Age Responses of Cultured Mammalian Cells to Cytotoxic Drugs

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SUMMARY

A number of cytotoxic drugs (mitotic poisons, chemical mutagens, bifunctional alkylating agents, inhibitors of DNA, RNA, or protein synthesis, for a total of 18 agents) have been tested for differences in their lethal effects through the generation cycle of HeLa and Chinese hamster cells in culture. The patterns of age response of cells to each group of agents show some features in common: mitotic poisons, chemical mutagens, and inhibitors of DNA synthesis appear to be most effective on cells in S phase, alkylating agents in M and G1, and inhibitors of protein synthesis at the G1/S transition, whereas inhibitors of RNA synthesis elicit an X-ray-like age response, i.e., show greatest activity on cells in M and at the G1/S transition. Further differences in lethal action have been defined by microscopic observations of the treated cells; these permit additional distinctions to be made between groups of agents. In the case of asynchronous cell populations, all the agents but 3 give rise to sigmoidal concentration-survival curves (an initial shoulder followed by an exponential decline). The exceptions are nitrogen mustard, which elicits an exponential curve, and hydroxyurea and pederine, which give rise to exponential curves followed by a plateau at about 45% survival level.

INTRODUCTION

While there is considerable knowledge of the biochemical modes of action of a number of the cytotoxic drugs presently used in cancer chemotherapy, the state of knowledge regarding the mechanisms by which these agents exert their lethal effects is meager. The known biochemical effects may or may not be unique, and may or may not be related to their lethal effect. The latter is clearly pertinent to their use in cancer chemotherapy; accordingly, it is necessary to investigate the action of such drugs at the cellular as well as at the biochemical level. Recently, Bruce et al. (11, 12) classified a number of chemotherapeutic agents on the basis of a comparison of their lethal effect on actively dividing murine lymphoma cells with that on predominantly nondividing normal bone marrow cells. These authors postulated that agents placed in Class 2 of their classification system were "phase specific," that is, that they exert their lethal effect mainly on cells at a specific phase of the cell generation cycle. While variations in the sensitivity (defined in terms of loss of colony-forming ability) of cultured mammalian cells through the division cycle to X-rays are well established (for a review, see Ref. 54), the determination of such age responses of cells to chemical agents is only at a beginning; the state of this field of investigation has recently been summarized (36).

There are 2 main reasons for studying the age responses of cells to lethal agents. First, our knowledge of the biochemical events which take place in the various phases of the division cycle is increasing; if cells are shown to be particularly sensitive to a given agent during one phase of the cycle, such a finding may delineate the possible biochemical processes, interference with which leads to cell death. Conversely, if a well-defined biochemical action of a drug is already established, then the finding that cells are sensitive in lethal terms to that drug at a given phase of the cycle may point to the occurrence of a particular biochemical event in that phase. Second, even without an increase in our understanding of the lethal action of drugs in biochemical terms, the determination of age responses should allow an improved rationalization of chemotherapy. For example, with regard to combination drug therapy, it would be possible to choose a combination in which each drug exerts its lethal action primarily on a different phase of the cell cycle so that, if the drugs were administered simultaneously, the lethal effect on an asynchronous population of dividing cells would be maximized. If drugs in combination were administered separately over a given period of time, then the temporal sequence could be chosen so that an individual drug is given at a time at which a partially synchronized cell population would be at a phase in the cycle sensitive to that drug. For this argument, it was necessary to demonstrate that tumor cells in vivo exhibit age responses to lethal agents similar to those of mammalian cells in vitro. This has recently been done with a murine lymphoma cell line with respect to both X-rays and certain cytotoxic drugs (42).

This communication describes the age responses of HeLa S3 and of Chinese hamster V79 cells—established cell lines widely used in cell culture studies—to a variety of cytotoxic drugs, chosen to represent a broad

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MATERIALS AND METHODS

Monolayer cultures of HeLa S3 cells were grown in Medium N16 supplemented with a 1:2 mixture of horse and human serum to yield a final serum concentration of 30%, v/v (27). Synchronous populations were obtained by harvesting mitotic cells after the method of Terasima and Tolmach (56). Following this procedure, the cells rapidly reattach to the surface of culture dishes with a resultant multiplicity of about 2 cells/microcolony at the time of treatment. The median interdivision time of cells was usually about 21 hr; in 1 set of experiments reported here, it was decreased to 19 hr.

