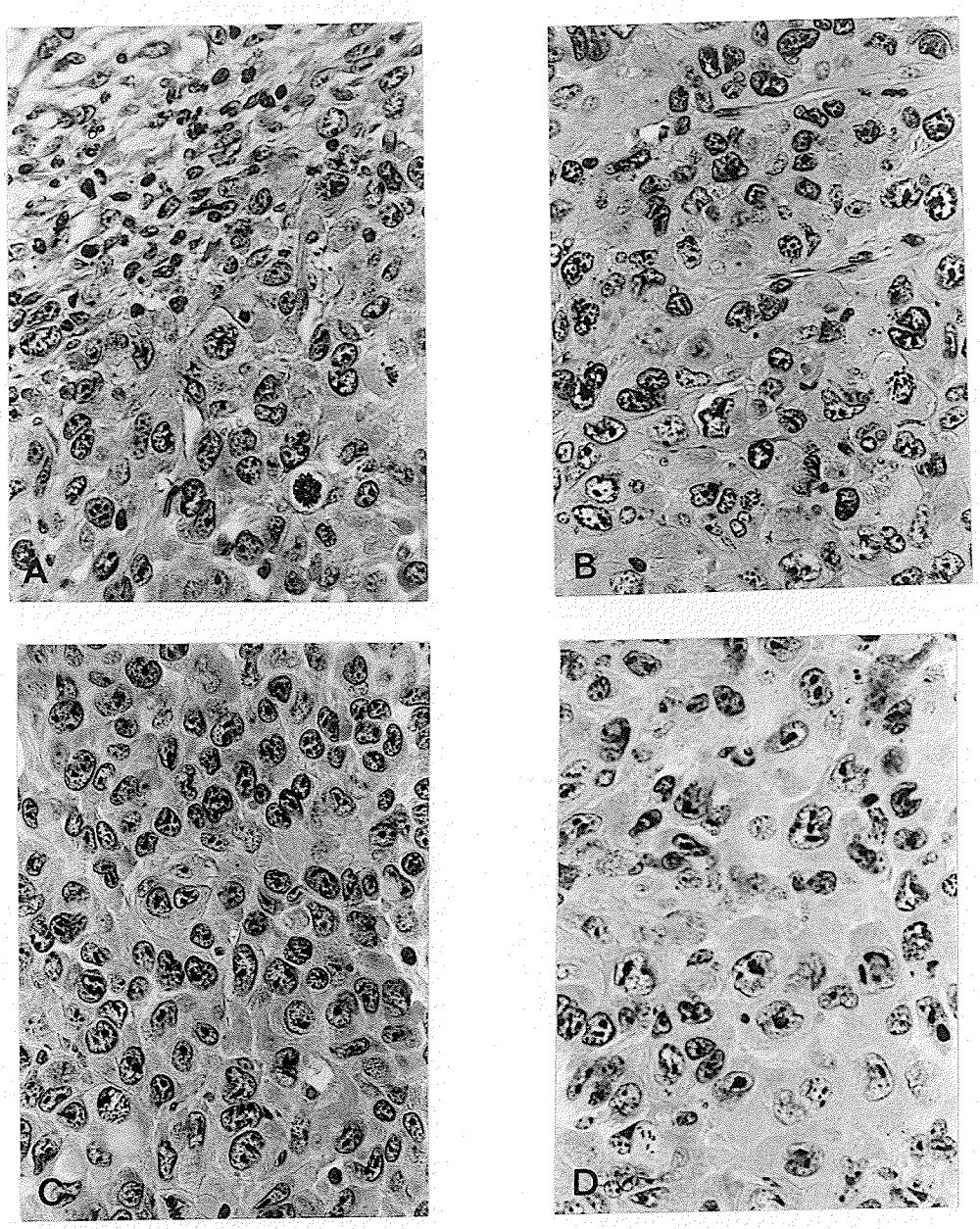


the LI's were apparently highest on the second post-irradiation day; however, those differences were never significant. When considered over the entire three-day period of observation, the LI's of all groups of irradiated mice were lower than normally, the reductions ranging from 28 to 70% of control levels. The LI's were significantly lower than control values on each day, as well as over the combined three-day period. All differences were significant at the 1% level. On the third post-irradiation day, the LI's of both the 1000R and 1500R groups of animals were significantly lower than that of the 500R group ($p<0.05$).

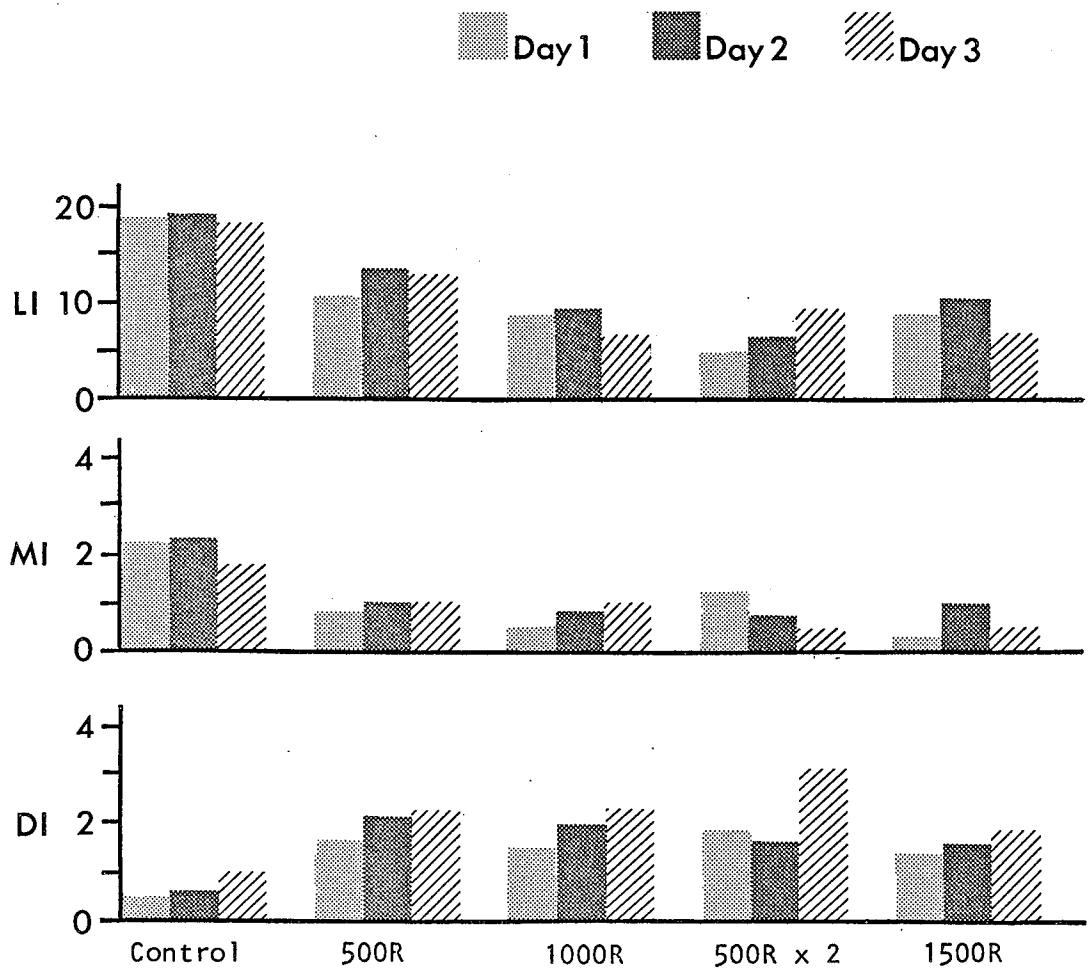
b. Mitotic Index (MI). The tumour MI's were significantly reduced (from control levels) throughout the three-day period of observation with all doses or irradiation ($p<0.01$). When the data were combined over the three-day period, a significant difference was not apparent between the tumour MI's of any of the irradiated groups of animals. On a day-to-day basis, however, there was one difference between groups, namely that of the MI of that group which received the fractionated dose of irradiation. In that case, the MI was significantly higher than those of any other irradiated group ($p<0.05$).

c. Degenerating Index (DI). Although slight undulations in the DI's of the tumour occurred over the three-day experimental period, the only difference that was significant was in that group receiving the fractionated dose of irradiation; here, the DI was significantly higher on the third post-irradiation day than either on the first or second days. Moreover, on that third day, the DI of this group was significantly higher than that of any other group. Range of the tumour DI of all four

TABLE 2 - THE EFFECT OF VARIOUS DOSAGES OF X-IRRADIATION ON THE CELL KINETICS OF THE B16 MELANOMA OVER A THREE-DAY PERIOD

	DAYS AFTER IRRADIATION	CONTROL	500R	1000R	500R x 2	1500R
		Mean S.D.	Mean S.D.	Mean S.D.	Mean S.D.	Mean S.D.
LABELING INDEX	1	18.3 ± 2.0	10.5 ± 1.1	8.6 ± 1.5	5.2 ± 0.8	8.5 ± 1.5
	2	18.9 ± 2.3	13.7 ± 2.0	9.4 ± 0.8	6.7 ± 2.0	10.2 ± 4.2
	3	17.9 ± 1.8	12.8 ± 2.1	6.8 ± 1.8	9.1 ± 1.6	7.0 ± 5.0
MITOTIC INDEX	1	2.2 ± 0.4	0.8 ± 0.3	0.5 ± 0.3	1.2 ± 0.4	0.4 ± 0.1
	2	2.3 ± 0.4	1.0 ± 0.2	0.8 ± 0.2	0.7 ± 0.2	1.0 ± 0.1
	3	1.8 ± 0.5	1.0 ± 0.3	1.0 ± 0.2	0.6 ± 0.2	0.5 ± 0.2
DEGENERATING INDEX	1	0.6 ± 0.3	1.7 ± 0.2	1.6 ± 0.2	1.9 ± 0.5	1.4 ± 0.4
	2	0.7 ± 0.2	2.1 ± 0.7	2.0 ± 0.2	1.7 ± 0.2	1.6 ± 0.3
	3	1.0 ± 0.4	2.2 ± 0.4	2.2 ± 0.6	3.1 ± 0.2	1.9 ± 0.2

CHART 13 - THE EFFECT OF VARIOUS DOSAGES OF X-IRRADIATION ON THE CELL KINETICS OF THE B16 MELANOMA OVER A THREE-DAY PERIOD



irradiated groups was between 88 and 208% greater than that of the control animals. These differences were significant at the 1% level of confidence, apart from 1000R on the first day, 1500R on all three days, and 500 x 2 on the second day ($p<0.05$).

Summary. The tumour LI's were significantly lower in all irradiated animals, as compared to control animals, on each of the three days following irradiation. Comparing between irradiated groups, on the third day, the LI's of both the 1000R and 1500R groups were lower than that of the 500R group. The tumour MI's were likewise reduced from control levels in all irradiated groups over the three-day period. Just the MI of the 500R x 2 group was higher on the first day than those of the other irradiated groups. The DI's became augmented on each day in all irradiated groups, on the third day more so in the 500R x 2 group than of the other irradiated animals.

The Effect of X-irradiation on the Survival of B16 Melanoma Bearing Mice

The animals of the irradiated groups seemingly survived for a slightly longer period than did the non-irradiated control mice (Table 3), as both TL50 and the MST became prolonged with increasing dosage. However, including in the calculation all animals that received a particular dosage, only the 1000R group actually survived significantly longer than the non-irradiated control animals ($p<0.05$). When those animals which died (likely from other causes) prior to 19 days were eliminated from the calculations, three groups of irradiated animals survived longer than the non-irradiated control mice, namely the 1000R, 1500R, and 500R x 2 groups. The differences

derived thusly were significant at the 1% level of confidence. Of interest is the fact that the MST of the fractionated group (1000R) was slightly longer than that of the 500R group, yet briefer than that of the 1000R group receiving the dosage as a single unit.

TABLE 3 - THE EFFECT OF VARIOUS DOSES OF X-IRRADIATION ON THE SURVIVAL OF C57B1/6J MICE BEARING THE MALIGNANT B16 MELANOMA

	RANGE		TL_{50}		MST		N	
RADS	GROUP 1	GROUP 2	GROUP 1	GROUP 2	GROUP 1	GROUP 2	GROUP 1	GROUP 2
DOSAGE								
0	19-50	---	30.5	---	33.4± 8.2	---	42	---
500	24-55	---	29.5	---	35.9±11.1	---	7	---
1000	23-63	---	43.5	---	46.0±12.0	---	12	---
500 x 2	13-53	31-53	36.5	38.5	37.2±11.7	39.4±7.4	8	7
1500	11-62	48-62	49.0	52.0	43.5±20.3	54.2±5.6	8	6

NOTE: GROUP 1 - All animals in a given group at a particular dosage.

GROUP 2 - Only those animals in a given group at a particular dosage which survived as long or longer than the earliest death recorded for untreated animals (i.e. longer than 19 days).

ARA-C IN COMBINATION WITH X-IRRADIATION

The purpose of this aspect of the investigation was to determine the effect of x-irradiation on the ara-C synchronized B16 melanoma cell population and, simultaneously, on the duodenum of the C57Bl/6J mouse. Dosage and schedule of administration of ara-C were identical to those employed for the previous experiments, as were the quality and method of administration of x-irradiation. In each of these experiments, there were at least 20 mice. Four mice were killed 24 hours (one day), 48 hours (two days), or 72 hours (three days) following the irradiation. Both duodenal and tumour tissues were prepared as previously indicated. A minimum of eight mice were maintained for each experimental group to ascertain survival data. Moreover, for each experimental group of mice, there were 16 other mice which received the ara-C injections but were not irradiated. Four of these mice were killed at the time of irradiation of the "combined treatment" groups, and four each 24, 48, and 72 hours later. Preparation of duodenal and tumour tissues was as for the combination treated mice.

SERIES I

In the first series of experiments, the mice received a single dose of 1000R x-irradiation at one of five specific times following the last injection of ara-C (see below). That dosage of x-irradiation was selected because it had been ascertained to depress both the tumour labeling and mitotic indices, increasing at the same time the tumour

degenerating index, at least over a three-day experimental period. Further, the 1000R dose did not result in the "early death" of any of the animals, yet it did augment the MST.

X-irradiation was given either four, 14, 16, 18, or 20 hours following the last injection of ara-C. The peak in the LI of the B16 melanoma was observed to occur 10 hours following the last ara-C injection. This peak was assumed to represent the mid-point of the S phase (Gibson and Bertalanffy, 1972). The cell cycle time of the B16 melanoma cells, following ara-C, was assumed to remain unchanged at 14 hours, with S=7.5 hours, G_1=3.8 hours, G_2=1.3 hours, and M=1.4 hours (Bertalanffy and Gibson, 1972). Therefore, the cohort of cells, released from the ara-C blockage, would presumably be in late G_1 at four hours, early G_2 at 14 hours, M at 16 hours, mid-G_1 at 18 hours, and late G_1 at 20 hours. The various combinations were designated 1000R at four, 1000R at 14 and so on.

Irradiation was not administered at six and eight hours, as it was assumed that by that time the cohort of cells would still be in late G_1, or at the same point in the cell cycle as it was at four hours. Irradiation was neither administered at ten or twelve hours after the last injection, because at those times the cohort of cells would be in the S phase (as signified by peaks in the labeling indices). At both points, most of the cells would hence be radioresistant.

<u>The Effect of 1000R X-irradiation following Ara-C on the Duodenum</u>

1. Morphology (Figure 10). Obvious damage to the duodenal mucosa was apparent following all combinations of ara-C and

Figure 10 The Duodenum of the C57Bl/6J mouse on each of three days following 1000R x-irradiation administered either four or 14 hours following the last injection of ara-C (H & E).

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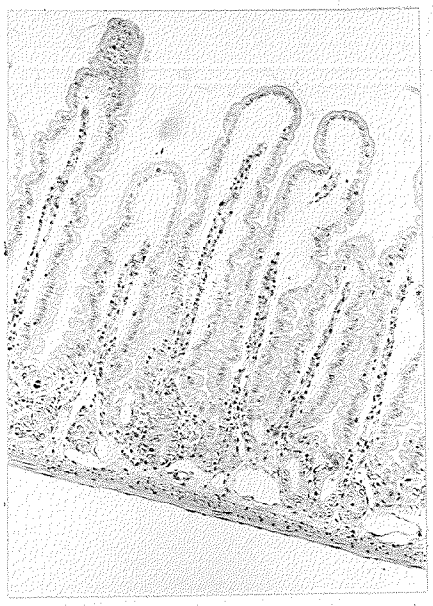
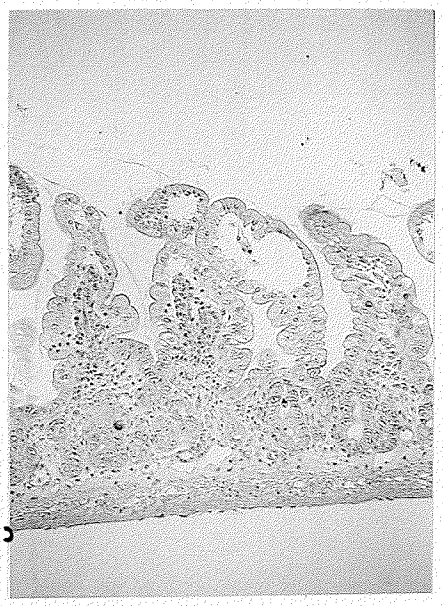
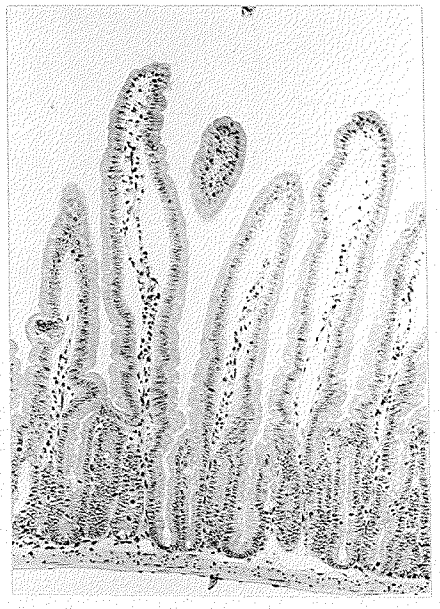






x-irradiation. That damage appeared to be most severe when mice were irradiated four hours following the last ara-C injection, less when they were irradiated at later times. One day following 1000R at four hours, both crypts and villi were shortened and the lamina propria appeared sparse. Crypt cellularity was extremely reduced, and the nuclei of the remaining crypt cells were swollen and irregular in both size and shape. The crypts were disorganized also on the second post-irradiation day. The lamina propria was still sparse, and distended blood vessels were now numerous. By the third day, the crypts were more cellular than on the previous day, yet the stromal damage appeared to be more severe. These changes, well exemplified in the 1000R at four hours group, were also noted with the other combinations of irradiation and ara-C, though they were not nearly as severe. Moreover, the extent of damage seemed to be less pronounced when there was a longer interval between the last ara-C injection and the administration of irradiation, perhaps ascribable to a recovery of the cell populations.

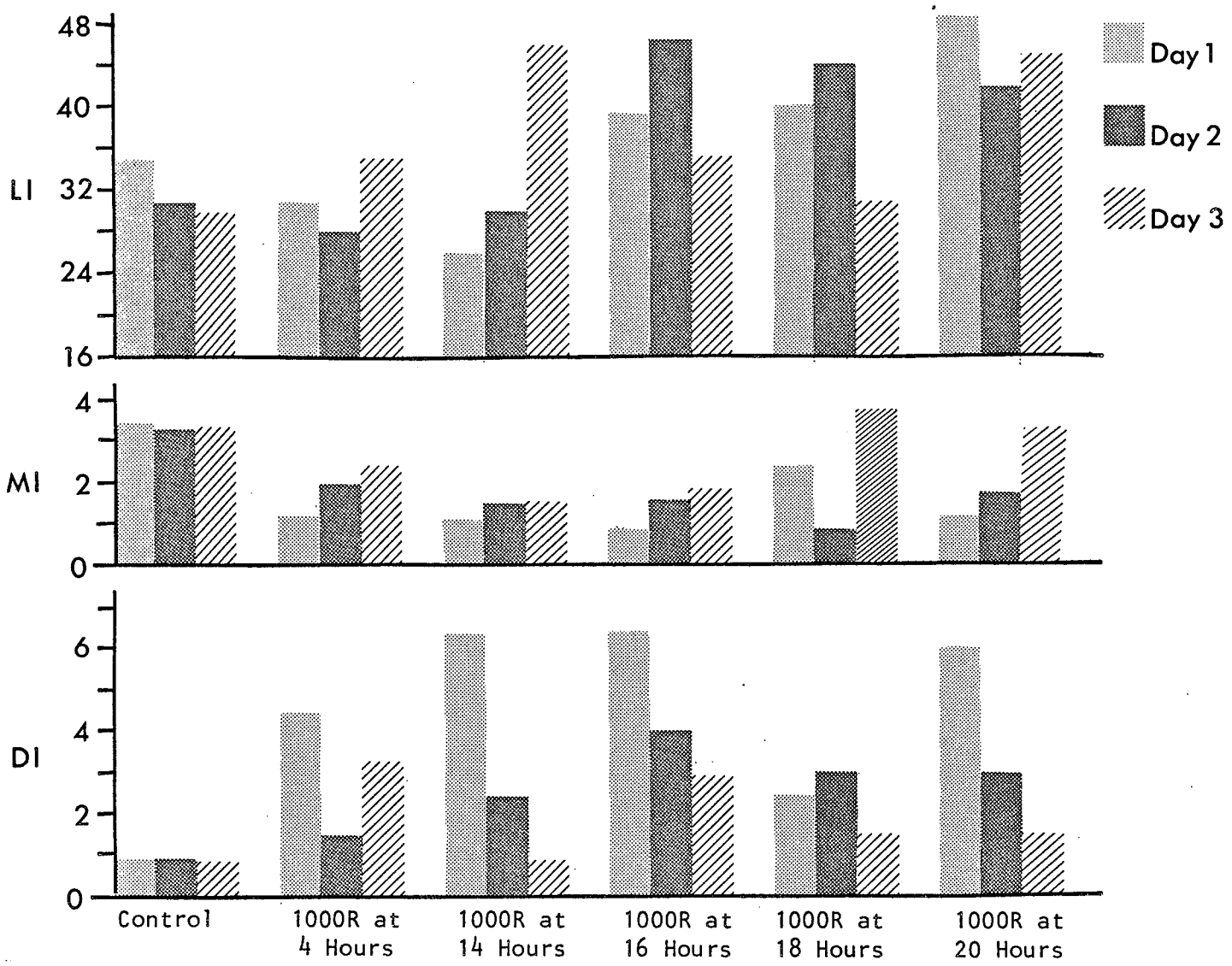
2. Duodenal Indices (Table 4 and Chart 14).

a. Labeling Index (LI). None of the LI's of the groups, treated or untreated, changed from day-to-day, and the duodenal LI's of the treated groups did therefore not differ from those of control animals on any single day or over the three-day period combined. There were, however, some differences between the LI's of the treated groups. On the first post-irradiation day, the LI's of both the 1000R at four, and 1000R at 14 groups were significantly lower than that of the 1000R at 20 group. On the third day, the LI of the 1000R at four group was significantly

TABLE 4 - THE EFFECT OF 1000R X-IRRADIATION AT VARIOUS TIMES FOLLOWING THE LAST OF 8 i.p. INJECTIONS OF 12.5mg/kg ARA-C GIVEN AT TWO-HOUR INTERVALS ON THE CELL KINETICS OF THE C57B1/6J MOUSE DUODENUM OVER A THREE-DAY PERIOD

	DAYS AFTER IRRADIATION	CONTROL 0	HOURS AFTER LAST INJECTION WHEN IRRADIATED 4 HOURS	14 HOURS	16 HOURS	18 HOURS	20 HOURS
		Mean S.D.	Mean S.D.	Mean S.D.	Mean S.D.	Mean S.D.	Mean S.D.
LABELING	1	35.1 ± 9.7	31.0 ± 9.6	25.2 ± 7.1	38.1 ± 2.2	40.0 ± 6.1	49.1 ± 5.9
INDEX	2	31.2 ± 6.0	27.7 ± 16.7	46.5 ± 14.2	46.5 ± 5.1	44.1 ± 9.9	42.2 ± 7.2
	3	30.0 ± 9.0	34.7 ± 6.5	45.7 ± 4.6	35.1 ± 3.8	36.8 ± 4.5	44.8 ± 2.4
MITOTIC	1	3.5 ± 0.4	2.1 ± 0.4	1.1 ± 0.3	0.8 ± 0.8	2.3 ± 0.6	1.1 ± 0.2
INDEX	2	3.3 ± 0.5	2.0 ± 1.2	1.5 ± 0.7	1.6 ± 1.1	0.8 ± 0.3	1.7 ± 1.4
	3	3.3 ± 0.4	2.4 ± 1.5	1.5 ± 0.8	1.7 ± 1.0	8.8 ± 2.4	3.2 ± 1.0
DEGENERATING	1	0.7 ± 0.3	4.3 ± 1.1	6.2 ± 1.8	6.4 ± 2.6	2.4 ± 0.6	6.0 ± 2.2
INDEX	2	0.9 ± 0.4	1.6 ± 0.4	2.4 ± 1.8	4.0 ± 1.0	3.0 ± 1.4	2.9 ± 1.4
	3	0.7 ± 0.2	3.2 ± 3.2	0.9 ± 0.7	2.9 ± 1.6	1.4 ± 0.8	1.4 ± 0.2

CHART 14 - THE EFFECT OF 1000R X-IRRADIATION AT VARIOUS TIMES FOLLOWING THE LAST OF EIGHT i.p. INJECTIONS OF 12.5 mg/kg ARA-C GIVEN AT TWO-HOUR INTERVALS ON THE CELL KINETICS OF THE C57B1/6J MOUSE DUODENUM OVER A THREE-DAY PERIOD



decreased over those of all other treatment groups, except the 1000R at 14 group. The latter, in turn, was higher than both the 1000R at 16 and 1000R at 18 groups. All differences were significant at the 5% level of confidence. Although there were slight changes in the LI's within each combination over the three-day period, comparison between groups did not reveal any consistent pattern of change.

b. Mitotic Index (MI). The only significant change in the MI over the three-day period of observation was in the 1000R at 18 group. There was a marked increase from the second to the third days ($p<0.05$). The MI's of the groups irradiated at four, 14, 16 and 20 hours were all significantly different from that of the control animals, but only on the first post-irradiation day, when they were decreased ($p<0.05$). Yet, the MI of the 1000R at 18 group was significantly lower than the control MI on the second day, while it was significantly higher than normal on the third post-irradiation day ($p<0.05$).

c. Degenerating Index (DI). The DI of the duodenum increased significantly over the three-day period following all combinations of treatment. In four of the groups (1000R at four, 14, 16, and 20 hours), the greatest augmentation of the DI occurred on the first post-irradiation day, when the increase was significant at the 1% level of confidence. Also, on that day, the DI of the 1000R at 18 group, which was then only slightly higher than control values, was significantly lower than the DI of the 1000R at 14, 16, and 20 hours ($p<0.05$). On the second day, just the DI of the 1000R at 16 group remained significantly higher than control values ($p<0.05$), while on the third day the DI's of all treatment groups were again within the normal range.

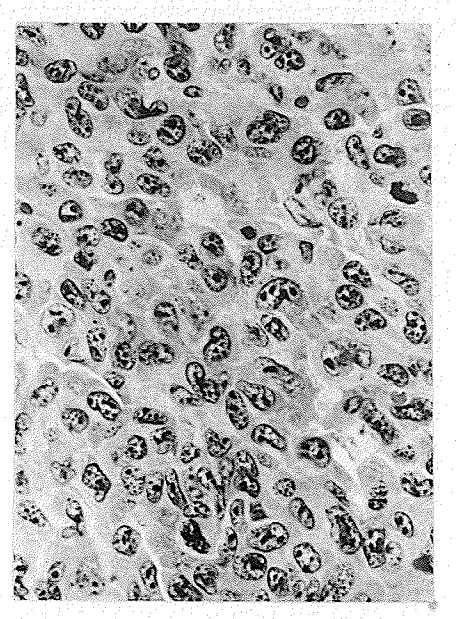
Summary. Over the three-day period following 1000R administered at different times after the last injection of ara-C, the LI's of the duodenum in all of the treatment groups remained unchanged from control values. Slight increases in some groups, with concomitant slight decreases in other groups, however, resulted in some differences in the LI's between treated groups. Thus, the LI of the 1000R at four hours group was lower than that of 1000R at 20 hours group on the first post-irradiation day, and lower than that of three other groups (16, 18, and 20 hours) on the third day. The MI's were significantly reduced from control levels with all combinations, apart from the 1000R at 18 group, on the first day. Subsequently, the only group different from control levels was the 1000R at 18 hours. The MI of that group was reduced on the second day, but higher on the third day. The DI was augmented over control values in four groups on the first and second days, but had returned to normal on the third.

The Effect of X-irradiation following Ara-C on the B16 Melanoma

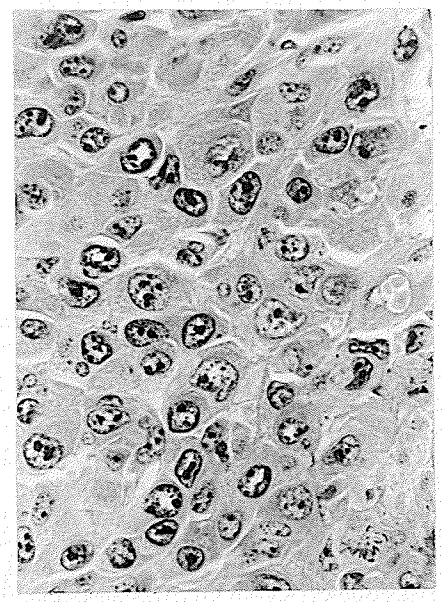
1. Morphology (Figures 11). As in the case of tumours irradiated alone, following the combination treatment of ara-C and irradiation, some giant nuclei and nuclei displaying margination of chromatin were noted. But any other morphological differences between treatment groups were not observed. Again the treated tumours appeared to be less cellular than untreated tumours, but it could not be ascertained if this was due to an enlargement of the cells or to greater intercellular spaces.

Figure 11 The B16 melanoma on each of three days following 1000R x-irradiation administered 14 hours following the last injection of ara-C (H & E, x800).

Figure 11



Day 1



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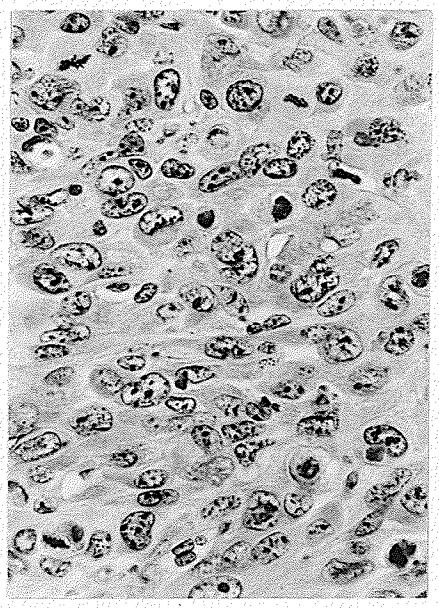

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2. Indices of the B16 Melanoma (Table 5 and Chart 15).

a. Labeling Index (LI). The tumour LI's were significantly reduced from control levels in all treatment groups over the three-day period, whether combined or considered on a day-to-day basis ($p<0.01$). The greatest decrease in the LI (approximately 90%) occurred in the 1000R at 14 group, where the LI was significantly lower than the LI's of the 1000R at 16 and 20 hours groups on the first post-irradiation day. It was also significantly lower than the 1000R at 16, 18, and 20 groups on the second post-irradiation day ($p<0.05$). Significant differences were not apparent between the LI's of the five treatment groups on the third day. Moreover, on a day-to-day basis, there were not any differences in the LI's of any single group, whether treated or untreated. The apparent variations in the LI's which did occur on a day-to-day basis were inconsistent, when compared between different groups.

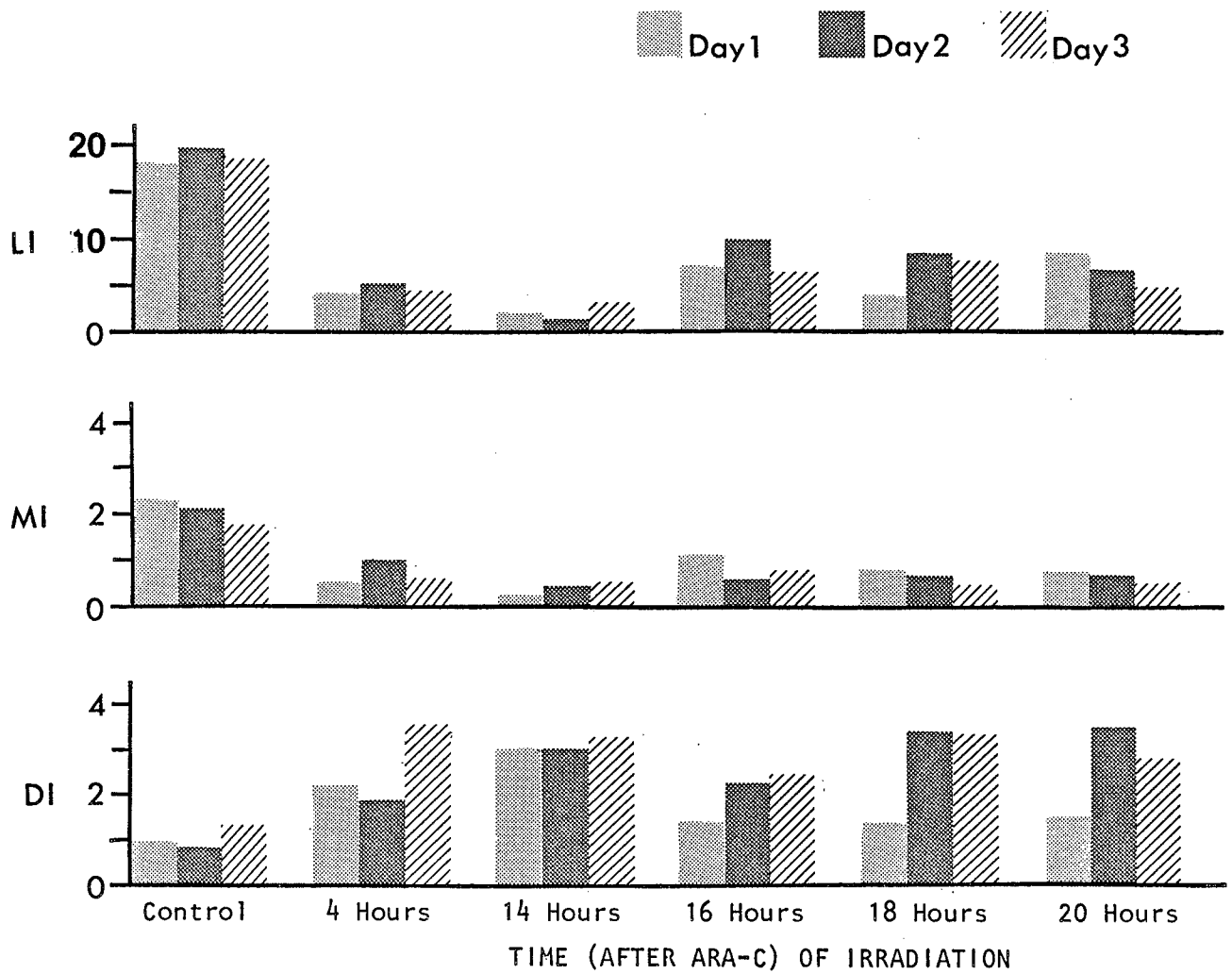
b. Mitotic Index (MI). Similar to the LI, the MI's were also significantly reduced from control values in all treatment groups over the three-day period, both combined as well as on each post-treatment day ($p<0.01$). The greatest decrease in the tumour MI was again in the 1000R at 14 group. However, that MI was significantly lower than just that of the 1000R at 16 group on the first post-treatment day ($p<0.05$). There did not appear to be any trend in the increases or decreases of the MI's over the three-day period. Nonetheless, it is of interest to note the similarity in the MI's of the 1000R at 16, 18, and 20 hours groups.

c. Degenerating Index (DI). The DI's of four treated groups augmented from control levels over the three-day period, but these increases were not consistent on a day-to-day basis. Thus, on the first

TABLE 5 - THE EFFECT OF 1000R X-IRRADIATION AT VARIOUS TIMES FOLLOWING THE LAST OF 8 i.p. INJECTIONS OF 12.5mg/kg ARA-C GIVEN AT TWO-HOUR INTERVALS ON THE CELL KINETICS OF THE B16 MOUSE MELANOMA OVER A THREE-DAY PERIOD

	DAYS AFTER IRRADIATION	CONTROL	4 HOURS	14 HOURS	16 HOURS	18 HOURS	20 HOURS
		Mean S.D.	Mean S.D.	Mean S.D.	Mean S.D.	Mean S.D.	Mean S.D.
LABELING	1	18.1 ± 2.0	4.3 ± 1.3	2.0 ± 1.0	7.3 ± 4.1	4.0 ± 0.7	8.1 ± 4.8
INDEX	2	20.0 ± 3.7	5.8 ± 1.0	1.5 ± 0.4	9.5 ± 2.5	7.3 ± 0.4	6.8 ± 3.0
	3	18.4 ± 5.1	4.8 ± 1.4	2.8 ± 0.5	6.2 ± 1.9	6.0 ± 0.6	5.2 ± 1.6
MITOTIC	1	2.3 ± 0.4	0.5 ± 0.2	0.2 ± 0.1	1.1 ± 0.4	0.8 ± 0.2	0.8 ± 0.2
INDEX	2	2.1 ± 0.4	1.0 ± 0.4	0.4 ± 0.2	0.7 ± 0.2	0.7 ± 0.1	0.7 ± 0.1
	3	1.8 ± 0.9	0.6 ± 0.1	0.5 ± 0.1	0.8 ± 0.4	0.5 ± 0.1	0.5 ± 0.1
DEGENERATING	1	1.0 ± 0.4	2.2 ± 0.5	3.0 ± 0.3	1.4 ± 0.6	1.4 ± 0.8	1.5 ± 0.8
INDEX	2	0.9 ± 0.2	1.9 ± 0.7	3.0 ± 1.5	2.2 ± 0.1	3.4 ± 0.7	3.5 ± 0.9
	3	1.3 ± 0.8	3.6 ± 0.8	3.2 ± 0.7	2.4 ± 0.9	3.3 ± 0.6	2.8 ± 0.9

CHART 15 - THE EFFECT OF 1000R X-IRRADIATION AT VARIOUS TIMES FOLLOWING THE LAST OF EIGHT i.p. INJECTIONS OF 12.5 mg/kg ARA-C GIVEN AT TWO-HOUR INTERVALS ON THE CELL KINETICS OF THE B16 MELANOMA OVER A THREE-DAY PERIOD



post-treatment day, the DI of just the 1000R at 14 group became significantly enhanced ($p<0.01$). Moreover, the DI of that group was significantly higher than those of the 1000R at 16, 18, and 20 hours groups ($p<0.05$), but did not exceed that of the group irradiated at four hours. On the second day, the DI's of animals irradiated at 14, 18, and 20 hours were all higher than control levels ($p<0.01$), while the group irradiated at four hours displayed a slight decrease in the DI, so that it was now significantly lower than both the 18 hours and 20 hours groups. By the third post-treatment day, the DI's of four of the treated groups (1000R at four, 14, 18, and 20 hours) were significantly enhanced over that of the control animals ($p<0.01$), while that of the 1000R at 16 group was within the normal range. The DI of the 1000R at 16 hours group, then, was not significantly increased over the three-day period of observation.

Summary. The LI's and MI's of the five treated groups decreased significantly, from control values, on all three post-treatment days, while the DI's increased in all the groups except that group irradiated 16 hours following the last ara-C injection. The LI of the group irradiated at 14 hours was lower than those of two other treated groups (1000R at 16 and 20 hours) on the first day, and lower also than those of three other groups (1000R at 16, 18, and 20 hours) on the second day. A similarity was noted in the MI's of the groups irradiated either 16, 18, or 20 hours after the last injection of ara-C. The DI's increased over control values in all treated groups over the three-day period, except in that group which received the irradiation 16 hours following the last injection. In this latter case, the DI was not different from the control.

The Effect of 1000R X-irradiation following Ara-C on the Survival of Mice Bearing the B16 Melanoma

As is evident from Table 6, just one group of ara-C administered animals, subsequently irradiated, did not survive as long or longer than the control animals. That group, which was irradiated four hours following the last injection of ara-C, had a MST of 19.5 ± 12.4 days, compared to a control MST of 33.4 ± 8.2 days. This MST (of the treated group) was significantly shorter than that of the control animals as well as briefer than those of animals treated with any of the other regimens ($p<0.01$). It should be noted that 75% of the animals irradiated at four hours died sooner than the earliest death recorded for control animals, that is, prior to 19 days after tumour transplantation and within eight days after the treatment.

When all animals within a particular treatment group were included in calculations of survival, only the group which was irradiated 18 hours following the last ara-C injection was noted to have survived longer than the control animals ($p<0.05$). Any differences could not be ascertained between any of the treated groups, in terms of MST. When just those animals in a particular treatment group which survived as long or longer than the earliest death recorded of untreated animals were included in the calculations, three groups of animals survived significantly longer than the control animals, namely the 1000R at 14 hours group ($p<0.01$), 1000R at 16 hours group ($p<0.05$), and 1000R at 18 hours group ($p<0.01$). Of these three groups, the longest MST (51 ± 13.8 days) was achieved by the group irradiated at 14 hours. However, that was not significantly longer than the MST of any other treated group.

TABLE 6 - THE EFFECT OF X-IRRADIATION FOLLOWING ARA-C ON THE SURVIVAL OF C57B1/6J MICE BEARING THE B16 MELANOMA

	RANGE		TL_{50}		MST		N	
	GROUP 1	GROUP 2	GROUP 1	GROUP 2	GROUP 1	GROUP 2	GROUP 1	GROUP 2
CONTROL	19-50	---	30.5	---	33.4± 8.2	---	42	--
1000R AT 4 HRS.	13-64	20-64	14.8	20.0	19.5±12.1	33.0±11.6	20	5
1000R AT 14 HRS.	16-74	21-74	48.5	49.5	44.7±18.7	51.0±13.8	11	9
1000R AT 16 HRS.	13-68	33-68	36.0	44.0	39.1±16.5	45.4±11.2	10	8
1000R AT 18 HRS.	14-82	33-82	39.0	39.5	45.6±18.0	48.5±15.7	12	11
1000R AT 20 HRS.	27-75	---	39.0	---	42.3±17.0	---	--	--

NOTE: GROUP 1 - All animals in a particular treatment group.

GROUP 2 - Only those animals in a particular treatment group which survived 19 days or longer.

SERIES II

In the second series of the combination treatment experiments, groups of mice were administered a 500R dose of x-irradiation either four or 14 hours following the last ara-C injection. The reasons for this regimen were as follows. When a 1000R dose followed four hours after the last injection of ara-C, 75% of the mice died within eight days of treatment. It was hoped that reducing the irradiation dose by one-half would improve the therapeutic ratio, that is, that the therapeutic effect of the lower x-ray dosage on the tumour would not be different from that of the larger dose, reducing at the same time the detrimental effect on the duodenum. A 500R dose at 14 hours was necessary for comparative purposes, as further experimentation was planned with fractionated doses of 500R, with the first dose of x-irradiation being given 14 hours following the last ara-C injection.

The Effect of 500R at Four or 14 Hours after Ara-C on the Duodenum

1. Morphology. Both combination treatments of x-irradiation and ara-C induced obvious damage to the duodenal mucosa. However, that damage appeared to be much more severe when the irradiation was administered four hours after the last injection of ara-C. In both instances, the most pronounced damage was apparent on the first post-irradiation day. The changes in both the crypts, as a whole, as of the nuclei, were very similar to those described following 1000R x-irradiation.

2. Duodenal Indices (Table 7 and Chart 16)

a. Labeling Index (LI). The LI's of the two treatment groups did not differ significantly from that of the control LI over the three-day period combined, and, on a day-to-day basis, the only significant differences occurred on the second post-irradiation day; the group irradiated at 14 hours displayed a higher LI than that of the control animals ($p<0.05$).

b. Mitotic Index (MI). The duodenal MI's of both treated groups were within the normal range of the first post-irradiation day. Only the group irradiated 14 hours following the last injection differed from the control on the remaining two days. In this latter case, the MI was significantly higher than those of both the control animals and the animals irradiated four hours following the last injection ($p<0.05$).

c. Degenerating Index (DI). The DI's of both treatment groups were significantly higher than that of control animals on the first post-irradiation day ($p<0.05$), but they did not differ from control values on the second or third days. Hence, they were not different from control values over the combined three-day period of observation.