Monolayer cultures of Chinese hamster cells, Subline V79-753B-SL1, were grown in medium HUT, supplemented with fetal calf serum at a concentration of 15% (18, 20). Exponentially growing cells were synchronized by a pretreatment exposure of 3 to 4 hr to 2.5 mM hydroxyurea (19, 21, 38) which selectively kills cells in the S phase (52, 53). Following this treatment with hydroxyurea, the surviving cell population comprises cells mainly in the G1 phase together with some cells in G2 and M (for a more complete discussion of this synchronization method, see Ref. 42). The cell multiplicity after overnight growth and before exposure to hydroxyurea is about 3; the residual multiplicity at the time of treatment is uncertain (see Ref. 19 for discussion of this question). The doubling time of the Chinese hamster cells is 8 to 9 hr.

The drugs the action of which was investigated are listed in Table 1, together with their molecular weights and their source. The subdivision into various classes is made for convenience and in no way implies that a given drug has only one biochemical mode of action or that the biochemical mode of action listed is necessarily related to its lethal action on cells. All the drugs, except actinomycin D, nitrogen and sulfur mustards, and nitrous acid, were dissolved in distilled water and stored in the frozen state. These stock solutions were diluted with cold 0.9% NaCl solution (Puck’s D1 for treatment of HeLa; Dulbecco’s NaCl solution for Chinese hamster cells) just before the start of an experiment and maintained cold (4°C) up to the time of administration. At set times after collection and plating of mitotic HeLa cells or after removal of hydroxyurea from Chinese hamster cells, 0.5 ml drug solution was added to each culture dish (60-mm size, Falcon Plastics, Oxnard, Calif.) containing 4.5 ml medium to yield the appropriate drug concentration. The activity of a drug (e.g., hydroxyurea, vinblastine, or sul-
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fur mustard; see also Ref. 41) may strongly be affected by the particular medium and/or serum supplement in which tests are performed. During the exposure, the cells were incubated at 37° in an atmosphere containing 5% CO₂. After a given time of exposure (3 hr in most cases; see Table 2), the medium was removed by gentle

Chart 1. Age response of HeLa cells to 0.05 μg/ml colchicine (△ — △), 0.3 μg/ml vinblastine (○ — ○), or 0.1 μg/ml vincristine (□ — □). Duration of exposure was 3 hr. Abscissa, times at which exposure began after collection of mitotic cells; box (top), approximate distribution of cells in different phases of the mitotic cycle.

Chart 2. A, age response of Chinese hamster cells to 0.05 μg/ml colchicine (○), 25 μg/ml demecolcine (□), or 25 μg/ml griseofulvin (●). The age response to 750 rads 220-kV X-radiation (■ — ■) is also given for comparison. Duration of drug exposure was 3 hr. Δ (in upper half of the first decade), survival to 2.5 mM hydroxyurea. Abscissa, times at which the exposure began after removal of hydroxyurea; box (top), approximate distribution of cells in different phases of the mitotic cycle. B, age response of Chinese hamster cells to 0.3 μg/ml vinblastine (● — ●) or 0.1 μg/ml vincristine (○ — ○). Conditions of treatment were as described in A.

Chart 3. Age response of HeLa cells to 300 μg/ml hydroxylamine (○ — ○) or 5 μg/ml nitrous acid (□ — □). Duration of exposure was 3 or 0.5 hr, respectively. Abscissa, time at which exposure began after collection of mitotic cells; box (top), approximate distribution of the cells in different phases of the mitotic cycle.

Chart 4. Age response of HeLa cells to 0.5 μg/ml sulfur mustard (○ — ○), 0.5 μg/ml nitrogen mustard (□ — □), or 7.5 μg/ml uracil mustard (▲ — ▲). Duration of exposure was 10 min for sulfur mustard and 0.5 hr for nitrogen and uracil mustard. Abscissa, times at which exposure began after collection of mitotic cells; box (top) approximate distribution of cells in different phases of the mitotic cycle.

aspiration, the dishes were washed once with the appropriate NaCl solution, and fresh medium was added. In the instance of nitrous acid, the stock solution was prepared by dissolving potassium nitrite in acetate buffer at pH 4.0, and diluting, prior to the experiments, with the same cold buffer. The treatment was then performed by removing all medium from the dishes and adding 5 ml nitrous acid solution at the desired concentration. Control experiments with acetate buffer at pH 4.0 (no drug) indicated that cell viability is not affected by treatments of less than 0.5 hr (which is the length of exposure used for this agent), although cell progression through the cycle is temporarily arrested.
Sulfur mustard and actinomycin D were dissolved in absolute ethanol, nitrogen mustard was dissolved in 0.01 N HCl, and the solutions were stored frozen. Immediately prior to each experiment, nitrogen mustard and sulfur mustard were further diluted in cold NaCl solution so that 0.5 ml could be added to each dish to give the required final concentration. The diluted stocks were kept at ice temperature and usually discarded after 1 hr. In the case of sulfur mustard, at the end of the desired exposure, the medium was removed and replaced directly with fresh medium, no washes being necessary since the drug has an extremely short chemical half-life in aqueous solution at room temperature or 37° (41).