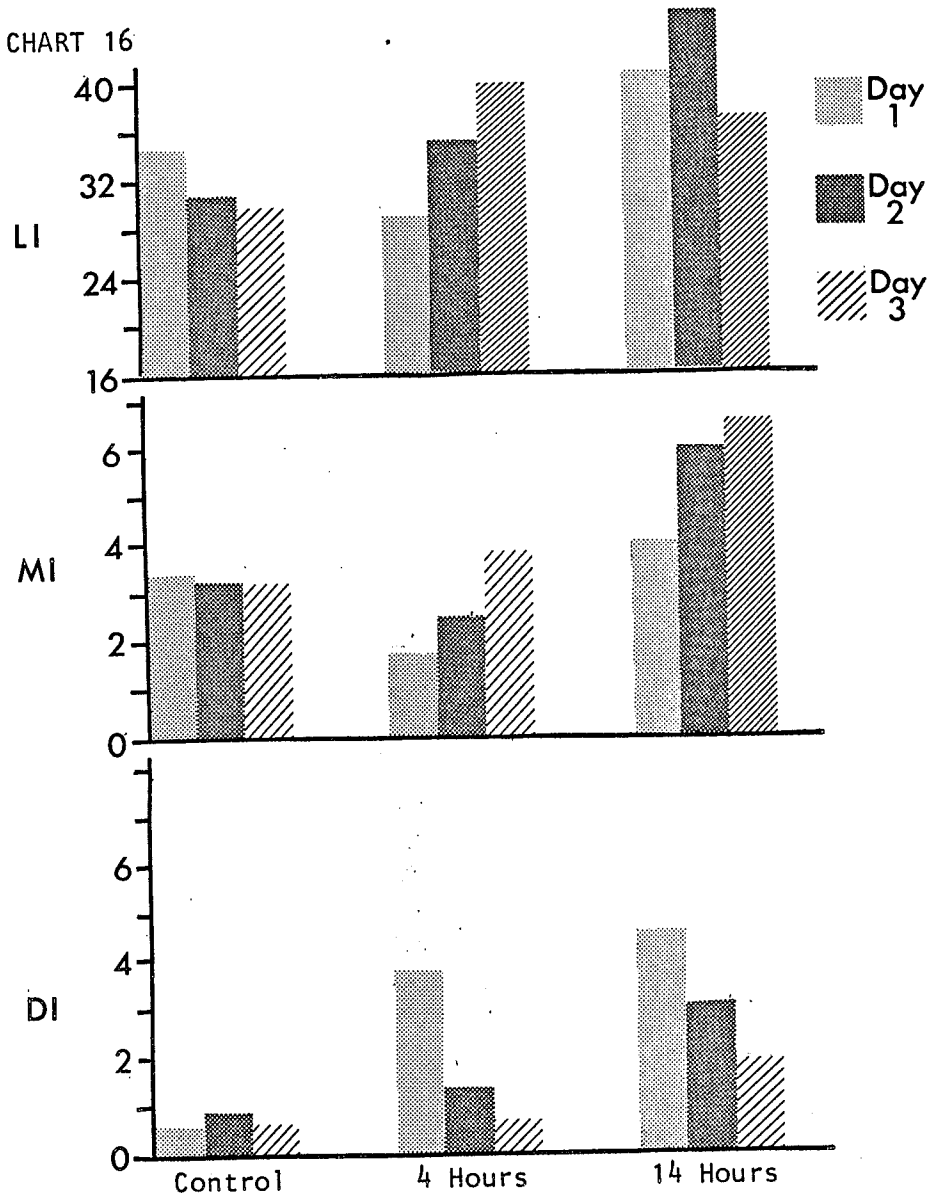
Summary. Neither the LI's nor the MI's of the two treated groups differed from each other or from the control animals on the first post-irradiation day. On the second day, both the LI and the MI of the 500R at 14 hours group were higher than those of the four hours group receiving 500R. On the third day, the MI of the group irradiated at 14 hours was higher than that of control animals as well as that of the other treated group. The DI's of both treated groups were increased, over control values, only on the first post-treatment day.

THE EFFECT OF 500R X-IRRADIATION AT FOUR HOURS OR AT 14 HOURS FOLLOWING THE LAST OF EIGHT i.p. INJECTIONS OF 12.5 mg/kg ARA-C GIVEN AT TWO-HOUR INTERVALS ON THE CELL KINETICS OF THE C57B1/6J MOUSE DUODENUM OVER A THREE-DAY PERIOD

TABLE 7

	DAYS AFTER IRRADIATION	CONTROL		4 HOURS		14 HOURS	
		Mean	S.D.	Mean	S.D.	Mean	S.D.
LI	1	35.1	± 9.7	28.8	± 9.1	41.0	± 7.5
	2	31.2	± 6.0	35.6	± 11.2	45.9	± 5.2
	3	30.0	± 9.0	40.0	± 7.8	36.7	± 16.3
MI	1	3.5	± 0.4	1.8	± 0.9	4.0	± 1.3
	2	3.3	± 0.5	2.4	± 0.4	6.0	± 0.8
	3	3.3	± 0.4	3.8	± 1.4	6.5	± 2.3
DI	1	0.7	± 0.3	3.8	± 1.5	4.6	± 1.9
	2	0.9	± 0.4	1.2	± 0.2	3.1	± 1.1
	3	0.7	± 0.2	0.9	± 0.5	1.9	± 1.4

CHART 16



The Effect of 500R at Four Hours or 14 Hours after Ara-C on the B16 Melanoma

1. Morphology. Differences were not apparent in the microscopic anatomy of the B16 melanoma between the 500R dose and the 1000R dose.

2. Indices of the B16 Melanoma (Table 8 and Chart 17)

a. Labeling Index (LI). The LI's of both treated groups were significantly decreased over the three-day period, as well as on each single day following treatment ($p<0.01$). Differences did not occur in the LI's of the treated groups from one day to the next nor were there any differences between the two groups.

b. Mitotic Index (MI). There was a general decrease, from control values, in the MI of tumours of animals irradiated four hours following the last injection of ara-C. That difference was significant on the first and second days following irradiation ($p<0.05$). In contrast, the MI of the group irradiated 14 hours following ara-C was significantly depressed only on the third post-irradiation day ($p<0.05$). Further, the MI of this group was significantly higher than that of the group irradiated at four hours on both the first ($p<0.01$) and second days ($p<0.05$). The MI's of neither the treated nor untreated animals changed from one post-treatment day to the next.

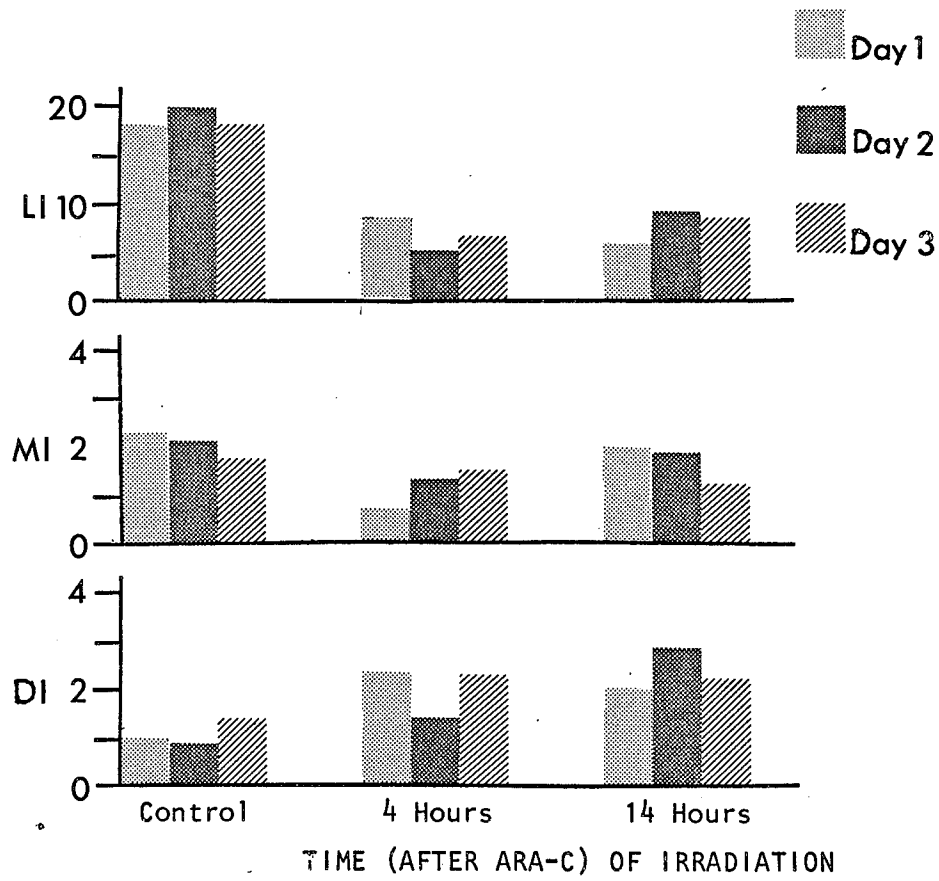
c. Degenerating Index (DI). Although there was an apparent increase, from control values, in the DI's of the treated groups over the three-day period, the differences were insignificant. There were, however, some larger differences on individual post-treatment days. Thus, the DI of the group irradiated at four hours was significantly higher than

THE EFFECT OF 500R X-IRRADIATION AT FOUR HOURS OR AT 14 HOURS FOLLOWING THE LAST OF EIGHT i.p. INJECTIONS OF 12.5 mg/kg ARA-C GIVEN AT TWO-HOUR INTERVALS ON THE CELL KINETICS OF THE B16 MELANOMA OVER A THREE-DAY PERIOD

TABLE 8

	DAYS AFTER IRRADIATION	CONTROL	4 HOURS	14 HOURS
	1	18.1 ± 2.0	8.4 ± 3.0	6.3 ± 2.1
LI	2	20.0 ± 3.7	5.2 ± 3.4	9.4 ± 2.3
	3	18.4 ± 5.0	7.3 ± 1.0	8.4 ± 1.6
	1	2.3 ± 0.4	0.7 ± 0.2	2.0 ± 0.4
MI	2	2.1 ± 0.4	1.3 ± 0.2	1.9 ± 0.4
	3	1.8 ± 0.9	1.4 ± 0.2	1.2 ± 0.4
	1	1.0 ± 0.4	2.4 ± 0.5	2.0 ± 1.2
DI	2	0.4 ± 0.2	1.4 ± 0.1	2.7 ± 0.8
	3	1.3 ± 0.8	2.3 ± 0.1	2.1 ± 1.1

CHART 17



control values on the first post-irradiation day, while the DI of the 14 hours group was significantly higher than that of controls on the second post-irradiation day ($p<0.05$). The only significant difference between the two treated groups was on the second day, when the DI of the 14 hours group was significantly higher than that of the four hours group ($p<0.05$).

Summary. The LI's of both treated groups were lower than control values on each post-treatment day. The MI of the 500R at four hours group, on the first two days, was lower than both the control values and the 14 hours group receiving the same dosage. On the third post-treatment day, it was lower only than that of control animals. The DI was increased, on the first day, only in the 500R at four hours group, and on the second day, just in the 500R at 14 hours group. The DI's of both groups were within normal limits on the third day.

The Effect of 500R at Four or 14 Hours following Ara-C on the Survival of Mice Bearing the B16 Melanoma

The MST of nine mice which received 500R four hours after the last ara-C injection was 33.7 ± 11.8 days with a TL_{50} of 34.5 days. When the two mice which died (likely of other causes) prior to 19 days were excluded from the calculations, the MST was 38.9 ± 6.6 days, the TL_{50} 35.5 days. The MST and TL_{50} of eight mice irradiated with 500R 14 hours following the last ara-C injection were 39.8 ± 16.1 days and 36 days, respectively. When the one "early death" was excluded from the calculations, these two values were 43.4 ± 13.4 days and 38.5 days. Thus, regardless of whether "early deaths" were included or excluded, neither group of animals survived longer than control animals.

SERIES III

The two groups of mice in this series received the first 500R dose of x-irradiation 14 hours following the last ara-C injection, and a second 500R dose a factor of 14 hours later. Thus, one group was irradiated 14 and 28 hours after the last ara-C injection, the second group 14 and 42 hours after the last injection of the drug. These intervals were based on the generation time of the B16 melanoma cells, which was reported to be 14 hours (Bertalanffy and Gibson, 1972). The two treatments were designated shorter or longer fraction intervals..

The Effect of Fractionated 1000R X-irradiation Doses following Ara-C on the Duodenum

1. Morphology (Figures 12A and 12B). When the fractionated doses of irradiation followed the administration of ara-C, the damage to the duodenal mucosa was extremely severe, more so on the first post-irradiation day than on subsequent days. Although both crypts and villi appeared to be more extensively damaged on the first day following the shorter fraction interval, on subsequent days the duodenal mucosa of mice receiving the longer fraction interval appeared to be more severely damaged.

2. Duodenal Indices (Table 9 and Chart 18)

a. Labeling Index (LI). Changes, from normal values, in the LI's did not occur in either of these two fractionated groups, over the three-day period whether considered combined or by single days, nor were

Figure 12 The duodenum of the C57Bl/6J Mouse on each of three days following a fractionated 1000R dose administered following the last ara-C injection (H & E, x200).

Figure 12A The duodenum of mice administered 500R at 14 hours and again at 28 hours following the last ara-C injection.

Figure 12B The duodenum of mice administered 500R at 14 hours and again at 42 hours following the last ara-C injection.

2

B

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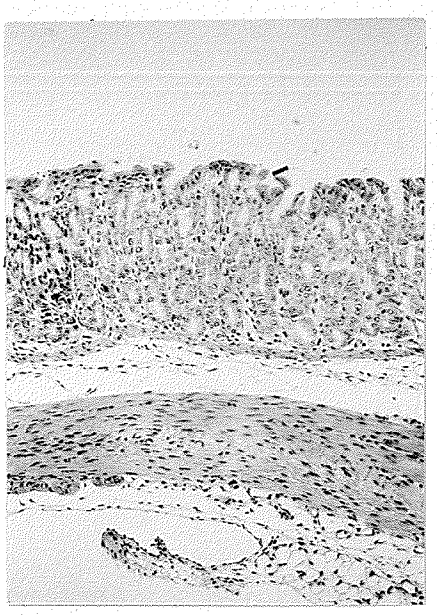
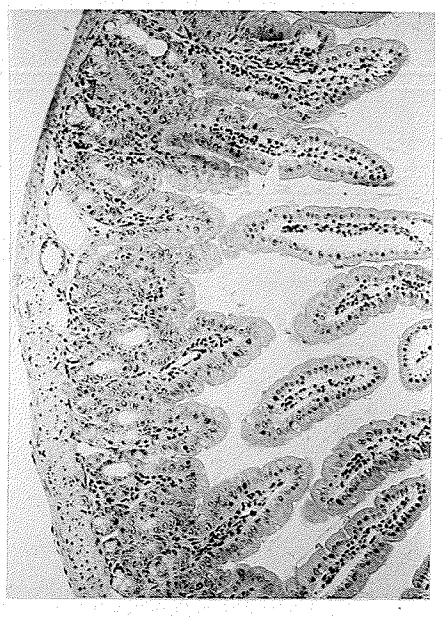
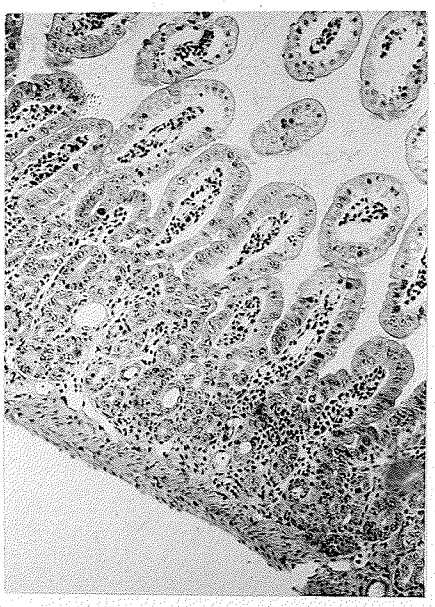
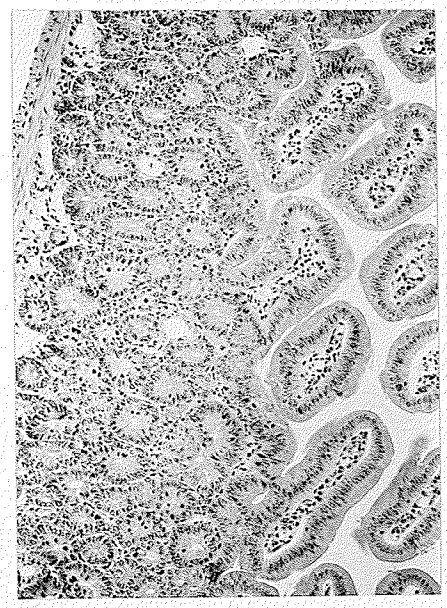


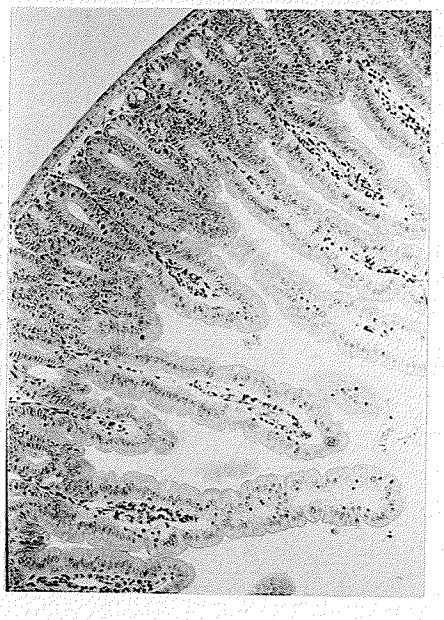


Day 2



y 3





the LI's of the two treated groups different from one another throughout the period of observation.

b. Mitotic Index (MI). The duodenal MI's of the treated groups were not different from control values on the first two post-treatment days. However, on the second day, the MI of the group receiving irradiation at the shorter fraction interval was significantly lower than that of the group receiving irradiation at the longer fraction interval ($p<0.05$). The MI's of both treated groups became apparently augmented from the second to the third days, however, just the MI of the group receiving irradiation at the shorter fraction interval increased significantly ($p<0.05$). Moreover, the MI's of both treated groups were now significantly higher than that of control animals ($p<0.05$).

c. Degenerating Index (DI). On the first post-irradiation day, the DI's rose significantly, from control values, following both regimens of irradiation combined with ara-C. Moreover, the DI of the group that received the irradiation at the shorter fraction interval was significantly higher than that of the group which received irradiation at the longer fraction interval. The DI of the 500R at 14 and 28 hours group declined significantly from the first day to the second, and remained then within normal limits. The DI of the 500R group at 14 and 42 hours remained significantly higher than that of the control group on the second day but was again within the normal range on the third day. All differences were significant at the 5% level of confidence.

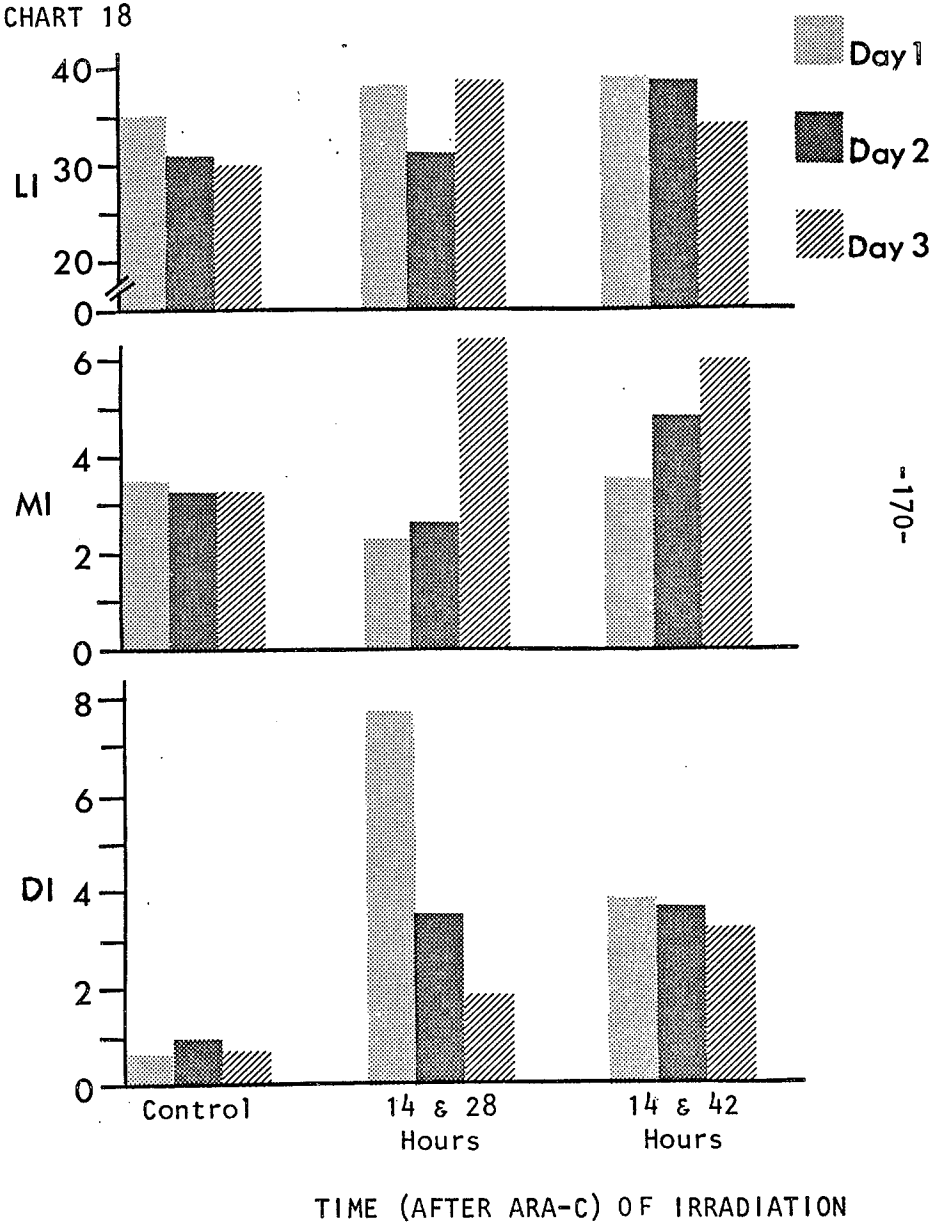
Summary. Changes in the LI's did not occur on any of the three days following either treatment, and the MI's were unchanged on the first

THE EFFECT OF FRACTIONATED 1000R DOSES FOLLOWING THE LAST OF EIGHT i.p. INJECTIONS OF ARA-C GIVEN AT TWO-HOUR INTERVALS ON THE CELL KINETICS OF THE C57Bl/6J MOUSE DUODENUM OVER A THREE-DAY PERIOD

TABLE 9

	DAYS AFTER IRRADIATION	CONTROL		14 & 28 HOURS		14 & 42 HOURS	
		Mean	S.D.	Mean	S.D.	Mean	S.D.
LI	1	35.1	± 9.7	37.7	± 13.9	38.7	± 3.1
	2	31.2	± 6.0	32.0	± 8.3	38.3	± 2.0
	3	30.0	± 9.0	38.0	± 6.3	34.6	± 13.0
MI	1	3.5	± 0.4	2.2	± 0.7	3.6	± 1.0
	2	3.3	± 0.5	2.7	± 1.7	4.8	± 2.0
	3	3.3	± 0.4	6.2	± 1.8	6.0	± 2.3
DI	1	0.7	± 0.3	7.7	± 3.3	3.9	± 1.4
	2	0.9	± 0.4	3.5	± 2.6	3.7	± 0.8
	3	0.7	± 0.2	1.9	± 0.8	3.1	± 2.1

CHART 18



second days. Although the MI of the shorter interval group was lower than that of the longer interval group on the second day, it increased significantly from the second day to the third, so that the MI's of both groups were now higher than control values. The DI's were significantly augmented in both groups on the first post-treatment day, the shorter interval group more so than the longer interval group. By the second day, only the DI of the longer interval group was higher than normal, and on the third day the DI's of both treatment groups were again within the normal range.

The Effect of a Fractionated Dose of X-irradiation following Ara-C on the B16 Melanoma

1. Morphology (Figures 13A and 13B). Following the combined regimens of irradiation and ara-C, the tumours appeared to be less cellular, and many of the nuclei were enlarged or swollen. It could not be determined if the "reduced cellularity" was brought about by enlarged cells or greater intercellular spaces.

2. Indices of the B16 Melanoma (Table 10 and Chart 19)

a. Labeling Index (LI). The LI's of both treatment groups were significantly lower than control values over the combined three-day post-irradiation period, as well as on each day after irradiation ($p<0.01$). None of the LI's (of treated or untreated groups) changed from day-to-day, nor were there any differences between the two treated groups on any day.

b. Mitotic Index (MI). There were no differences in the MI's of the two treated groups over the three-day period combined, but

Figure 13 The B16 melanoma on each of three days following a fractionated 1000R dose administered following the last ara-C injection (H & E, x800).

Figure 13A The B16 melanoma of mice administered 500R at 14 hours and again at 28 hours following the last ara-C injection.

Figure 13B The B16 melanoma of mice administered 500R at 14 hours and again at 42 hours following the last ara-C injection.

Fig 13

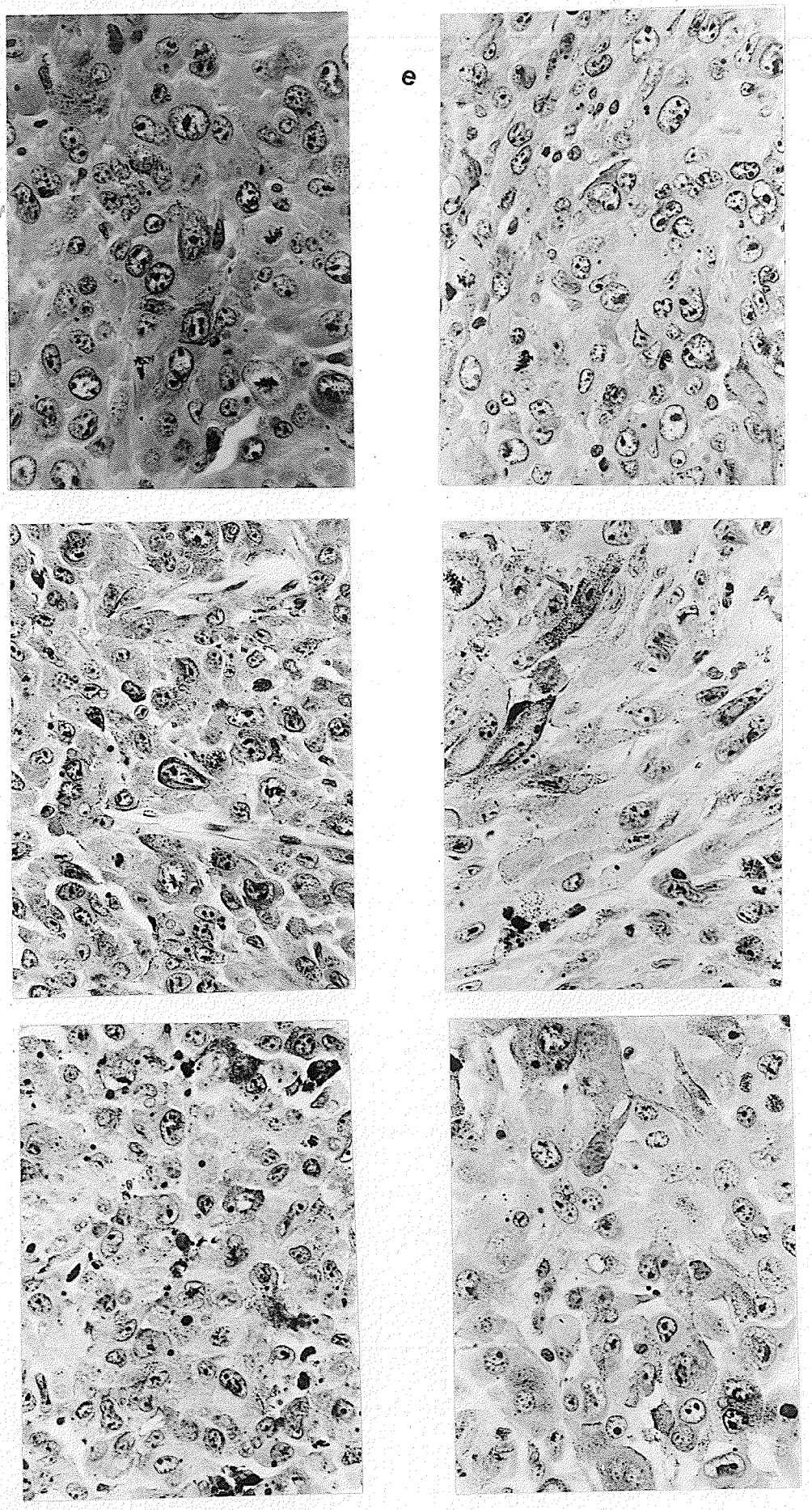
B

e

Day 1

Day 2

Day 3



this can be explained on the basis of the day-to-day changes. On the first post-treatment day, the MI's of both treated groups were significantly augmented from control values ($p<0.05$). A decrease in the MI's from the first to the second day returned both indices to normal, and a further decline on the third day decreased the MI's to a significantly lower level than control values ($p<0.01$). None of the changes in the MI's, from day-to-day, were significant.

c. Degenerating Index (DI). The tumour DI's of both treated groups were significantly augmented over control values over the entire three-day period ($p<0.05$). On the first day, only the DI of the animals which received irradiation at 14 hours and 28 hours was significantly higher than control values ($p<0.01$). On the second and third days, the DI's of both treated groups were significantly higher than control values ($p<0.01$).

Summary. The LI's of the two treated groups were decreased, from control values, over the three-day period following treatment. The DI's were increased during the same period. The MI's were augmented on the first post-treatment day, were within the normal range on the second day, and declined on the third day.

The Effect of Fractionated Dose of X-irradiation following Ara-C on the Survival of Mice Bearing the B16 Melanoma

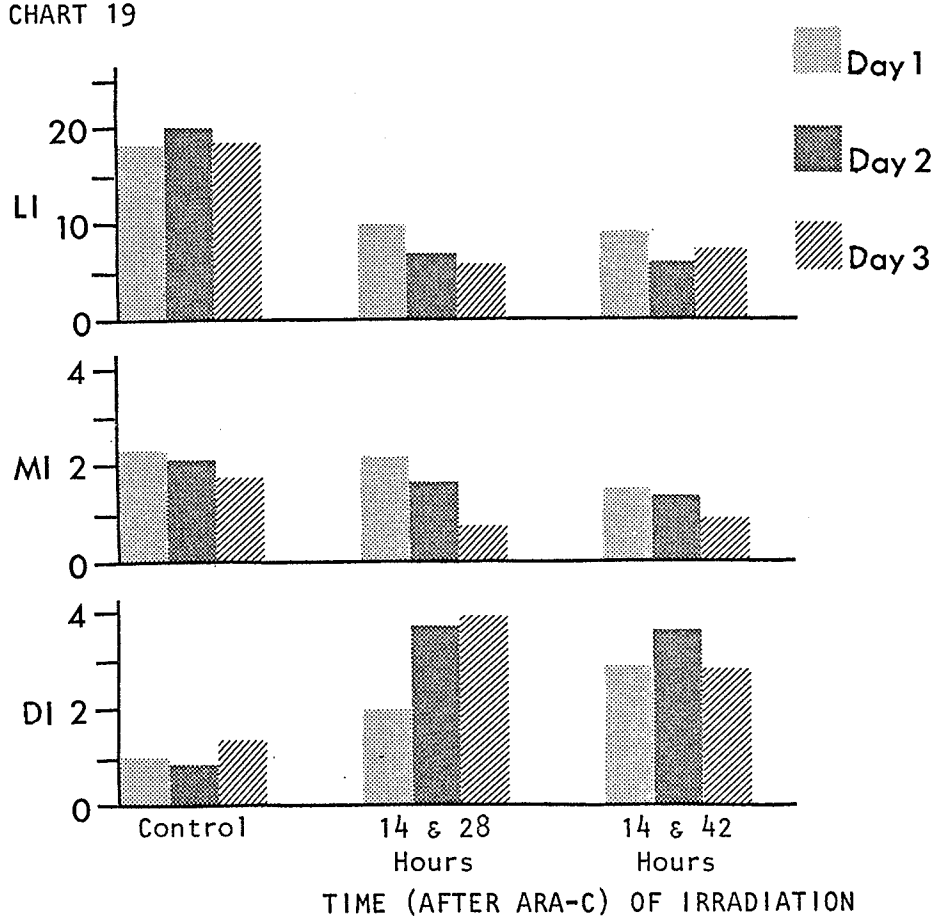
The MST and TL_{50} of eight animals irradiated 14 and 28 hours after the last ara-C injection were 36.4 ± 15 days and 35 days, respectively. When three "early death" animals were eliminated from the calculations,

THE EFFECT OF FRACTIONATED 1000R DOSES FOLLOWING THE LAST OF EIGHT i.p. INJECTIONS OF ARA-C GIVEN AT TWO-HOUR INTERVALS ON THE CELL KINETICS OF THE B16 MELANOMA OVER A THREE-DAY PERIOD

TABLE 10

	DAYS AFTER IRRADIATION	CONTROL		14 & 28 HOURS		14 & 42 HOURS	
		Mean	S.D.	Mean	S.D.	Mean	S.D.
	1	18.1	± 2.0	10.3	± 3.2	9.4	± 2.2
LI	2	20.0	± 3.7	7.6	± 2.8	5.8	± 1.1
	3	18.4	± 5.1	5.2	± 0.6	6.2	± 1.4
	1	2.3	± 0.4	2.2	± 0.7	1.6	± 0.4
MI	2	2.1	± 0.4	1.7	± 0.7	1.5	± 0.2
	3	1.8	± 0.9	0.7	± 0.2	0.9	± 0.2
	1	1.0	± 0.4	2.0	± 0.6	2.9	± 0.7
DI	2	0.9	± 0.2	3.7	± 0.8	3.6	± 0.7
	3	1.3	± 0.8	3.8	± 1.4	2.8	± 0.5

CHART 19



these two values were 43.3 ± 9 days and 38 days. There were no "early deaths" among the eight animals that received the irradiation at the longer fraction interval (that is, 500R at 14 and 42 hours); the MST was 38.4 ± 7.8 days, the TL_{50} 35.5 days. The animals which received either course of fractionated x-irradiation following ara-C did not, therefore, survive longer than the control animals.

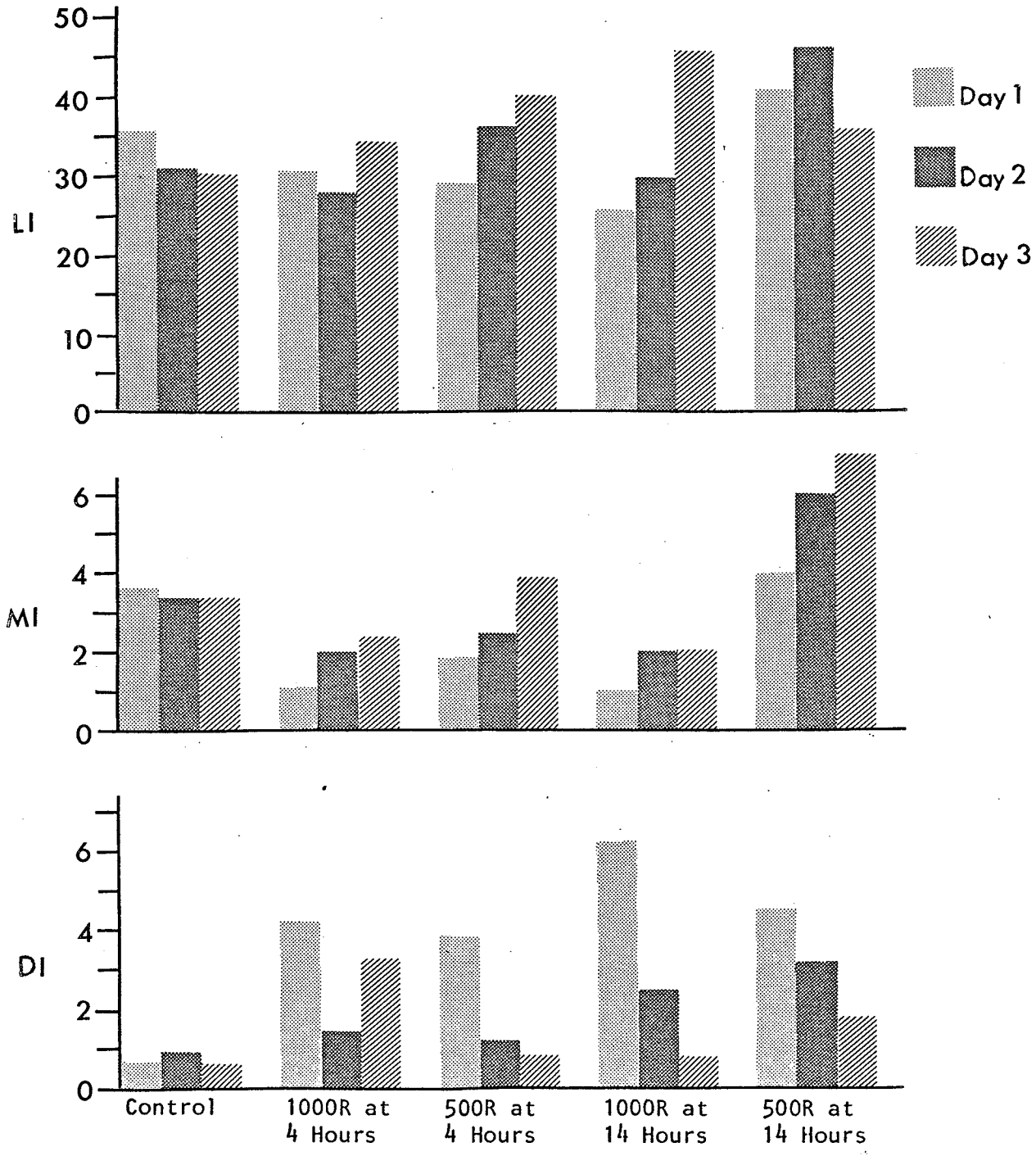
COMPARISONS BETWEEN SELECTED TREATMENT GROUPS

COMPARISON 1

This comparison was between treatments in which the dosage of x-irradiation differed, but in which the time of exposure to x-irradiation after the last injection of ara-C was identical. Thus, the effects of a 1000R dose of x-irradiation at four or 14 hours after the last injection of ara-C were compared to the effects of a 500R dose of x-irradiation at those two points.

1. Duodenal Indices (Chart 20). Differences were not apparent between any of the indices of the two irradiation doses when they were administered four hours after the last ara-C injection. When the animals were irradiated 14 hours after the last ara-C injection, however, the duodenal LI's were significantly lower in that group which received 1000R on both the first and second post-treatment days ($p<0.05$ and $p<0.01$, respectively). The LI of the 1000R group was not different from that of the 500R group on the third day. The MI of the 1000R at 14 hours group was significantly lower than the MI of the 500R at 14 hours group on all

CHART 20 - COMPARISON OF THE EFFECTS OF TWO DIFFERENT DOSES OF X-IRRADIATION GIVEN AT THE SAME TIME FOLLOWING ARA-C ON THE CELL KINETICS OF THE C57B1/6J MOUSE DUODENUM

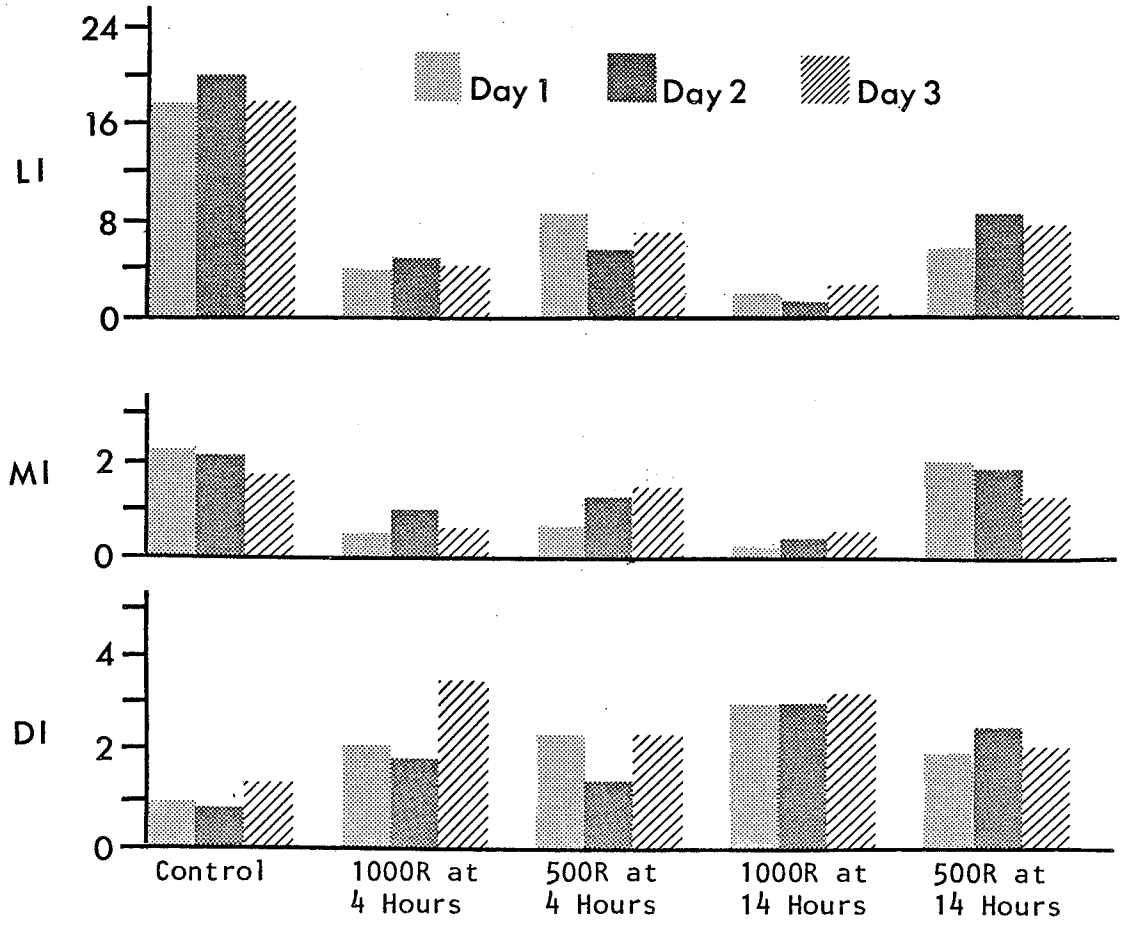


three days. These differences were significant at the 5% level of confidence on the first day, and at the 1% level of confidence on the last two days. The DI's did not differ between these groups on any of the three post-treatment days.

2. Indices of the B16 Melanoma (Chart 21). The only difference in any of the indices of animals irradiated four hours after the last ara-C injection occurred on the third post-treatment day. On that day, the MI of the group which received a dose of 1000R was significantly lower and the DI significantly higher than those of animals administered 500R. Both differences were significant at the 5% level of confidence. When animals were irradiated 14 hours following the last injection of ara-C, both the LI and the MI of animals that received 1000R were lower, on all three days, than those of animals administered 500R. The difference of the LI on the first post-treatment and MI on the third post-treatment days were significant at the 5% level of confidence; all other differences were significant at the 1% confidence level. The DI's of the two treated groups differed just on the first post-treatment day, when that of the 1000R at 14 hours group was significantly higher ($p<0.05$).

3. Survival. Differences in the MST's of any of the treatment groups were not apparent. However, as noted previously, 75% of the animals irradiated with 1000R four hours following the last ara-C injection died prior to the day of the earliest recorded death of untreated tumour bearing animals. When a 500R dose was administered four hours following the last injection, two of nine (22%) animals died prior to 19 days. A 1000R dose 14 hours following the last injection also resulted in two "early deaths" (of 11 mice), or 18%. When the dose of irradiation

CHART 21 - COMPARISON OF THE EFFECTS OF TWO DIFFERENT DOSES OF X-IRRADIATION GIVEN AT THE SAME TIME FOLLOWING ARA-C ON THE CELL KINETICS OF THE B16 MELANOMA



was reduced to 500R, one of nine (11%) animals died before 19 days. Only that group administered 1000R at 14 hours following ara-C survived longer than control animals ($p<0.01$), and that group only when "early deaths" were excluded.

COMPARISON 2

The effects of a single dose of x-irradiation were compared to those of a fractionated dose following ara-C administration. Comparisons were thus made among the following three groups:

a. 1000R 14 hours following the last ara-C injection.

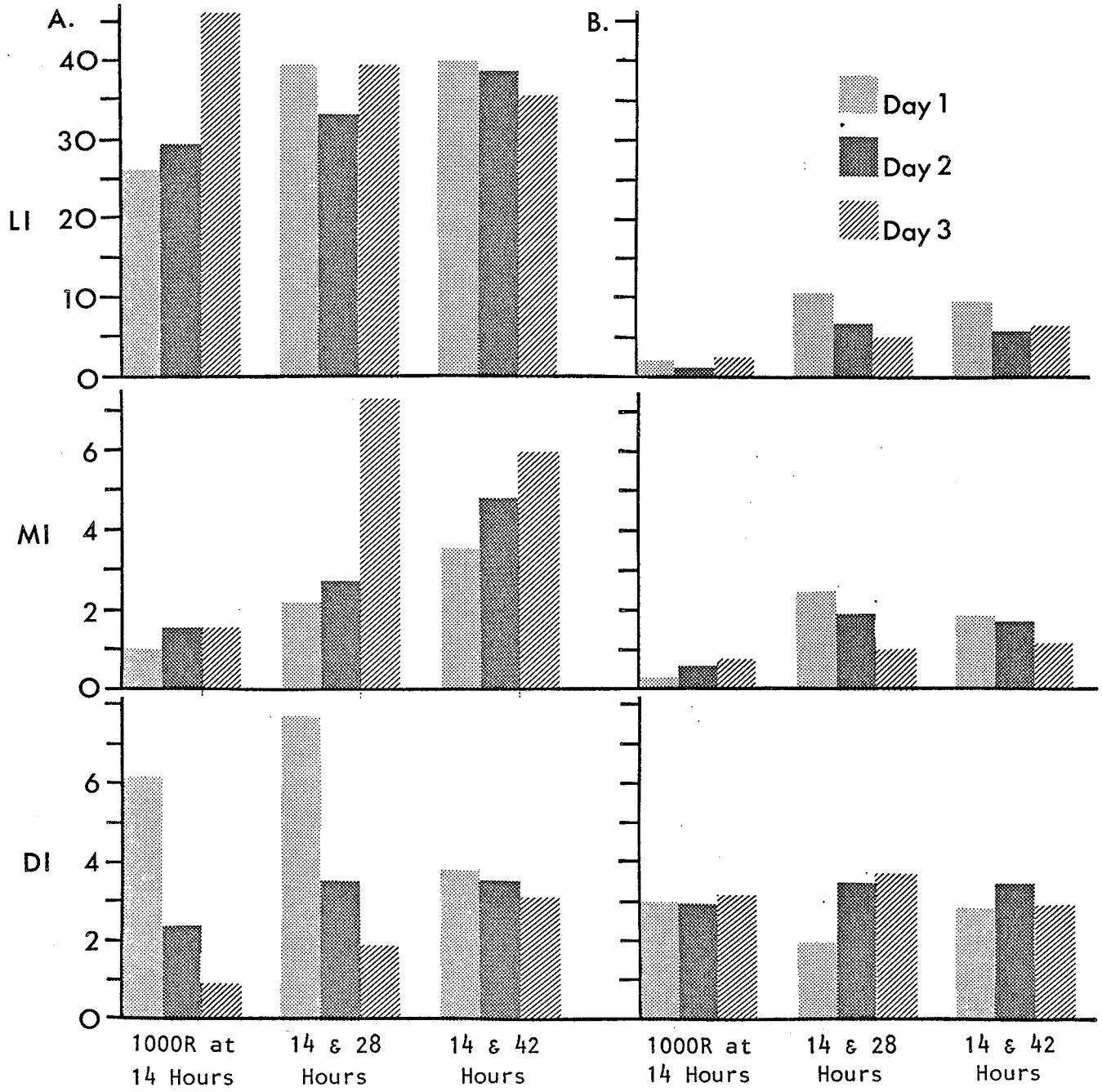
b. 500R 14 hours following the last ara-C injection and a second 500R dose 28 hours following the last injection.

c. 500R 14 hours following the last ara-C injection and a second 500R dose 42 hours following the last injection.