Stock solutions of actinomycin D were diluted in NaCl solution prior to each experiment and then further diluted in culture medium. Treatment was effected by replacing the medium in a dish with medium containing the drug. After an exposure of 30-min duration, the medium was removed, the cells were washed once with NaCl solution, and fresh medium was added. One hr later, this washing procedure was repeated to diminish as much as possible residual action of this drug (21). Since actinomycin D and nogalamycin are sensitive to visible light, all operations with these drugs were performed under a dim, red light. With both sulfur mustard and actinomycin D, the final alcohol concentration (below 1%) was too low to affect cell survival.

Cell survival was determined by counting the macroscopic colonies in the dishes, in duplicate or triplicate, following the required length of incubation (10 to 11 days for HeLa; 8 to 9 days for Chinese hamster cells) after the method of Puck et al. (48). Plating efficiencies lay between 75 and 100% for HeLa cells and between 85 and 100% for Chinese hamster cells.

RESULTS

In all age-response experiments described below, the drug concentrations and the lengths of exposure were chosen on the basis of preliminary experiments in order to present a well-defined age response and to overcome as much as possible any threshold observed when the surviving fraction was followed as a function of duration of exposure to a given drug concentration.

In all the charts depicting the results of age response experiments, the surviving fraction is plotted against the time at which drug exposure commenced, given in hr after collection of mitotic cells (HeLa) or hr after the end

Chart 5. Age response of HeLa cells to 10 mM hydroxyurea (O) or 0.1 µg/ml pederine (Δ). Duration of exposure was 3 hr. Abscissa, times at which exposure began after collection of mitotic cells; box (top), approximate distribution of cells in different phases of the mitotic cycle.

Chart 6. Age response of HeLa cells to 0.14 µg/ml actinomycin D (O) or 1.0 µg/ml nogalamycin (Δ). The age response to 750 rads 220-kV X-radiation (□ --- □) is also given for comparison. Duration of drug exposure was 0.5 and 3 hr respectively. Abscissa, times at which exposure began after the collection of mitotic cells; box (top), approximate distribution of cells in different phases of the mitotic cycle.

Chart 7. Age response of Chinese hamster cells to 1.0 µg/ml nogalamycin (O --- O). The age response to 750 rads 220-kV X-radiation (□ --- □) is also given for comparison. Duration of drug exposure was 1 hr. △ --- △ (in upper half of the first decade), survival to 2.5 mM hydroxyurea. Abscissa, times at which the exposure began after removal of hydroxyurea. The approximate distribution of cells in different phases of the mitotic cycle is indicated in Chart 2.
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Age Responses to Mitotic Poisons. The age response of HeLa cells exposed to colchicine (0.05 μg/ml) for a period of 3 hr is shown in Chart 1 (triangles). For ease of comparison, Chart 1 includes the age response curves of HeLa cells exposed for 3 hr to vinblastine (0.3 μg/ml; circles) or vincristine (0.1 μg/ml; squares) taken from previous work (36). In terms of molarity, the concentrations of colchicine and vincristine are almost the same, while that of vinblastine is 3 times higher. These concentrations for all 3 drugs are about 10 times those required to produce arrest of treated cells at or close to mitosis (13, 33, 46). As in the case of vincristine, colchicine is most effective as a lethal agent on HeLa cells 9 to 17 hr after collection; that is, cells in the S phase are most sensitive to the drug. G1 cells do not exhibit any sensitivity to colchicine (or to vincristine) as they do to vinblastine (higher concentrations of colchicine or vincristine did not reveal any fine structure in the G1 portion of the age-response curve; results not shown). In contrast to their sensitivity to vinblastine, mitotic cells are relatively resistant to discrete 3-hr exposures to colchicine, as they are to vincristine (unpublished results).

Two other mitotic poisons, demecolcine (Colcemid) and griseofulvin, were tested for their lethal effect on HeLa cells, but no loss of cell survival was observed in either case following 3 to 6 hr exposures at concentrations up to 25 μg/ml.