1. Duodenal Indices (Chart 22,A). Differences were not apparent on any of the three post-treatment days, between the LI's or DI's of the mice that received a single dose of irradiation, when compared to those which received the fractionated dose. The MI of the single dose group, however, was significantly lower than that of the group administered the fractionated dose at 14 and 42 hours after ara-C on both the first ($p<0.05$) and second ($p<0.01$) post-treatment days. On the third day, the MI of the group that received a single dose was significantly lower than those of both groups administered the fractionated dose ($p<0.01$).

2. Indices of the B16 Melanoma (Chart 22,B). Differences did not occur in any of the indices between the single dose and fractionated dose groups on the third post-treatment day, nor did the DI differ on the

CHART 22 - COMPARISON OF THE EFFECTS OF A 1000R DOSE 14 HOURS FOLLOWING ARA-C WITH TWO 500R DOSES AT 14 AND 28 HOURS OR AT 14 AND 42 HOURS ON THE CELL KINETICS OF THE C57B1/6J MOUSE DUODENUM (A) AND B16 MELANOMA (B)



first or second days. However, on both the first and second post-treatment days, both the LI and the MI of the single dose group were significantly lower than those of both fractionated groups. The differences were significant at the 1% level of confidence, apart from the LI on the second day, where the difference was significant at the 5% confidence level.

3. Survival. There were two (of 11) "early deaths" in the group which received the single irradiation dose, two deaths (of eight) in the group administered the fractions at 14 and 28 hours, and none in the group which received the fractions at 14 and 42 hours. Excluding these early deaths from the calculation, the MST of the treated groups was 51.0 days, 43.3 days and 38.4 days, respectively. Thus, the mice which received the single dose of x-irradiation survived significantly longer than animals administered the fractionated doses at 14 and 42 hours ($p<0.05$).

COMPARISON 3

The effects of x-irradiation alone, ara-C alone, and x-irradiation following ara-C were compared. The dosage of 1000R was selected for these comparisons, based on the following reasons: (a) that dosage did not result in the "early death" of any of the animals, yet it did augment the MST; (b) the tumour cell labeling and mitotic indices were depressed, and the degenerating index enhanced over the three-day period; and (c) that dosage was also employed in the "combination treatments" and was more effective (in terms of tumour indices) than either a 500R dose alone or a fractionated 1000R dose. The ara-C dosage and schedule of

administration were as previously related. Duodenal and tumour indices, following ara-C, were determined for animals killed at times identical to those killed following the combination treatments. For example, when the irradiation followed 14 hours after the last ara-C injection, the animals were killed 24, 48, or 72 hours after irradiation, or 38, 62, and 86 hours following the last injection of ara-C. Similarly, animals receiving ara-C alone were killed 38, 62, or 86 hours following the last injection. Combination treatments selected for comparison were 1000R at 14, 16, or 18 hours after the last ara-C injection.

1. Duodenal Indices. Comparison of the magnitude of any apparent differences may be deduced from Chart 23, while the trend and significance levels are tabulated in Table 11.

a. Labeling Index (LI). On the first post-treatment day, the LI's of animals which had received only ara-C were significantly higher than those of animals administered 1000R x-irradiation alone ($p<0.05$), 1000R 14 hours following the last injection ($p<0.01$), and 1000R 16 hours after ara-C ($p<0.01$). Differences were not evident on the first post-treatment day, between the LI's of animals that were only irradiated as compared to those which were irradiated following ara-C. On the second post-treatment day, the LI of those animals administered irradiation alone was significantly higher than that of animals which received 1000R 14 hours following the last ara-C injection. On the third day, the LI of this latter group was higher than that of animals which had received ara-C alone. Both these differences were significant at the 5% level of confidence. Any other differences in the LI's of the various groups were not noted on the second or the third post-treatment days.

CHART 23 - COMPARISON OF THE EFFECT OF X-IRRADIATION ALONE, ARA-C ALONE, AND ARA-C FOLLOWED BY X-IRRADIATION ON THE CELL KINETICS OF THE C57B1/6J MOUSE DUODENUM

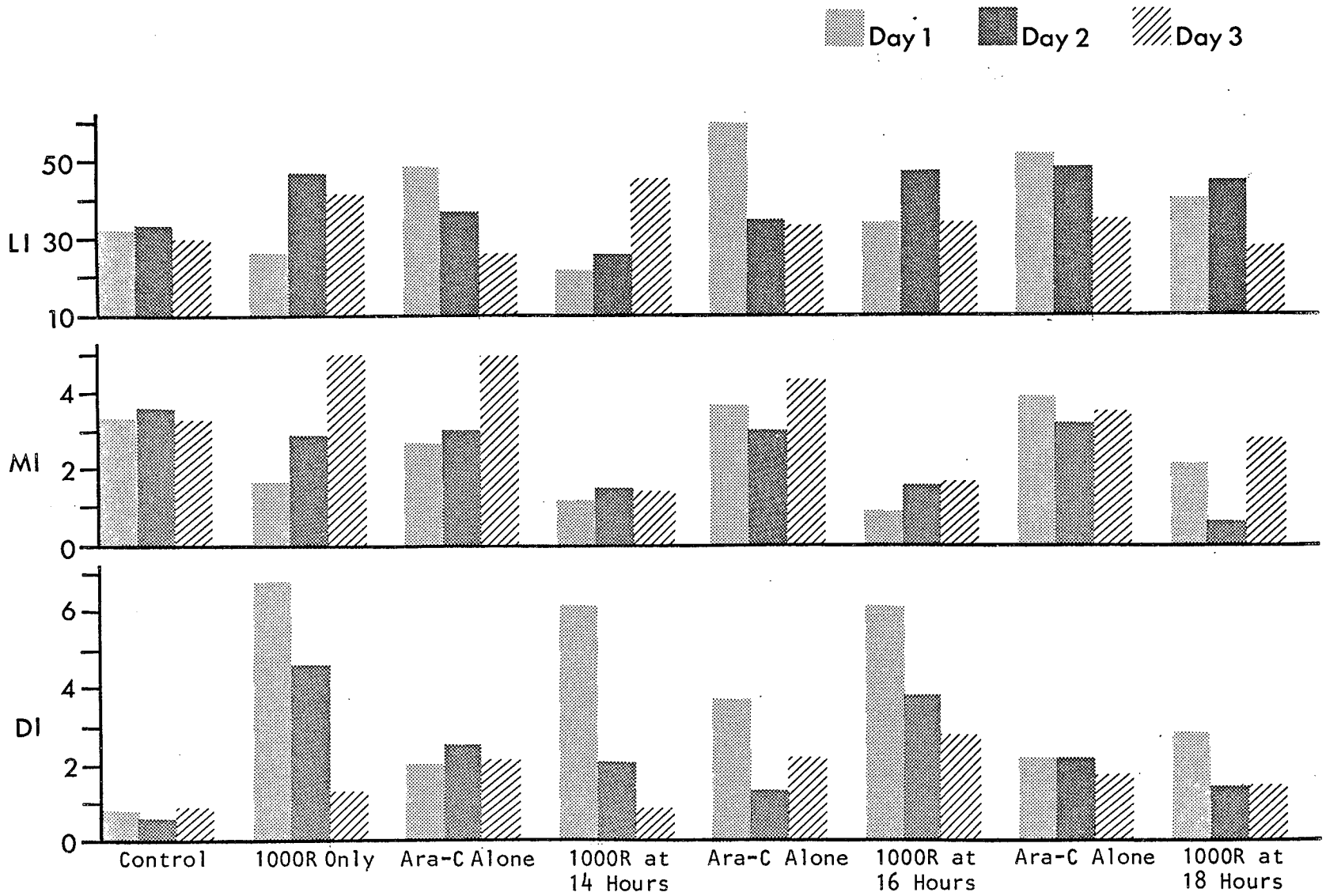


TABLE 11 - COMPARISON OF THE EFFECT OF X-IRRADIATION ALONE, ARA-C ALONE, AND ARA-C FOLLOWED BY X-IRRADIATION ON THE CELL KINETICS OF THE C57B1/6J MOUSE DUODENUM

	DAY 1	DAY 2	DAY 3
	Ara-C > 1000R (05)	Ara-C ≈ 1000R	Ara-C ≈ 1000R
	1000R ≈ 1000R at 14 hrs.	1000R > 1000R at 14 hrs. (05)	1000R ≈ 1000R at 14 hrs.
	1000R ≈ 1000R at 16 hrs.	1000R ≈ 1000R at 16 hrs.	1000R ≈ 1000R at 16 hrs.
LI	1000R ≈ 1000R at 18 hrs.	1000R ≈ 1000R at 18 hrs.	1000R ≈ 1000R at 18 hrs.
	Ara-C > 1000R at 14 hrs. (01)	Ara-C ≈ 1000R at 14 hrs.	Ara-C < 1000R at 14 hrs. (05)
	Ara-C > 1000R at 16 hrs. (01)	Ara-C ≈ 1000R at 16 hrs.	Ara-C ≈ 1000R at 16 hrs.
	Ara-C ≈ 1000R at 18 hrs.	Ara-C ≈ 1000R at 18 hrs.	Ara-C ≈ 1000R at 18 hrs.
	Ara-C > 1000R (05)	Ara-C ≈ 1000R	Ara-C ≈ 1000R
	1000R ≈ 1000R at 14 hrs.	1000R ≈ 1000R at 14 hrs.	1000R > 1000R at 14 hrs. (01)
	1000R ≈ 1000R at 16 hrs.	1000R ≈ 1000R at 16 hrs.	1000R > 1000R at 16 hrs. (01)
MI	1000R ≈ 1000R at 18 hrs.	1000R > 1000R at 18 hrs. (01)	1000R ≈ 1000R at 18 hrs.
	Ara-C > 1000R at 14 hrs.	Ara-C ≈ 1000R at 14 hrs.	Ara-C > 1000R at 14 hrs. (01)
	Ara-C > 1000R at 16 hrs. (01)	Ara-C ≈ 1000R at 16 hrs.	Ara-C > 1000R at 16 hrs. (05)
	Ara-C ≈ 1000R at 18 hrs.	Ara-C > 1000R at 18 hrs. (01)	Ara-C ≈ 1000R at 18 hrs.
	Ara-C < 1000R (01)	Ara-C ≈ 1000R	Ara-C ≈ 1000R
	1000R ≈ 1000R at 14 hrs.	1000R > 1000R at 14 hrs. (05)	1000R ≈ 1000R at 14 hrs.
	1000R ≈ 1000R at 16 hrs.	1000R ≈ 1000R at 16 hrs.	1000R ≈ 1000R at 16 hrs.
DI	1000R > 1000R at 18 hrs. (01)	1000R ≈ 1000R at 18 hrs.	1000R ≈ 1000R at 18 hrs.
	Ara-C < 1000R at 14 hrs. (01)	Ara-C ≈ 1000R at 14 hrs.	Ara-C ≈ 1000R at 14 hrs.
	Ara-C < 1000R at 16 hrs. (05)	Ara-C < 1000R at 16 hrs. (05)	Ara-C ≈ 1000R at 16 hrs.
	Ara-C ≈ 1000R at 18 hrs.	Ara-C ≈ 1000R at 18 hrs.	Ara-C ≈ 1000R at 18 hrs.

KEY: Ara-C = 8 i.p injections of 12.5 mg/kg given at 2-hour intervals.

1000R = Dosage of irradiation (no ara-C).

1000R at 14 hrs. (or 16 or 18) = 1000R dose of x-irradiation at 14 hours following last ara-C injection.

(05) (01) = 5% or 1% level of confidence.

< and > = less than, greater than.

≈ = No significant difference.

b. Mitotic Index (MI). The duodenal MI of the animals which received only ara-C was higher than those of several other treatment groups over the entire three-day post-treatment period. Thus, on the first post-treatment day, the MI of the ara-C group was greater than those of animals which received only irradiation ($p<0.05$) or irradiation (1000R) at 14 hours ($p<0.01$), on the second day it was greater than that of the 1000R at 18 hours group ($p<0.01$), and on the third day greater than those of both the 1000R at 14 hours and 16 hours groups ($p<0.01$ and $p<0.05$, respectively). On the first post-treatment day, the MI of animals which received irradiation alone did not differ from those of animals administered both irradiation and ara-C. On the second day, it was higher than that of the 1000R at 18 hours group, and on the third day higher than both the 1000R at 14 and 16 hours groups. These differences were significant at the 1% level of confidence.

c. Degenerating Index (DI). On the first post-treatment day, the DI of the animals which had received just ara-C was significantly less than those of three other groups, namely, the 1000R group ($p<0.01$), 1000R at 14 hours group ($p<0.01$) and 1000R at 16 hours group ($p<0.05$). On the second day, the DI of the ara-C group was lower only than the 1000R at 16 hours group ($p<0.05$), and on the third day it did not differ from any of the other groups. The DI was significantly higher, on the first post-treatment day, following irradiation alone than when irradiation was combined 18 hours following ara-C ($p<0.01$). On the second post-treatment day, the DI was greater following irradiation alone than when irradiation was given 16 hours after ara-C ($p<0.05$). Differences did not occur between

the DI's of the different groups on the third post-treatment day.

Summary. Comparison of the indices between the various treatment groups revealed that there were three times as many similarities as differences, and that the number of differences was twice as great on the first day as on the second or third post-treatment days. Both the LI and the MI of the groups which received ara-C alone were higher (particularly on the first day) than those of groups just irradiated, but also higher than two of the three groups which received irradiation following ara-C. The DI of the ara-C group was lower than those of the latter groups on the first day. When duodenal indices of the irradiated group (1000R) were compared to those of animals where irradiation accompanied ara-C, in all cases where a difference existed, the index of the group irradiated alone was higher than that of animals irradiated after ara-C. Thus, the LI of the former group was larger than that of the 1000R at 14 hours group on the second day, the MI was greater than one group on the second day and two groups on the third day, and the DI was higher than that of one group on the first day and another group on the third day.

2. Indices of the B16 Melanoma (Chart 24 and Table 12)

a. Labeling Index (LI). The LI of the B16 melanoma of animals administered ara-C alone was greater (on all three days) than that of animals which received only irradiation (1000R), but also of animals which received a 1000R dose either 14, 16 or 18 hours after the last ara-C injection. All differences were significant at the 1% level of confidence. The only variance in the LI's of the animals which received x-irradiation alone, when compared to x-irradiation following ara-C, occurred on the

first two post-treatment days. The LI of the animals irradiated 14 hours following the last ara-C injection was significantly lower than that of animals which received just the irradiation ($p<0.05$).

b. Mitotic Index (MI). When the MI of animals which received ara-C alone was compared to those of animals administered irradiation alone or both, the indices were almost identical to those reported for the LI's. The only difference (LI versus MI) was that the MI of animals which received only ara-C did not differ, and that on the third day only, from that of animals which were only irradiated. There were no differences, on any day, between the MI's of animals which were administered only irradiation, and animals which received ara-C followed by irradiation.

c. Degenerating Index (DI). On the first post-treatment day, the DI of mice that received only ara-C did not differ from that of mice merely irradiated or administered ara-C followed by irradiation. On the second day, the DI of mice which received only ara-C was higher than that of animals administered irradiation alone but lower than that of mice which received 1000R 18 hours following ara-C. These differences were significant at the 5% level of confidence. On the third day, the DI of the ara-C group was again higher than that of the 1000R group ($p<0.05$), but was now lower than that of all three groups of mice which received irradiation following ara-C ($p<0.01$). There were only three differences, over the entire three-day period, between the DI's of mice which received irradiation alone when compared to mice administered irradiation following ara-C. On the first day, the DI of the former group was significantly lower than that of animals irradiated at 14 hours, while on the second and third days the

CHART 24 - COMPARISON OF THE EFFECT OF X-IRRADIATION ALONE, ARA-C ALONE, AND ARA-C FOLLOWED BY X-IRRADIATION ON THE CELL KINETICS OF THE B16 MELANOMA

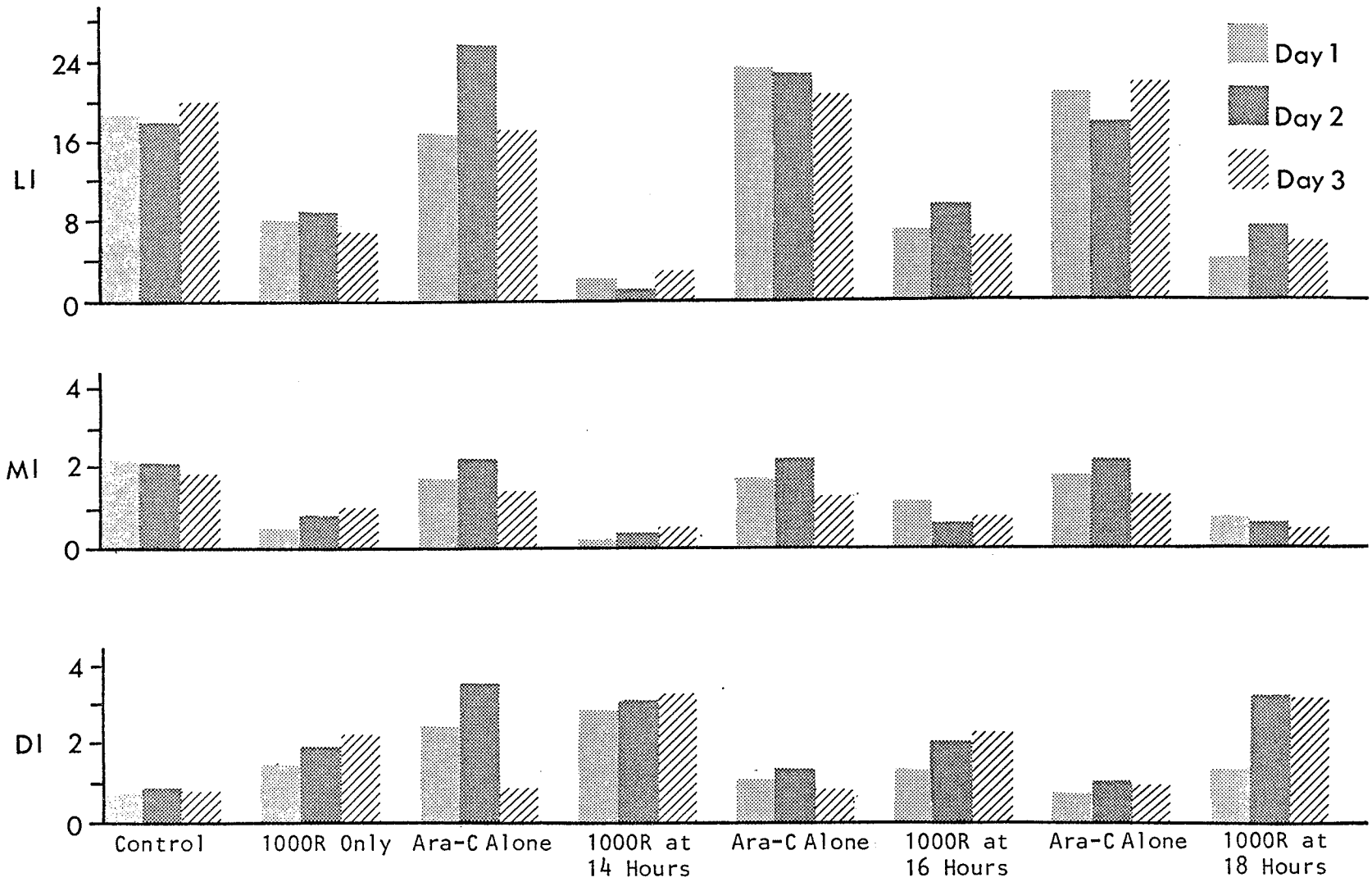


TABLE 12 - COMPARISON OF THE EFFECT OF X-IRRADIATION ALONE, ARA-C ALONE, AND ARA-C FOLLOWED BY X-IRRADIATION ON THE CELL KINETICS OF THE MALIGNANT B16 MOUSE MELANOMA OVER A THREE-DAY PERIOD

	DAY 1	DAY 2	DAY 3
	Ara-C > 1000R (01)	Ara-C > 1000R (01)	Ara-C > 1000R (01)
	1000R > 1000R at 14 hrs. (05)	1000R > 1000R at 14 hrs. (05)	1000R ≈ 1000R at 14 hrs.
	1000R ≈ 1000R at 14 hrs.	1000R ≈ 1000R at 16 hrs.	1000R ≈ 1000R at 16 hrs.
LI	1000R ≈ 1000R at 18 hrs.	1000R ≈ 1000R at 18 hrs.	1000R ≈ 1000R at 18 hrs.
	Ara-C > 1000R at 14 hrs. (01)	Ara-C > 1000R at 14 hrs. (01)	Ara-C > 1000R at 14 hrs. (01)
	Ara-C > 1000R at 16 hrs. (01)	Ara-C > 1000R at 16 hrs. (01)	Ara-C > 1000R at 16 hrs. (01)
	Ara-C > 1000R at 18 hrs. (01)	Ara-C > 1000R at 18 hrs. (01)	Ara-C > 1000R at 18 hrs. (01)
	Ara-C > 1000R (01)	Ara-C > 1000R (01)	Ara-C ≈ 1000R
	1000R ≈ 1000R at 14 hrs.	1000R ≈ 1000R at 14 hrs.	1000R ≈ 1000R at 14 hrs.
	1000R ≈ 1000R at 16 hrs.	1000R ≈ 1000R at 16 hrs.	1000R ≈ 1000R at 16 hrs.
MI	1000R ≈ 1000R at 18 hrs.	1000R ≈ 1000R at 18 hrs.	1000R ≈ 1000R at 18 hrs. (01)
	Ara-C > 1000R at 14 hrs. (01)	Ara-C > 1000R at 14 hrs. (01)	Ara-C > 1000R at 14 hrs. (01)
	Ara-C > 1000R at 16 hrs. (05)	Ara-C > 1000R at 16 hrs. (01)	Ara-C > 1000R at 16 hrs. (05)
	Ara-C > 1000R at 18 hrs. (01)	Ara-C > 1000R at 18 hrs. (01)	Ara-C > 1000R at 18 hrs. (05)
	Ara-C ≈ 1000R	Ara-C > 1000R (01)	Ara-C < 1000R (05)
	1000R < 1000R at 14 hrs. (05)	1000R ≈ 1000R at 14 hrs.	1000R ≈ 1000R at 14 hrs.
	1000R ≈ 1000R at 16 hrs.	1000R ≈ 1000R at 16 hrs.	1000R ≈ 1000R at 16 hrs.
DI	1000R ≈ 1000R at 18 hrs.	1000R < 1000R at 18 hrs. (05)	1000R < 1000R at 18 hrs. (05)
	Ara-C ≈ 1000R at 14 hrs.	Ara-C ≈ 1000R at 14 hrs.	Ara-C < 1000R at 14 hrs. (01)
	Ara-C ≈ 1000R at 16 hrs.	Ara-C ≈ 1000R at 16 hrs.	Ara-C < 1000R at 16 hrs. (01)
	Ara-C ≈ 1000R at 18 hrs.	Ara-C < 1000R at 18 hrs. (01)	Ara-C < 1000R at 18 hrs. (01)

KEY: Ara-C = 8 i.o. injections of 12.5 mg/kg given at 2-hour intervals.

1000R = dosage of irradiation (no ara-C).

1000R at 14 hrs. (or 16 or 18) = 1000R dose of x-irradiation at 14 hours following last ara-C injection.

(05)(01) = 5% or 1% level of confidence.

< and > = less than, greater than.

≈ = No significant difference.

DI of this former group was less than that of animals irradiated at 18 hours ($p<0.05$).

Summary. With just one exception, both the LI's and the MI's of the B16 melanoma of animals treated with ara-C were higher than those of animals which received either 1000R x-irradiation alone or 1000R combined with ara-C 14, 16 or 18 hours following the last injection. The DI of the mice which received ara-C alone was higher (on two days) than that of animals just irradiated, but lower (on the third day) than all three groups of mice administered both irradiation and ara-C. When indices of animals receiving irradiation alone were compared to those of animals administered both irradiation and ara-C, the LI of the irradiated group was lower than that of the 1000R at 14 hours group on two days. The MI's between these same groups did not differ on any day. The DI of the irradiated group was lower than that of the 1000R at 14 hours group on the first day only. Moreover, the DI's of irradiated animals were lower than those of animals irradiated 18 hours following ara-C on both the second and third post-treatment days. There were no other differences between any of the indices, on any of the three days, neither between groups which received irradiation alone, nor groups administered ara-C plus irradiation.

3. Survival (Table 13). The only difference in the MST's between the various groups was apparent when "early deaths" were excluded from the results. The mice irradiated 14 hours following the last ara-C injection survived longer ($p<0.05$) than those which received ara-C alone, but not longer than those animals administered 1000R x-irradiation only. There was one early death in the ara-C group, none in the 1000R

group, two in each of the 1000R at 14 hours and 16 hours groups, and one in the 1000R at 18 hours group.

TABLE 13 - THE EFFECT OF X-IRRADIATION ALONE, ARA-C ALONE, AND ARA-C FOLLOWED BY X-IRRADIATION ON THE SURVIVAL OF MICE BEARING THE B16 MELANOMA

TREATMENT	RANGE		TL_{50}		MST		N	
	GROUP 1	GROUP 2	GROUP 1	GROUP 2	GROUP 1	GROUP 2	GROUP 1	GROUP 2
ARA-C	14-48	25-48	40.5	40.5	36.1 ± 12.0	38.6 ± 9.6	10	9
1000R	23-63	---	43.5	---	46.0 ± 12.0	---	12	--
1000R AT 14 HRS.	16-74	21-74	48.5	49.5	44.7 ± 18.7	51.0 ± 13.8	11	9
1000R AT 16 HRS.	13-68	33-68	36.0	44.0	39.1 ± 16.5	45.4 ± 11.2	10	8
1000R AT 18 HRS.	14-82	33-82	39.0	39.5	45.6 ± 18.0	48.5 ± 15.7	12	11
CONTROL	19-50	---	30.5	---	33.4 ± 8.2	---	42	--

NOTE: GROUP 1 - All animals in a particular treatment group.

GROUP 2 - Only those animals in a particular treatment group which survived as long or longer than the earliest recorded death for untreated animals (i.e. longer than 18 days).

CHAPTER V

DISCUSSION

Gibson and Bertalanffy (1972) reported that a particular regimen of ara-C administration produced synchrony of the growth fraction of the malignant B16 melanoma. It was the purpose of this investigation to determine the effect of x-irradiation on that synchronized cell population. Subsidiary problems were to determine the effect of ara-C alone, x-irradiation alone, and ara-C in combination with x-irradiation on the cell kinetics of the C57Bl/6J mouse duodenum and malignant B16 melanoma cell populations, and on the survival of C57Bl/6J mice bearing the tumour.

Following 8 i.p. injections of 12.5 mg/kg ara-C, given at two-hour intervals, the duodenal mucosa of the C57Bl/6J mouse exhibited obvious morphological damage. As soon as four hours after the last injection, the crypts were shortened, less cellular, and the nuclei of crypt cells were swollen, irregular in size and shape, with dispersed chromatin. Degenerating cells were abundant. Leach et al (1969) reported very similar findings in the duodenal mucosa of mice following doses of up to 360 mg/kg (LD_{100}) of ara-C. These investigators noted that the most severe damage occurred four hours after the conclusion of the injections. It was characterized by severe degenerative changes, including numerous karyorrhectic cells and vacuolization of deep crypt cells. Cohen et al (1972) likewise reported that, four to six hours following injections of bleomycin, the crypt cell nuclei were irregular in shape, enlarged, and

contained less stainable chromatin material. In the present study, these acute cellular changes were observed up to 28 hours following the last ara-C injection, at which time the lamina propria appeared sparse and infiltrated with cells, most of which were leukocytes. This is in agreement with Leach et al (1969) who likewise observed the villus stroma of mice to be infiltrated with inflammatory cells (lymphocytic and histiocytic elements) following near lethal doses of ara-C. In the present study, changes of the villus epithelium were not evident throughout the 116-hour course of the experiment. The changes in the crypts, apparent at 28 hours, lessened with time. The crypts became eventually longer and more cellular, with the nuclei of crypt cells gradually approaching normalcy. By 76 hours, the entire mucosa appeared normal. Several other investigators noted likewise that sublethal doses of various chemotherapeutic agents did not bring about any changes in the duodenal villi (Leach et al, 1969; Cohen et al, 1972; Dethlefsen and Riley, 1973). Dethlefsen and Riley (1973) correlated the lengthening of crypts with the evidence of mucosal recovery after injections with hydroxyurea (HU), noting, however, that although the crypts were still shorter between 24 to 48 hours following termination of the injections, the acute cellular responses were completed. Following sublethal doses of ara-C to mice, Leach et al (1969) observed that the duodenal mucosa had reverted to essentially normal by 72 hours and that there was no more observable damage by 120 hours.

It must be emphasized that interpretation of the duodenal indices (following ara-C) cannot be complete unless they are correlated with crypt cellularity. For example, the normal duodenal LI was 33%, that is, approximately 30 labeled cells and 60 unlabeled cells per crypt. Four hours

following ara-C, the crypt cellularity was drastically reduced, to an extent that the LI, though appearing normal, now represented three labeled cells and six unlabeled cells per crypt. Thus, although the proportions of labeled cells were identical, the absolute numbers of labeled cells were not. Crypt cellularity remained drastically reduced for at least 28 hours. It increased very slowly thereafter.

The mean LI of the duodenal crypts, in the normal C57Bl/6J mouse, was 32.9% following a 15-minute pulse label with H^3-TdR. That figure correlated well with LI's of mouse duodenal crypts of 31 to 37% reported by other investigators (Fry et al, 1961; Lesher, 1961 and 1967; Hagemann et al, 1970a and b; Lesher and Lesher, 1974). At four, 10, 12, and 14 hours following the last injection of ara-C, the LI of the duodenum remained within normal limits. Complete, or nearly complete, inhibition of the duodenal LI in mice was reported to have occurred shortly after the administration of HU (Dethlefsen and Riley, 1970), bleomycin (Cohen et al, 1970), and of ara-C (Leiberman et al, 1970; Verbin et al, 1972a). The observed duration of that inhibition varied among these studies, however. Thus, Dethlefsen and Riley (1973) reported that inhibition of the LI following HU lasted four to five hours, with the LI increasing to a peak (well above control values) at eight hours. Lenaz and Phillips (1970) observed that the rat duodenal LI was reduced at three hours following ara-C, but that recovery was evident by six hours. As the effect of ara-C may vary with the dose (Leach et al, 1969), some of the observed variation was undoubtedly due to the different dosages administered. Moreover, if there was any initial inhibition of the LI in the present study,

its recovery was complete by four hours following the last ara-C injection. By 16 and 18 hours, the LI was approximately 56% and 62%, respectively, almost double the control values. This was followed by a subsequent decline of the LI, so that by 28 hours it had again returned to normal limits. A second peak, when the LI again reached 60%, occurred at 40 hours. It gradually declined thereafter to control values by 68 hours, and remained from then on within control limits.

The degree of synchrony can be determined according to the formula of Sinclair and Morton (1965), modified by Gibson (1972):

$$L_{max} - L_{min} = S.I.$$

where L_{max} is the maximum fraction of cells labeled (LI_{15}) following ara-C administration, L_{min} is the minimum fraction of labeled cells following the peak percentage of labeled cells, and SI is the synchrony index. In the present study, the SI of the first peak (at 18 hours) was 31.2, that of the second peak was 29.1. When these SI values were adjusted to take into account that only 55 to 60% of the crypt cells comprise the actual growth fraction (Lesher and Bauman, 1969), the two indices were 40.8 and 48.5. The real magnitude of the LI synchrony was obscured however, because the minimal labeling following both peaks was within control limits. The peak LI's of approximately 60%, might indicate that virtually all the cells of the growth fraction were synthesizing DNA at that time, provided the size of growth fraction remained unaltered. Several investigators observed that ara-C was lethal to some cells in S when it was administered, and that cells in G_1 were prevented from entering S. Ara-C thus caused an accumulation of cells at the G_1-S boundary (Kim and Eidnoff, 1965; Kit

et al, 1964; Bremerskov et al, 1970; Bhuyan et al, 1973). It was further reported that ara-C caused a minor accumulation of cells in G_2 by blocking S cells from reaching mitosis (Bremerskov et al, 1970; Wheeler et al, 1972). Cells recovering from the ara-C-induced G_1-S block would enter S at increased rates (Tobey and Crissman, 1972). This might explain the peak of the LI at 18 hours, as observed in the present study, after the last injection. If that peak of the LI was assumed to represent the midpoint of the S phase, with a duration of 7.5 hours in the C57Bl/6J mouse duodenum (Hagemann et al, 1970), then the G_1-S block terminated at approximately 14.3 hours following the last ara-C injection. The second peak of the LI (at 40 hours) occurred 22 hours after the first peak, implying an interval almost twice as long as the 13.1 hours of the total duration for a cell to pass through the entire cycle in the mouse duodenum (Hagemann et al, 1970). Several possible explanations offer themselves. Following release of cells from the G_1-S block, the cell cycle time of that synchronized cohort was greatly extended. Gibson and Bertalanffy (1972), employing the identical dosage and schedule of administration of ara-C as was used in the present study, reported that the cell cycle time was not lengthened in malignant B16 melanoma cells, despite a high degree of synchrony of the cells. Moreover, although lengthening of the cell cycle was reported following administration of various drugs, a full doubling of the cell cycle time was never observed. Therefore, it would seem very unlikely that ara-C caused the generation time of duodenal crypt cells to increase by almost 170%. A second possible reason for the delay is based on the actual points of times (following ara-C) when the LI data were ascertained.

As that particular aspect of the investigation was just a pilot study, the animals were not killed at regular two- or four-hour intervals following ara-C, but rather at times matched to correspond to the times at which animal groups would be killed following x-irradiation. Consequently, duodenal indices were determined at 20, 28, and 38 hours, but not at any intermediate intervals. Similar lapses occurred also between 44 and 62 hours and between 68 and 86 hours. If cell synchrony had been maintained, based on a 13-hour cell cycle time, peaks would have been expected to reoccur at 31 and 44 hours. A definite peak was in fact observed at 40 hours. If, on the other hand, the ara-C treatment caused a reduction of the generation time of the proliferating crypt cells, then, based on the observed peaks at 18 and at 40 hours, another peak would be expected at 29 hours. The cell cycle time would then be just 11 hours in length, two hours shorter than normally. Gibson and Bertalanffy (1972) observed an initial synchrony of B16 melanoma cells following ara-C, however, the degree of synchrony decayed quite rapidly with time. Twenty-four hours after the last ara-C injection, the SI was less than one-half the SI at 10 hours. In the present study, even if it is conceded that a peak of the LI occurred at 29 hours, the magnitude of the peak at 40 hours still remains difficult to explain. Part of that increase of the LI at that time was most certainly ascribable to the synchrony caused by ara-C. Yet, two further factors may have contributed to augment this synchrony-induced increase of the LI. Firstly, all other factors being equal, a shortening of the generation time of crypt cells could augment the LI, but only, of course, if a reduction occurred of non-S phases of the cycle. It is

probable that the G_1 phase was shortened, thus allowing cells to reach S sooner than normally. Secondly, the LI may also have been augmented by an actual increase in the size of the growth fraction of duodenal crypt cells, as a "delayed" response, to compensate for the acute cellular damage by ara-C. Thus, the extensive loss of crypt cells, so evident shortly after ara-C administration, but not at any later times, could have resulted in the movement of cells from the maturing and/or functional compartments into the proliferative compartment. Such an increase in the relative size of the growth fraction, along with the shortening of the cell cycle, could account for both the rapid recovery of cellularity in the crypts as well as for some of the increase in the LI. That possibility was previously suggested by Lesher et al (1961), in reference to recovery of crypts following x-irradiation.

Synchrony of duodenal crypt cells was thus achieved by utilizing identical dosage and regimen of ara-C administration initially devised by Gibson and Bertalanffy (1972) to produce synchrony of the B16 melanoma cell population. Such a synchrony of intestinal crypt cells, or the time of occurrence of the synchrony, have not been previously reported. The significance of this phenomenon becomes apparent when further treatment, following ara-C synchronization, is planned with cell cycle specific (CCS) agents. For instance, if an S phase specific agent were administered 18 hours following the last ara-C injection, that is, at the time of the peak of the duodenal LI, it might virtually kill all cells of the duodenal growth fraction. Although G_0-type cells might be recruited into the growth fraction to repopulate the epithelium (Lesher, 1961), the effects of the

second dose would, nonetheless, be devastating. The time of occurrence of synchrony of the crypt cell population relative to synchrony of the malignant cell is particularly important. If the synchrony in both populations occurred at different times, a regimen might be utilized to enhance cell killing in the malignant cell population (by a CCS agent), while the intestinal crypt cells, with maximal synchrony at different times, would be largely spared.

The duodenal MI was almost zero four hours following the last ara-C injection, and remained depressed for 18 hours. Subsequently, it returned to control values, where it remained for the duration of the experimental period. The MI, therefore, did not display a comparable pattern of peaks and troughs which were peculiar of the LI. Leach et al (1969) reported likewise that severe inhibition of the MI occurred in the small intestine of mice following ara-C, and noted that by 24 hours the inhibition had become much less severe, with recovery being evident at 72 hours. Verbin et al (1972a) noted that, although the MI of the rat small intestine was abolished two and one-half hours following ara-C, 12 hours after the drug the level of MI had attained three times the control value. It subsequently decreased, attaining the control level by 16 to 24 hours. Gibson and Bertalanffy (1972) observed a slight overshoot of the MI of the B16 melanoma (to 3.5 from a control value of 2.3) eight hours following the peak in the LI. If, as reported, ara-C delayed the entry of S cells into mitosis (Bremerskov et al, 1970; Wheeler, 1972), the 18-hour inhibition of the MI in the present study appears plausible. The fact that peaks of the MI did not occur following either of the peaks in the LI may again be interpreted by the actual times at which the MI data were ascertained.

The duration of the S phase of C57Bl/6J duodenal crypt cells was reported to be 7.5 hours and the G_2 phase 0.5 hours (Hagemann et al, 1970); onset of mitosis might hence be expected to occur approximately four hours following the peak of the labeling index. The first peak in the LI occurred at 18 hours, and therefore a peak in the MI would not be expected earlier than 22 hours. Similarly, a MI peak following the LI second peak (at 40 hours) might be expected at 44 hours. In actual fact, (see Chart 6, page 124) at 44 hours after ara-C, the MI was slightly above control values.

The DI of the mouse duodenum was vastly augmented four hours after the last injection of ara-C. It rapidly declined subsequently to come within the normal range by 20 hours. Dethlefsen and Riley (1970) reported similar findings in the mouse small intestine following injections of HU, and suggested that the killed cells were very rapidly eliminated from the crypts. The circumstance that the DI had returned to normal while crypt cellularity was still decreased would seem to support this notion.

The duodenal indices, though indicative themselves of cell kinetic changes following ara-C, became still more meaningful indicators when correlated with the morphological changes. Thus, although the LI appeared to be within normal limits four hours after the last injection, because crypt cellularity was vastly reduced, the absolute number of labeled cells was drastically lower than normal. The enhanced DI at four hours was a clear indication of that reduction in crypt cellularity. As it would be expected that ara-C would preferentially kill proliferating cells, as opposed to maturing or functional cells, the decreased cellularity was probably largely due to a reduction in the size of the growth fraction. This should have resulted in a decreased LI, which obviously was not the

case, however. It is likely then, that cells were added to the growth fraction, perhaps during, and certainly shortly after onset of cell kill by ara-C. By 14 hours the LI was still within normal limits, while the MI and DI began to show evidence of recovery. Crypt cellularity was still notably lower, as would be expected. Even though cells might have been added to the growth fraction, this cell compartment was still very much reduced and therefore incapable of repopulating the crypts within this 14-hour period. Based on a normal growth fraction of 55 to 60%, the peak in the LI at 18 hours might signify that the entire growth fraction was synthesizing DNA at that time. This is highly unlikely, as some cells enter S even in the presence of high concentrations of ara-C. This, then, is a further indication that nonproliferating cells must have been recruited to enter the proliferative compartment. At 28 hours, when all indices were within normal limits, crypt cellularity was still very much reduced. This would be expected, as this time was still within the duration of one generation time of the peak in the LI, and a complete repopulation could hence not be expected within this short time period. Following the second peak in the LI (at 40 hours) crypt cellularity had gradually returned to normal, so that by 52 hours the crypts were morphologically normal and cellularity appeared to be restored. All other duodenal indices were normal also by that time.

The LI of the untreated B16 melanoma averaged 18.6 over a five-day period (day 10 to day 15 of tumour growth) and did not differ on a day-to-day basis. This is in agreement with Gibson (1972) who likewise reported the LI to be 20.2% on day 10 of tumour growth. Four hours

TABLE 14 - INDICES OF THE B16 MELANOMA AT VARIOUS TIMES FOLLOWING THE LAST OF EIGHT i.p. INJECTIONS OF 12.5 mg/kg ARA-C GIVEN AT TWO-HOUR INTERVALS.

		LABELING INDEX (LI_{15})		MITOTIC INDEX		DEGENERATING INDEX	
		A	B	A	B	A	B
CONTROL		18.6	20.2	2.17	2.53	0.90	0.80
HOURS AFTER	4	6.3	6.8	0.14	0.02	6.52	2.26
LAST INJECTION	10	41.1	45.1	0.48	0.61	5.96	5.96
	12	37.0	39.0	0.53	0.61	8.32	8.57
	14	18.7	28.7	0.86	1.40	8.71	6.98
	16	18.4	13.23	2.51	2.83	4.14	3.92
	18	17.3	4.4	2.10	3.49	4.90	5.61
	20	13.8	7.3	1.83	2.37	3.45	3.89
	28	23.0	17.7	1.06	2.48	2.91	1.37

A - present study.

B - Gibson and Bertalanffy (1972).

following the last ara-C injection, the number of labeled cells had significantly decreased to 6.3%, while at 10 and 12 hours it was 41.1 and 37%, respectively. These latter two values are not significantly different from those reported by Gibson and Bertalanffy (1972), employing the same dosage and schedule of administration of ara-C as used in this study (Table 14). By 14 hours, in the present study, the LI was again within the normal range, where, with some oscillation to either side of the control values, it remained

for the duration of the experimental period. This is in contrast to the data by Gibson and Bertalanffy (1972) indicating that the LI was greatly reduced (to 4.4 and 7.3%) 18 and 20 hours after the last injection, but at 22 and 24 hours, was again within the normal limits. It was suggested that this second "peak", following the trough, represented a residual, and thus decayed, cohort of cells from the first synchrony. Further, as this second peak was 14 hours after the first peak, it was assumed that the duration of the normal 14-hour cell cycle had not been affected by the ara-C. Although the peaks in the LI at 10 hours, in the present study compared to that of Gibson and Bertalanffy (1972), are not significantly different, the SI in this study was 27 while in the latter study it was 40. The SI's, when adjusted to take into account that only 53% of cells of this tumour comprised the growth fraction, were 51 (in this study) compared to 77 (Gibson and Bertalanffy, 1972). Again, as in the case of the duodenum, the low SI was ascribable to the circumstance that the LI was not drastically reduced following synchrony. This does not alter the fact that approximately 40% of the (53%) growth fraction cells were labeled 10 hours after the last ara-C injection. Thus, in terms of absolute numbers of labeled cells, the observations of the present study did not differ from those of Gibson and Bertalanffy (1972). The absence of troughs in the LI (at 18 and 20 hours) remains unexplained. The duration of the cell cycle of the B16 melanoma on day 10 of tumour growth was reported to be 14 hours, with S = 7.5 hours, G_1 = 3.8 hours, G_2 = 1.3 hours, and M = 1.4 hours (Gibson and Bertalanffy, 1972). If the peak in the LI at 10 hours is again assumed to represent the mid-point of the S phase, then the G_1-S block ended approximately 6.5 hours after the last injection of ara-C.

The peak of the LI at 10 hours would again be attributed to those cells, blocked at the G_1-S boundary, being released from this block and passing as a cohort into the S phase of the cell cycle. In the present study, there was no evidence of a synchronized population of tumour cells (which would be represented by a peak in the LI) following this initial episode at 10 hours. This is in agreement with Gibson and Bertalanffy (1972) who reported a second "peak" in the LI (following a "trough"), which was, however, within the normal limits.

There are several differences in the response between the tumour cell population to this regimen of ara-C and the duodenal cell population. In the first place, the duration of the G_1-S block was estimated to be 6.5 hours in the tumour compared to approximately 14 hours in the duodenum. This difference cannot be explained on the basis of a difference alone in the generation times of the two populations (which is less than an one-hour difference), or on the basis of a difference in the duration of the S phase, as the latter is identical in both cell populations, specifically 7.5 hours (Hagemann et al, 1970; Bertalanffy and Gibson, 1972). Secondly, in the duodenum, a second peak in the LI was observed 22 hours following the first peak, while, in the tumour, after the first peak at 10 hours, further evidence of cell synchrony was not apparent. Even though indices were not determined during the period between the two peaks, the likelihood suggests itself that a peak may have occurred in the duodenal LI during that time, implying that synchrony did not decay rapidly following ara-C, but rather caused a shortening of the cell cycle time, with peaks occurring approximately 11 hours apart. Thirdly, the LI of the tumour was depressed

immediately following the last injection of ara-C, while that of the duodenum remained still around the control values almost to the time of the first LI peak. As was pointed out, these responses of the two cell populations cannot be explained on the basis of differences in the cell cycle alone. The differences, then, must be due to differences in the responses of the two cell populations to the ara-C treatment. It is also not unreasonable to assume that factors governing cell kinetic responses may vary in a normal population as compared to a malignant cell population, regardless of whether the cell populations are being influenced by an external agent. Moreover, Gibson and Bertalanffy (1972) suggested that the cells of the B16 melanoma could consist of two distinct cell populations accentuated by treatment with ara-C; a fairly rapidly dividing population of cells with a generation time of 14 hours, and a second, more slowly proliferating fraction with a much longer generation time.

It is obvious that the different responses of these two cell populations can be employed to advantage when planning therapeutic regimens. For example, an S phase specific agent, administered 10 hours following the last ara-C injection, would be expected to enhance cell killing in the tumour, as at that time 40% of the 0.53 growth fraction tumour cells were engaged in DNA synthesis. Conversely, the synchronized cohort of duodenal crypt cells were at that time likely blocked still at the G_1-S boundary and hence would remain spared from the lethal effect of the CCS agent.

The MI of 2.17% of the untreated B16 melanoma on day 10 of tumour growth ascertained in this study, did not differ from that reported by Gibson and Bertalanffy (1972). Up to 14 hours after the last injection of ara-C,

the MI was significantly below control values. It recovered thereafter to slightly overshoot control values at 16 hours and then returned to control limits by 18 and 20 hours. These results are very similar to those reported by Gibson and Bertalanffy (1972) although in the latter study the overshoot of the MI at 18 hours was higher than in the present investigation. If the mid-point of S is assumed to be 10 hours, then the onset of mitosis might be expected to occur normally 5.1 hours later ($\frac{1}{2}S + G_2$), that is, at 15 hours. The predicted interval could be slightly longer than the actual values if the ara-C caused some accumulation of cells in G_2, as suggested by Bremerskov et al, 1970. Thus, the peak of the MI, at 16 hours, could have been brought about by such a delay caused by the accumulation of some cells in G_2.

Four hours following the last ara-C injection the DI was 6.5, hence significantly higher than control values, and also higher than that previously reported by Gibson and Bertalanffy (1972). The DI remained elevated for 38 hours in the present study. The values obtained at most points during that period were not different from those of Gibson and Bertalanffy (1972). Ara-C is known to be cytotoxic to S-phase cells (Lark and Lark, 1964; Cohen, 1966). The circumstance that the DI level remained high in the tumour much longer than in the duodenum, could be ascribable to the much more rapid removal of killed cells from the duodenum than from the tumour. Although definite morphological changes in the tumour were not apparent following treatment with ara-C, this explanation would seem unlikely, inasmuch as other investigators asserted that killed tumour cells are also rapidly removed (Goldfeder and Ghosh, 1973). It was

ascertained that, following ara-C treatment, cells must accumulate in S and remain there for a sufficient time before they die (Yataganas, 1974). However, that phenomenon does not offer a likely explanation for the longer duration of the enhanced DI, because a lethal accumulation of cells in the S phase would greatly affect the DI within the same generation time of the occurrence of that accumulation. Yet the duration of the increased DI in the present instant spanned at least two generation times. Again, the fact that the DI remained still augmented 38 hours following the cessation of the ara-C treatment was suggestive that a second population of cells existed in the B16 melanoma tumour with a much longer generation time in addition to the fairly rapidly proliferating fraction.

Regarding survival times, untreated C57Bl/6J mice bearing the B16 melanoma survived 19 to 50 days following transplantation, with a MST of 33.4 ± 8.2 days. Gibson and Bertalanffy (1972) reported that C57Bl/6J mice bearing this tumour survived from 15 to 46 days, with a MST of 29 ± 6.9 days. This insignificant difference was likely due to slight modifications in the transplantation procedure. The MST was not significantly increased in animals which were treated with ara-C, and one of the 10 animals in that series died before the time of the earliest recorded death of control animals. This "early death" was ascribed to the direct toxic effect of ara-C, producing, in the animal, the GI syndrome similar to that observed in irradiated animals. That the animals did not survive longer following ara-C treatment would indicate that however severe the immediate effects of the drug may have been on the cell kinetics of the tumour, after the effect of the drug had ceased, the tumour continued to grow and

eventually killed its host. This was reflected in all three tumour indices, which had returned to control values within two days of the last ara-C injection.

For the x-irradiation, the mice were anaesthesized and the tumour was exposed to the ionizing rays through a 2.5 by 1.5 cm aperture in a lead shield. As the tumour had been implanted s.c. in the left mid-abdominal area, it was likely that the underlying duodenum was also irradiated. Administered dosages were 500R, 1000R, or 1500R, as single exposures, or 1000R in two equal exposures of 500R 24 hours apart. The dosage rate was 143.5R per minute with a 30 cm F.S.D., 2.5 cm in diameter field size, and H.V.L. 2.8 mmCu. Duodenal and tumour indices were determined 24, 48, or 72 hours following the x-irradiation.

On the first day, following all doses of x-irradiation, obvious damage to the duodenal crypts was apparent, the degree of damage being dose-dependent (see Figure 8, pages 133 and 134). The irradiation damage was morphologically similar to that observed following ara-C treatment, notably, the shortened and less cellular crypts, with crypt cell nuclei appearing swollen and irregularly shaped. MF's were scarce while degenerating cells were abundant. Such changes were observed also by numerous other investigators (for example, Montagna and Wilson, 1955; Quastler, 1963; Bond et al, 1965), and that morphological changes following irradiation are dose-dependent is a well-known phenomenon. The epithelia of mice receiving the 1000R fractionated dose did not appear to be as severely affected as those of mice receiving a single 1000R dose. This would be expected, as some cellular recovery from damage sustained

by x-irradiation is known to occur during the 24 hours between dose fraction application. Although the crypts of animals which received either a 500R dose or 500R x 2 dose gave evidence of recovery on the second day, those of animals which received 1000R or 1500R, appeared unaltered from the first day, and the crypts may have been even less cellular on the second day. In all groups, there were not nearly as many degenerating cells on the second day as on the previous day. Dependent on the dose of x-irradiation, severe morphological changes in the crypts were reported to persist for one or two days following exposure (Montagna and Wilson, 1955; Quastler, 1963), however the number of pyknotic cells was greatly dependent on the dose. The degenerative process in the crypts was past its peak by the second or third day after exposure (McGrath, 1960). This was noted also in the present study, as, although damage to the crypt epithelium was still evident on the third day, the degree of that damage was very much reduced, more so even following 500R or 500R x 2 than after 1000R or 1500R dosages. Villus changes following x-irradiation occur later than crypt changes (Montagna and Wilson, 1955; Quastler, 1963; Bond et al, 1965), and usually become evident at about the time the crypts are commencing to exhibit evidence of repair, that is, at three and one-half to five days after irradiation (Quastler, 1963). Although the epithelial cells of the villi were seemingly undamaged over the three-day period of observation in this study, the villi as a whole appeared to be slightly shorter than normally. This could have been caused by damage to the stromal tissue or by loss of extracellular fluid into the intestinal lumen (Taketa, 1962).

It is well-known that the degree of inhibition of DNA synthesis following x-irradiation is dose-dependent (Hagemann and Lesher, 1971; Lesher and Lesher, 1970 and 1974). Particularly those cells in the S phase at the time of irradiation are affected (Lajtha et al, 1958; Ord and Stocken, 1972), and probably also cells in G_1 at the time of irradiation (Beltz et al, 1957; Bollum et al, 1960; van Lancker, 1959, 1960a and b). With doses up to 900R, this inhibition was reported to persist for 24 hours or less after irradiation, with the duration of the inhibition being longer at higher doses (Lesher and Lesher, 1970 and 1974). In the present study, just the duodenal LI of that group receiving a dose of 1500R was depressed, and that decrease was evident on both the first and second post-irradiation days. On the third post-irradiation day, the only difference in any of the LI's was again in the group irradiated with 1500R, where the LI was doubled from that of the second day. This latter LI was the only instance whereby the LI was higher than control values. Other investigators reported "compensatory overshoots" of the LI in the intestinal crypts following irradiation, with the time of the overshoot occurring anywhere between two to four days following exposure (Hagemann and Lesher, 1961; Lesher and Lesher, 1970 and 1974; Sigdestad et al, 1973; Göbbeler, 1973). In the present study, on the second post-irradiation day, the LI of both the 500R and 1000R groups had significantly increased from the previous day, but were not higher than control values. It is unknown whether these indices would have continued to augment during the 48 to 72 hour period. The duodenal LI of the mice which received the fractionated dose of irradiation was slightly, though not significantly,

higher than control values on each of the three days but did not change significantly on a day-to-day basis. Göbbeler (1973) noted also that the effect of a fractionated dose of irradiation was different from that of a single dose. Following a single 600R dose, the LI of mouse duodenal crypts remained severely depressed at six hours, and increased thereafter to overshoot control values by 48 hours. When two 300R doses were administered 18 hours apart, the LI's were 74%, 86%, and 80% of control values two and three days following the second exposure. When the fraction interval was increased to 48 hours, the LI's were 86%, 110%, and 105% on each of the three days, respectively. The LI's of the fractionated groups in the present study, slightly above control values, fell between the values for the two different fractionated groups reported by Göbbeler (1973).

Twenty-four hours following x-irradiation, the MI's of all groups were within the normal range. A dose-dependent inhibition of mitosis following irradiation is a well-known phenomenon (Elkind et al, 1963), ostensibly due to damage to the division mechanism of cells. Cells in mitosis at the time of irradiation complete mitosis, however, whereas cells preparing for division are prevented from entering the mitotic phase (Carlson, 1972; Dewey et al, 1972). The duration of the mitotic inhibition is likewise dose-dependent, and can vary also according to the type of irradiation. Thus, Poulokos and Osborne (1973) observed a complete absence of MF's for three days in rat intestinal crypts after 1000R administered to the exteriorized ileum, while Tsubouchi and Matsuzawa (1974) reported that the MI in the crypts of mice exposed to 1000R WBI had returned to control values by 14 hours. Lesher and Lesher (1974)

observed that the MI in mouse jejunal crypts was only slightly lower than normally 30 and 60 hours following partial abdominal irradiation with 600R or 900R. The slightly lower MI's observed on the first post-irradiation day in the present study were likely an expression of recovery in the MI following an initial inhibition, which almost certainly must have occurred immediately following exposure. On the second post-irradiation day, the MI of the 500R group increased, overshooting control values. A comparable augmentation occured also in the 1000R group on the third day. Lesher and Lesher (1974) observed likewise that the time at which peaks occurred of the MI of irradiated mice was dose-dependent. Following 600R, there was a peak at 66 hours, while after 900R the peak occurred at 80 hours. In the present study, the MI of the mice which received 1500R was slightly below control values on all three days, and did not vary significantly from one day to another. Any peak which might have occurred in the MI of this group would likely have been sometime after 72 hours, at which time the last data were collected. As noted previously, the LI of the mice that received 1500R became significantly augmented on the third post-irradiation day, and therefore any increase in the MI, which would be expected to occur several hours later, would not also be observed on the third day. The duodenal MI's of the mice which received the fractionated doses of irradiation exhibited a remarkably similar pattern, relative to control values, to the LI's of these groups of animals. Thus, on all three days, the MI's did not differ significantly from control values. Hagemann and Lesher (1971) reported that, following fractionated doses of WBI to mice, augmented proliferative cellularity of intestinal crypts

following irradiation injury would not be expected to occur for three to four days. In the present investigation, any increase was not observed in the MI during the three-day period of observation. It must also be noted that, although the generation time of intestinal crypt cells is likely accelerated following x-irradiation (Lesher et al, 1961; Lesher and Lesher, 1974), cells might be blocked in G_2 immediately following irradiation, the duration of that block being dose-dependent (Lesher, 1964). This could mean that the actual period until an increase in the MI occurred would be longer than the predicted time calculated from the generation time of the cells.

The duodenal DI of mice irradiated with doses of 500R, 500R x 2, and 1000R were all significantly increased on the first post-irradiation day, as would be expected, x-irradiation being cytotoxic. Other investigators noted that the maximal proportion of pyknotic cells occurred shortly after irradiation (four to six hours), and that pyknotic cells remained abundant throughout the entire first post-irradiation day (Patt and Quastler, 1964; Tsubouchi and Matsuzawa, 1974). In the present study, the number of pyknotic cells had decreased in all groups by the second day, so that just the mice which received the 1000R dose exhibited a higher DI rate than that of controls. A reduction in the number of pyknotic cells on the second post-irradiation day is a commonly reported occurrence, because degenerating crypt cells are known to become rapidly removed (Montagna and Wilson, 1955; Patt and Quastler, 1963). However, the survival of cells following x-irradiation is clearly dose-dependent, cell killing being enhanced by higher doses (Puck and Marcus, 1956). Cells may, however, still proceed

through several cell cycles before eventually dying. In the crypts of those mice irradiated with either 500R or 500R x 2, the peaks of cell killing occurred on the first post-irradiation day, and degenerating cells had been removed and disappeared from the crypts by the second day. Cells which were destined to recover from the sublethal damage had done so by that time. Following a dose of 1000R, degeneration of cells continued throughout the second post-irradiation day, likely because cells which had accumulated lethal damage kept on degenerating, perhaps after progressing through several cell cycles. By the third post-irradiation day, the DI's of all three groups were again within normal limits. This observation correlates well with the morphological evidence of cell regeneration in the crypts, as previously noted. The duodenal DI of the mice which received a 1500R dose did not follow a similar pattern to those of the other groups. In this former case, the DI remained within normal limits on all three post-irradiation days, although the diminished cellularity was more severe than in other groups. It would appear then that the degeneration of cells occurred at more rapid rates following the higher dose, so that the dead cells, after having been removed from the crypts, would obviously not be included in the cell counts.

The duodenal indices, as determined one, two and three days following x-irradiation, did not correlate perfectly with the observed morphological damage. Thus, on the first post-irradiation day, when the damage appeared to be very severe, the DI was generally augmented, which would be expected, but, with a single exception, neither the LI nor MI were reduced. It must be realized though, that the absolute numbers of labeled cells and

mitotic cells were very much reduced, as total crypt cellularity was diminished. Whereas approximately 25 to 30 cells of the 65 to 80 cells normally present in a longitudinal crypt section which included the lumen would ordinarily be labeled, just three or four of the roughly 10 cells present after irradiation would be labeled. Further, it would be expected that the x-irradiation would preferentially kill the proliferating cells, thus reducing the growth fraction, both in terms of absolute numbers and also in terms of the ratio of proliferating to non-proliferating cells. While it is true that the size of the growth fraction was indeed reduced, the proportion was not. This suggested that cells had actually been added to the growth fraction, presumably from the (previously) functional and/or maturational compartments. This same process likely continued also on the second and third post-irradiation days, when the crypt cellularity increased to return, and perhaps even surpass, the normal cell numbers. On the second day, the decline of the LI of those mice which received 1500R might signify that a longer recovery period was required following the higher as compared to the lower doses. The overshoot in the LI on the third day would further suggest a recruitment of cells to the proliferative compartment.

Following treatment of the B16 melanoma with 500R, 500R x 2, 1000R, and 1500R, both the LI's and the MI's were decreased, from control values, in all irradiated groups and on each of the three post-irradiation days. The DI's were augmented in all groups on each day. None of the indices exhibited changes from one day to another. Previous investigators observed that the degree of inhibition of both the LI and the MI of tumours was dose-dependent. However, it was not stated over what time period the LI

determinations were made (Goldfeder and Ghosh, 1973). In the case of mitosis, inhibition of cell division was observed over periods up to 20 hours or less (Brown and Berry, 1969). With regard to the DI, agreement does not seem to exist. Tannock and Howes (1973) observed pyknotic cells in mouse mammary adenocarcinoma tumour cords up to 24 hours following irradiation, but not thereafter, while van Peperzeel (1972) suggested that the elimination of radiation-killed cells in tumours was more rapid. In contrast, other authors did not observe any increase in the number of pyknotic cells shortly following irradiation (Klouwen, 1965; Hofer, 1970), but mentioned that the "killing phase" occurred only two to six days following exposure (Hofer, 1970).

Inasmuch as a decline of the LI and MI, with a concomitant increase in the DI would be indicative of a slowing of tumour growth, the survival patterns of irradiated B16 melanoma bearing C57Bl/6J mice were examined. All of the irradiated groups of tumour-bearing animals apparently survived for a longer period than did the non-irradiated controls (Table 3, page 145), and both the TL_{50} and MST became augmented with increasing irradiation dosage. It would appear then, despite the circumstance that differences between any of the irradiated groups in terms of the indices did not occur over the three-day period, there were differences in the growth characteristics of the tumour following the various dosages. One factor that could be partially responsible for the increased survival was that the growth of the tumour was slowed following irradiation in proportion to the dosage. Thus, although the LI or MI did not differ between groups over a three-day period, the inhibition of these indices may have been of

longer duration following higher than lower doses. It was reported that the generation time of a squamous carcinoma after irradiation increased (Brown and Berry, 1969; Brown, 1970). If that prolongation of the cell cycle was also dose-dependent, tumours irradiated with higher doses would be expected to grow more slowly. These all could be factors contributing to the increased volume doubling time (VDT), observed with irradiated tumours (Eddy and Casarett, 1972). Another factor, tumour regression, could have contributed to a longer MST following irradiation. That factor is also known to be dose dependent (van Peperzeel, 1972). It is logical that the more a tumour regressed following irradiation, the longer it would require to regrow (or repopulate) to its former size. Finally, following higher doses there may have been more extensive cell death occurring more rapidly than with lower irradiation doses, yet, it would not be reflected in any of the indices, if dead cells were rapidly removed from the cell population. The observation that the MST of the fractionated group (1000R) was longer than that of the 500R group, but shorter than the 1000R group receiving the dosage as a single unit, is not surprising, as both some repair of sublethal damage and repopulation of tumours occur between administration of the dose fractions (Howes and Suit, 1974). The present cell kinetic and survival data would therefore indicate, contrary to the opinion of some investigators who considered melanomas to be radioresistant (Desjardins, 1934; Pack 1948; Philips, 1963; Sugiura, 1963), that even single doses of x-irradiation caused some growth retardation of this moderately pigmented tumour. This observation is in agreement with Barranco et al (1971), reporting that three strains of melanoma cells,

containing varying amounts of pigment, exhibited a similar degree of radiosensitivity, and did not differ markedly from the radioresponse of non-pigmented Chinese hamster ovary cells. Although it was not determined whether the radiosensitivity of the B16 melanoma was more, less, or equal to that of other epithelial-derived tumour cells, the present data tend to support the suggestion by Biran et al (1973) that, because some types of melanoma may be radiosensitive, a trial course of radiotherapy would not be contraindicated.

On the basis of foregoing observations of the effect of x-irradiation on the B16 melanoma, the 1000R dose was selected for a combination treatment with ara-C. That dose was chosen because of its definite effect on the indices indicative of tumour growth retardation. Further, it augmented the survival time of mice bearing the tumour, without causing "early death" of any of the animals, ostensibly due to the GI syndrome. Because cells are not uniformly radiosensitive throughout the cell cycle, it was assumed that if cells could first be synchronized by ara-C, and the synchronized cohort irradiated while in the most radiosensitive phase of the cell cycle, enhanced cell killing might be achieved. A pilot study, in which 8 i.p. injections of 12.5 mg/kg ara-C were administered at two-hour intervals, revealed that a high degree of synchrony (as indicated by a peak in the LI) was achieved in the B16 melanoma 10 hours following the last injection of ara-C. That dosage and regimen of administration of ara-C was ascertained by Gibson and Bertalanffy (1972), devised to produce synchrony in the B16 melanoma. Synchrony in the duodenal crypt cells occurred in the present study 18 hours following the last ara-C injection.

Although a complete agreement does not prevail as to the relative radiosensitivities of all phases of the cell cycle, various investigations signified that the following principles appear to apply to most types of cells:

1. Cells in the S phase are not radiosensitive, and they are even less so in late S than in early S.
2. Cells in the M phase are the most radiosensitive.
3. Cells in G_1 are slightly less radiosensitive than M-phase cells, and their radiosensitivity increases as cells progress through G_1.
4. Cells in G_2 are radiosensitive, with the degree of radiosensitivity augmenting as cells progress through this phase.

It was assumed that the peak of the LI represented the mid-point of the S phase, and that the administration of ara-C left the duration of the cell cycle unaltered (Gibson and Bertalanffy, 1972). In the B16 melanoma, the t_c = 14 hours, with S = 7.5 hours, G_2 = 1.3 hours, M = 1.4 hours, and G_1 = 3.8 hours (Gibson and Bertalanffy, 1972). 1000R x-irradiation was given four hours, 14 hours, 16 hours, 18 hours, or 20 hours following the last ara-C injection, that is at times when it was expected that the cohort of tumour cells would either be in late G_1 phase (before synchrony), early G_2 phase, M phase, mid-G_1 phase, or late G_1 phase (after synchrony) respectively. Irradiation was not administered at six and eight hours following the last injection, because at that time the cells would still be blocked at the G_1-S junction, and would hence be comparable to the cohort at four hours. Irradiation was neither applied at 10 and 12 hours,

because at that stage the cells were accumulated in the radioresistant S phase. For similar reasons, and also the circumstance that the decay in synchrony was fairly rapid, irradiation was not administered beyond 20 hours following the last ara-C injection.

Regarding the cytodynamic data of the duodenum, the t_c = 13.1 hours, with S = 7.5 hours, G_2 = 0.5 hours, M = 1 hour, and G_1 = 4.1 hours. At both four and 14 hours following the last ara-C injection, most cells were likely in G_1, assumed from the prediction of the duration of the G_1-S block of 14 hours. During the period of 16, 18 and 20 hours after ara-C, the cohort of cells would be in the radioresistant S phase, because the peak of the LI occurred at 18 hours.

When animals were given a 1000R dose four hours following the last ara-C injection, tumour cell proliferation, as expressed by the labeling, mitotic, and also degenerating indices, was slowed over the subsequent three-day period, however not to any greater extent than when irradiation was given at 14, 16, 18 or 20 hours. In fact, this regimen may have been slightly less effective, because the LI was not depressed as much as that of the group irradiated at 14 hours. The DI was also lower than those of all other groups on the second post-irradiation day. The duodenal indices of this four-hour group did not differ significantly from those of other groups irradiated at later times. However, on a morphological basis, the damage to the mucosa was much more severe in this early group than in groups irradiated later. Thus, although the indices appeared similar, the absolute number of proliferating cells was much lower in that group (than in other groups), because the total crypt cellularity was greatly reduced.

This phenomenon would also account for the fact that the MST of the latter treatment group was approximately 19.5 days, that is, significantly shorter than the 33-day MST of untreated animals. The severity of the mucosal damage, then, was further substantiated by the fact that 75% of the animals died within eight days of the treatment. Moreover, 11 of the 15 "early deaths" occurred within four days of cessation of treatment. It is probable that these animals died of GI syndrome, similar qualitatively to the "acute intestinal radiation death" described by Quastler et al (1951). Symptoms of ruffled fur, lethargy, and diminished food and water intake were observed in these animals, and at autopsy, the frequent observation of gastric retention. All are symptomatic of the GI syndrome. The severe reduction in duodenal cellularity brought about by irradiation four hours after the last ara-C injection may have been due to a single or a combination of several factors. In the first place, it was observed that ara-C alone caused generally a fairly severe reduction of crypt cellularity, and this was particularly evident at four hours after the last injection. Irradiation at that time, when the proliferating cells were already reduced in number by ara-C, may have further diminished this cell population. The resultant depletion caused by this additive effect compromised the integrity of the mucosa to such an extent that death of the animal resulted. Another possibility is that, because the site of biochemical impact may be similar by ara-C and irradiation, this caused "additive effects" by the administration of the two agents within a short period of time. Thus, it was reported that ara-C inhibited the polymerase activity necessary for DNA replication (Drewinko et al, 1972). It could also be incorporated into

the DNA molecule, thereby causing strand breaks, particularly in cells in late S at the time of administration (Doering et al, 1966; Brown and Christie, 1966). Similarly, DNA might be the sensitive molecule in irradiation (Terasima and Tolmach, 1961), affecting the formation of the enzymes (both kinase and polymerase) required for DNA replication. Although cells can repair some damage caused by irradiation, a combined damage (likely to the same molecule) produced by ara-C and subsequent x-irradiation might be beyond the repair capacity of most cells. It is difficult to determine whether the ara-C-induced cell synchrony was a major factor in the enhanced cell killing by subsequent irradiation, as there are two opinions. On the one hand, if the cohort of duodenal cells was in the mid-G_1 phase at the time of irradiation, or at the G_1-S junction, enhanced cell killing by irradiation would be expected owing to the radiosensitivity of G_1 cells. On the other hand, Tsubouchi and Matsuzawa (1974) reported that pyknotic cell formation in mouse duodenal crypts was low in G_1 following 1000R WBI but high in both S and G_2. It was suggested that the greater irradiation resistance of G_1 cells was an important factor in the recovery of the crypt cell population following irradiation. If that was the case, the synchronizing effect of ara-C on duodenal crypt cells might not have been a crucial factor in cell killing when irradiation followed ara-C treatment.

Because the 1000R dose following four hours after ara-C administration resulted in the death of 75% of the animals, that irradiation dosage was subsequently reduced to 500R in an effort to maintain enhanced cell killing of the tumour cells yet without concomitant augmented cell killing of duodenal cells. Neither the duodenal nor the melanoma indices

differed between the animals receiving the two dosages or irradiation. Consequently, as it was also the case when 1000R followed four hours after ara-C, 500R at four hours was not as effective, in terms of delay of tumour cell proliferation, as were some of the other regimens with longer intervals between irradiation and ara-C administrations. The animals did tolerate the lower dose of irradiation more favorably than the higher dose, and only two of nine (22%) mice died prior to the time of the earliest recorded death of untreated animals. When the two "early death" animals were excluded from the calculations, a difference in the MST's of the two groups of animals was not apparent, and neither group survived longer than untreated animals. Whatever factors contributed to the enhanced severity of duodenal damage when 1000R followed ara-C were, therefore, lessened when the dose of irradiation was reduced. Thus, the therapeutic ratio was much higher when 500R followed ara-C at four hours than when 1000R was administered four hours after ara-C.

When 1000R x-irradiation was administered 14, 16, 18, or 20 hours after the last ara-C injection, the effect was not as lethal to the animals as was 1000R four hours following ara-C. Thus, no more than 20% of the animals died in any of the four groups prior to 19 days. Furthermore, as assessed on a morphological basis, the damage to the duodenal mucosa was not as severe when irradiation was applied at longer intervals after ara-C, and also the severity of the damage appeared to be less with longer periods between the last injection of ara-C and irradiation. In terms of the duodenal cell kinetics, the indices were remarkably similar in all these groups, with the possible exception of the 18 hour series. In the latter

instant, the differences of the indices were indicative of slightly less mucosal damage than inflicted in the groups irradiated at either 14, 16, or 20 hours after ara-C. For example, the duodenal DI of the 18 hour group was normal, while those of the other groups were elevated, particularly on the first post-treatment day. The peak of the duodenal LI occurred at 18 hours following ara-C, hence the cohort of cells would be expected to be in the S phase at that time, 18 hours, but also at 16 hours and 20 hours, because of the total duration of the S phase of 7.5 hours. Some of the cells would likely be in late G_1 at 16 hours, while at 20 hours, some would be in mitosis or in early G_1. At 14 hours, some cells would be in late G_1, that is, blocked at the G_1-S junction. This was substantiated by an apparent decrease of the LI at this time. Many mammalian cells are radiosensitive while in G_1, particularly in late G_1, but not while in S. Moreover, Tolmach (1967) reported that ara-C, when administered before x-irradiation, enhanced killing of HeLa cells, and that cells in G_1 were killed to a larger degree than cells in S. On this basis, the extent of duodenal cell killing would be expected to be highest when irradiation was administered 14 hours following ara-C, and lowest when administered at 18 hours. Cell killing following irradiation 16 and 20 hours after ara-C would be slightly more than at 18 hours, but less than at 14 hours. The DI (as a measure of degree of cell kill) was indeed substantially lower when irradiation was administered at 18 hours than when it was administered at 14, 16, or 20 hours. However, a difference was not apparent in the DI's of the latter three groups. This might have been ascribable to the circumstance that the "cohort" of crypt cells of the 16 hours and 20 hours groups consisted again

of an asynchronous cell population in all phases of the cycle. The high rate of cell killing of G_1 cells, and low rate of S cells (as observed in this study) contrast the results of Tsubouchi and Matsuzawa (1974) who reported that pyknotic cell formation of G_1 duodenal cells was low following x-irradiation.

In the present investigation the LI's of all four treated groups were remarkably similar, as were the MI's of groups irradiated 14, 16 or 20 hours after the last ara-C injection. The MI's of these latter groups remained depressed over the three-day period, more so on the first than on subsequent days. The group irradiated 18 hours following ara-C did not fit this pattern, however, because the MI was lower only on the second post-treatment day. These observations are in agreement with previous reports of a short mitotic delay of cells irradiated while in G_1 or M, and a long delay of cells irradiated while in S or G_2 (Lajtha et al, 1955; Sinclair, 1967; Leeper et al, 1970 and 1972a; Muller et al, 1973).

The damage to the duodenal mucosa produced by ara-C alone was severe, and this was reflected by the morphology, the duodenal indices, as well as by the fact that one animal died shortly following the treatment. Equally severe damage was also observed following a single 1000R dose of x-irradiation. Lesher and Lesher (1970) reported likewise that 60% of mouse jejunal cells were destroyed by a single 1000R dose. It would hence be expected that the mere combination of ara-C and irradiation might cause a virtual depletion of the intestinal mucosa, regardless of whether there was any interaction between the two agents. This was clearly not the case, however, when assessed on a morphological basis or on an evaluation of

changes of the duodenal indices. Further, when the survival patterns of the animals were examined, there were just two "early deaths" in the groups irradiated at 14 and 16 hours after ara-C, one in the group irradiated at 18 hours, and none at all in the group irradiated at 20 hours. This cannot be explained on the basis of the synchrony alone, induced by the ara-C, because the synchronizing effect would be expected to be radioprotective at 16, 18, and 20 hours, when the cohort of cells was in the radioresistant S phase, but not at 14 hours, when the majority of cells would be in the G_1 phase, which is radiosensitive. It seems likely that the time interval between the last ara-C injection and the x-irradiation was more crucial, because the severity of damage was less with longer time intervals between the two treatments.

Both the LI and the MI of the B16 melanoma in general were drastically reduced over the three-day period of observation following all four treatment combinations. However, the LI of the group irradiated 14 hours after the last ara-C injection was lower than those of two other groups on the first post-treatment day and also less than those of all three other treatment groups on the second post-treatment day. The MI's of the 16, 18, and 20 hours groups were all remarkably similar, while that of the 14 hours group was slightly lower than those of the latter groups. Also, the DI of the 14 hours group was higher on the first day than that of any other treated group. It can be predicted that at 14 hours after the last ara-C injection, the cohort of cells would be in the early part of the G_2 phase, while by 16 hours the cohort would have moved into the M phase, into mid-G_1 at 18 hours, and at 20 hours it would be in late G_1. On this basis, the

maximal effect of the combination treatment would be expected to occur when animals were irradiated 16 hours following the last injection of ara-C, while the cell cohort would be in the M phase, the most radiosensitive phase of many mammalian cell types. However, although the intergroup differences were not substantial, the cell kinetic data nonetheless suggest that the greatest impact on cell proliferation, growth retardation and/or regression in the B16 melanoma cells occurred when the irradiation followed 14 hours after the ara-C. When "early death" animals were excluded from the calculations and the survival patterns, the MST's were: 51 days (14 hours group), 45 days (16 hours group), 49 days (18 hours group), and 42 days (20 hours group). Just the group irradiated at 20 hours did not survive appreciably longer than untreated animals. The other three treated groups did exhibit survival times surpassing those of untreated animals, but the differences in the MST's between the treatment groups themselves were insignificant.

Both the indices of the B16 melanoma and the survival patterns of the treated animals signified the most effective regimen to be irradiation 14 hours following the last ara-C injection. The treatments were progressively less effective when irradiation was administered 16, 18, or 20 hours after the last injection. Several factors might be responsible, either wholly or partly, for these observations. Firstly, because the cohort of cells was in G_2 at the time of the irradiation in the 14 hours group, G_2 could be the most radiosensitive phase of the cell cycle of the B16 melanoma. However, this contrasts the general concensus of opinion asserting the M phase to be the most radiosensitive with most mammalian

cells (Terasima and Tolmach, 1961, 1963a and b; Ludovici et al, 1961). The second factor is based on the interval between the last ara-C injection and the irradiation treatment. Two mechanisms could here be involved. The cytotoxic effect of both ara-C and irradiation is (at least partially) due to the effects of these agents on the DNA molecule and/or the enzymes essential for DNA replication. It is hence logical that if the effects of the two agents were additive, these combined effects would be greater when the two agents were administered within short succession rather than after an extended period of time, in which case some repair of damage produced by the first agent might have occurred. Thus, the more time elapsed between the last injection of ara-C and subsequent irradiation, the less enhancement of cell killing would be expected. Related to the duration of time between ara-C and irradiation is the degree at which synchrony is maintained. Gibson and Bertalanffy (1972) observed that the ara-C induced synchrony of the B16 melanoma cell population decayed quite rapidly. On this basis, the cell population at 14 hours after ara-C would be more homogeneous, with respect to cell cycle position, than the cell populations would be at 16, 18, or 20 hours. Moreover, a cell population composed to a large degree of G_2 cells would be more radiosensitive than an asynchronous cell population where the cells are in any of the phases of the cell cycle.

On the basis of the aforementioned cell kinetic and survival data, further experimentation was conducted with irradiation following 14 hours after the last ara-C injection. In one series, in an attempt to reduce the number of "early deaths", the dose of irradiation was reduced from 1000R to 500R. In another, the 1000R dose was fractionated to two equal doses of

500R, the first administered at 14 hours after the last ara-C injection, the second either one cell cycle time (at 28 hours) or two cell cycle times later (at 42 hours).

Although, at least on a morphological basis, duodenal damage following the single 500R dose appeared to be comparable to that of the single 1000R dose, both the LI's and the MI's were depressed to a higher degree following the larger than the smaller dosage. The DI's did not differ between the two groups. Moreover, two (of 11) "early deaths" occurred with the larger dose of irradiation, but only one (of 9) with 500R. Thus, the survival of these animals would support the cell kinetic data, signifying that less damage occurred when the irradiation dose was reduced, as was likely to be expected.

The indices of the B16 melanoma were comparable to those of the duodenum following the two doses of irradiation. The LI's and MI's of the animals receiving a 1000R dose were generally lower than those of animals administered the 500R dose. The DI's were again very similar. This indicates that the larger dose of irradiation (following ara-C) exerted a greater impact on the tumour cell kinetics than did the smaller dose. This contention was further supported by the survival data, because when "early death" animals were excluded from the calculations, the MST of the mice that received 1000R was longer than that which received only 500R.

When the 1000R dosage was fractionated to two 500R doses, administered either at 14 and 28 hours or at 14 and 42 hours after ara-C, the damage to the duodenal mucosa was more severe than after a single 500R dosage. Moreover, the damage was greater with the shorter interval

fraction regimen than with the regimen allowing the longer fraction interval, at least on the first two post-treatment days. This was corroborated by the duodenal indices, and also by the fact that there were two (of eight) "early deaths" in the 500R at 14 and 28 hours group, and none in the 500R at 14 and 42 hours series. As previously explained, one peak of the duodenal LI occurred 18 hours following the last ara-C injection and a second at 40 hours. Further, it was suspected that still another peak might have occurred at 29 hours, although this could not be substantiated from the available data. On this basis, irradiation at 14, 28, and 42 hours all corresponded to the times at which the cohort of cells would be in the S phase. That phase is considered to be radioresistant with most mammalian cell types, yet it may be radiosensitive with duodenal crypt cells (Tsubouchi and Matsuzawa, 1974). Depending on whether duodenal S cells are radioresistant or else radiosensitive, ara-C-induced synchrony could therefore provide either a radioprotective effect or else a radiosensitizing effect, particularly at the time the second dose of x-irradiation was administered. This hypothetical model does not account for the fact, however, that the first dose of irradiation (at 14 hours) might have altered the cell kinetic pattern of the crypt cells. Thus, several investigators noted that irradiation blocked cells in G_2, the duration of the block being dose-dependent (Lajtha et al, 1955; Sinclair, 1967; Leeper et al, 1970 and 1972a; Muller, 1973). Hence, a single dose of irradiation can cause partial synchronization of a cell population. If such an irradiation-induced synchrony is actually superimposed on that produced by ara-C, it would be virtually impossible to predict the eventual outcome, in terms of the

effects on the cell kinetics. For example, a G_2 block, with a subsequent mitotic delay, would lengthen the cell cycle duration, and the peaks of the LI would hence be expected to occur somewhat later than 29 hours and 40 hours. Alternatively, Lesher and Lesher (1974) reported that the cell cycle of intestinal crypt cells was shorter following irradiation. On that basis, the peaks would be expected to occur sooner than 29 and 40 hours. Moreover, if irradiation and ara-C-induced synchronies were additive, the subsequent peaks in the LI would have to be higher than those observed following ara-C alone.

Whatever the consequences of the combined effects, a greater degree of damage to the mucosa was produced by 500R administered at 14 and 28 hours than at 14 and 42 hours. Again, the longer time interval between the administration of the irradiation doses may have allowed for some repair of cell damage induced by the first dosage.

Both the LI's and MI's of the B16 melanoma were lower when a single dose (1000R) of irradiation followed ara-C than when the same dose, fractionated to two 500R dosages, followed ara-C. The DI's of all groups were similar. This indicated that the single dose was more effective in causing tumour growth retardation then either of the two regimens where fractionated doses were administered. When "early death" animals were excluded from the calculations, mice that received a single 1000R dose exhibited a MST of 51 days, compared to a MST of just 43 days of animals administered a single 500R dose at 14 hours and again at 28 hours, and a MST of 38 days for those receiving a 500R dose at 14 hours and again at 42 hours. This would be expected from the recovery which occurs likely

between the fractionated doses and the decay of ara-C-induced synchrony with time. Most probably the irradiation dose administered at 28 or 42 hours was not to a synchronized cell population, but likely by that time it had again become asynchronous.

The impact of a single dose of 1000R was compared to the effect of 8 i.p. injections of 12.5 mg/kg ara-C, either alone or followed, at 14, 16, or 18 hours, by a 1000R dose of x-irradiation. It was to ascertain whether the effect of ara-C and irradiation combined was greater than, less than, or equal to the effect of either agent alone.

All comparisons were made 24, 48, or 72 hours following cessation of treatment, and the effect of ara-C alone at these times, as determined by extrapolation, was compared to control values. Differences were not observed of any of the indices (either duodenal or tumour). All other comparisons between ara-C alone and the various combinations of ara-C and irradiation were made at identical times following cessation of treatment.

Comparison between the duodenal indices of the various treatment groups revealed that the individual groups were more similar than dissimilar, particularly on the second and third post-treatment days. Moreover, the indices indicated that ara-C was only slightly less injurious to the duodenal epithelium than irradiation alone or when administered in combination with ara-C. It was difficult to assess the differences between irradiated groups and groups which received irradiation after ara-C, as the trend of the differences was the same of all three indices. Thus, where differences occurred, the LI's, MI's, <u>and</u> DI's were all higher in groups receiving irradiation after ara-C. These cell kinetic data

correlated also with the survival data, as there was one "early death" in the ara-C group, none in the irradiated group, two each in the 14 hours and 16 hours groups, and one in the 18 hours group. The fact that one animal died shortly following treatment with ara-C alone indicates that some of the deaths which occurred in the combination treated groups might have been brought about by ara-C alone. The combination treatments were, therefore, not significantly more cytotoxic than ara-C itself.

Both the tumour LI's and MI's of animals that received only ara-C were generally higher than those of mice which received irradiation alone or were treated by a combination of irradiation and ara-C. The DI of the ara-C group was lower than that of animals which received the combination treatment but higher than that of animals just irradiated. This suggests that irradiation alone or in combination with ara-C caused a greater impact on cell proliferation (i.e., more retardation and/or regression of tumour growth) than ara-C alone. The MST's of the various groups were as follows: ara-C = 39 days, 1000R = 46 days, 1000R at 14 hours = 51 days, 1000R at 16 hours = 45 days, and 1000R at 18 hours = 48 days. Thus, the MST of animals irradiated 14 hours following ara-C was longer than that of control animals, and also of animals which received only ara-C. It was not longer than that of any of the other treatment groups, however.

There was, therefore, enhanced retardation of the growth of the B16 melanoma when irradiation was administered following ara-C when compared to the group treated solely with ara-C. The combination of ara-C with irradiation, however, was not more effective (in terms of the MST's of the animals) than was irradiation alone, although the change of the indices of

the tumour over a three-day period could indicate rather significant gains. This (apparent) dichotomy may be explained on the basis of time, as the significant gains (noted by changes in the indices) were observed only over the entire three-day period. It seems likely that, following both types of treatment, the tumour cell population must have recovered fairly quickly from the impact. The slightly shorter MST of animals which were just irradiated may be indicative of more rapid recovery of the tumour following irradiation alone than following irradiation after ara-C.

This study, in addition to solving some relevant problems, also raised some interesting questions, the answers to which, once elucidated, might be of value in planning therapeutic regimens aimed at tumour cure. Because the therapeutic regimen is often the determining factor in the selection of a particular treatment measure, the effect of ara-C alone on the cell kinetics of the duodenum administered over an extended period of time must be determined. The regimen of ara-C employed in the present study definitely produced synchrony of duodenal crypt cells, yet that synchrony did not coincide in time with the synchrony of the B16 melanoma cells. In addition, a peak in the duodenal LI was suspected to occur approximately 29 hours following the last ara-C administration, but the present data cannot substantiate its actual occurrence. Answers to these two concerns could be ascertained if duodenal indices (following the regimen on ara-C) were determined at two-hour intervals over three or four days, followed by similar determinations at somewhat longer intervals, as for a period of one to two weeks. The determination of such indices over the extended period would be necessary to disclose when duodenal indices

returned to, and remained at normal levels. This would be indicative of the time required for a complete recovery of the mucosa.

Both duodenal and tumour indices should be determined at the same intervals and over the identical period of time, as suggested above, following irradiation alone and irradiation at 14 hours after the last ara-C injection. This would reveal the magnitude and trend of change immediately following treatment, as also the duration of the effect of the agents on both duodenum and the tumour. On the basis of the present observations, a treatment regimen would be suggested where 8000R to 10,000R x-irradiation be administered in fractions of 1000R each, the fraction interval being dependent on the recovery in both the duodenum and the tumour. These observations could be compared to those obtained by the same dosage of irradiation, fractionated in the same manner, but with each dose of irradiation being preceded 14 hours earlier by a regimen of ara-C administration. It could thereby be determined whether the gain observed following a single course of treatment would be accumulated over several courses of treatment, thus resulting in actual tumour cure, rather than mere amelioration.

CHAPTER VI

CONCLUSIONS

The principal purpose of this investigation was to determine the effect of x-irradiation on the cell kinetics of duodenal and malignant B16 melanoma cell populations, synchronized by 8 i.p. injections of 12.5 mg/kg cytosine arabinoside (ara-C) administered at two-hour intervals. Pilot studies were necessary to determine the dosage of x-irradiation to be employed, and also to ascertain whether synchrony of tumour cells could be readily achieved according to the method devised by Gibson and Bertalanffy (1972). Based on the observations of the experimental series, the following conclusions seem justified:

1. Eight i.p. injections of 12.5 mg/kg ara-C administered at two-hour intervals produced synchrony of both the cell population of the B16 melanoma and of the C57Bl/6J mouse duodenal crypt cells.

2. The ara-C-induced synchrony occurred earlier and decayed more rapidly in the tumour cell population than it did in the duodenal cell population.

3. Changes in the duodenal indices, following ara-C, correlated well with the observed morphological damage.

4. The regimen of ara-C administration did not exert any effect on the MST of C57Bl/6J mice bearing the malignant B16 melanoma, but was lethal to approximately 10% of the experimental animals.

5. X-irradiation with 500R, 1000R, 1500R, or 500R x 2 (24 hours apart) caused damage to the duodenal mucosa, the severity and duration of which were dose-dependent.

6. Changes in the duodenal indices following x-irradiation were indicative of a general damage but did not reflect a dose-dependency.

7. Changes of the indices of the B16 melanoma following x-irradiation were indicative of retardation of tumour growth and/or tumour regression. These changes were not dose-dependent.

8. The MST of irradiated mice appeared to be dose-dependent. Doses of 500R x 2, 1000R, and 1500R all increased the MST of mice bearing the tumour.

9. The moderately pigmented B16 melanoma, employed in the present investigation, cannot be considered to be a radioresistant tumour.

10. A 1000R dose of x-irradiation four hours following the last injection of ara-C was lethal to 75% of the animals, and such a treatment could not be considered for therapeutic purposes.

11. A 500R dose of x-irradiation four hours following the last injection of ara-C remained relatively ineffective in retarding the growth of the B16 melanoma.

12. The degree of damage to the duodenal mucosa augmented as the time between ara-C administration and irradiation was shortened.

13. In terms of retardation of tumour growth or regression of the tumour, a 1000R dose of x-irradiation 14 hours following ara-C proved to be the most effective regimen, although 1000R at 16 hours or at 18 hours were also effective treatments.

14. Generally, fractionation of an irradiation dose caused less duodenal damage than the identical dose administered by a single exposure, but the therapeutic gain was offset by less retardation or regression of the tumour.

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