The age response curves for Chinese hamster cells exposed for 3 hr to colchicine (0.05 μg/ml), demecolcine (25 μg/ml), or griseofulvin (25 μg/ml) are shown in Chart 2A. This chart also includes an X-ray (750 rad) age response as a check of synchrony (it is well known that the peak in resistance to X-rays occurs in the latter part of the S phase; see Ref. 54). For ease of comparison, the age responses of the Chinese hamster cells to vincristine and vinblastine, taken from previous work (36), are shown in Chart 2B. Apart from differences in concentration, all 3 drugs elicit an age response similar to that of vincristine; that is, cells show maximal sensitivity in the latter part of the S phase.

Age Responses to Chemical Mutagens. The age response of HeLa cells exposed for a period of 3 hr to hydroxylamine (300 μg/ml) or for 30 min to nitrous acid (5 μg/ml) are shown in Chart 3. The short treatment time was chosen for the latter agent because nitrous acid is used at pH = 4.0 (see “Materials and Methods”) and longer exposure of the cells at this pH in the absence of any agent would affect their viability. In any case, pre-
Table 2
Summary of survival data for various agents (HeLa cells)
The treatments were carried out on cells in exponential phase of growth. The multiplicity was 1.5 to 1.8 cells/microcolony.

<table>
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<tr>
<th>Agent</th>
<th>Exposure (hr)</th>
<th>D_{10} g/ml</th>
<th>D_{0} g/ml</th>
<th>n</th>
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<tr>
<td></td>
<td></td>
<td>m</td>
<td>m</td>
<td></td>
</tr>
<tr>
<td>Mitotic poisons</td>
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<tr>
<td>Vinblastine sulfate</td>
<td>3</td>
<td>2.7 x 10^{-7}</td>
<td>2.9 x 10^{-7}</td>
<td>8.5 x 10^{-8}</td>
</tr>
<tr>
<td>Vincristine sulfate</td>
<td>3</td>
<td>6.5 x 10^{-4}</td>
<td>7.1 x 10^{-4}</td>
<td>2.8 x 10^{-8}</td>
</tr>
<tr>
<td>Colchicine</td>
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<td>1.8 x 10^{-4}</td>
<td>2.0 x 10^{-8}</td>
</tr>
<tr>
<td>Demecolcin</td>
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<td>5.6 x 10^{-4}</td>
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<td>Griseofulvin</td>
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<td>Hydroxylamine HCl</td>
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<td>4.4 x 10^{-4}</td>
<td>6.3 x 10^{-3}</td>
<td>1.1 x 10^{-4}</td>
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<td>Potassium nitrite</td>
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<td>Bifunctional alkylating agents</td>
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<td>Nitrogen mustard</td>
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<td>7.2 x 10^{-7}</td>
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<tr>
<td>Uracil mustard</td>
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<td>7.3 x 10^{-4}</td>
<td>2.9 x 10^{-4}</td>
<td>1.7 x 10^{-4}</td>
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<td>Inhibitors of DNA synthesis</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>0.5</td>
<td>1.8 x 10^{-2}</td>
<td>1.4 x 10^{-7}</td>
<td>1.2 x 10^{-7}</td>
</tr>
<tr>
<td>Nogalamycin</td>
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<td>2.0 x 10^{-4}</td>
<td>2.7 x 10^{-4}</td>
<td>5.5 x 10^{-7}</td>
</tr>
<tr>
<td>Inhibitors of RNA synthesis</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycloheximide</td>
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<td>2.3 x 10^{-3}</td>
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<tr>
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<td>Streptovitacin A</td>
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<tr>
<td>Others</td>
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<tr>
<td>Phleomycin</td>
<td>3</td>
<td>5.3 x 10^{-7}</td>
<td></td>
<td>1.1 x 10^{-7}</td>
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</table>

Chart 11. Dose-survival curves for asynchronous HeLa cells exposed to colchicine (O), pederine (D), or cycloheximide (△). Duration of exposure was 3 hr.

Preliminary experiments indicated that the maximum biological effect, as measured in terms of cell killing, is achieved by about 20 min.) Chart 3 shows that these 2 chemical mutagens are most lethal to cells in the S phase. The great difference between the molar concentrations of the 2 drugs necessary to produce a lethal effect may reflect differences in stability as well as chemical reactivity.

The age responses of Chinese hamster cells to these 2 agents is essentially the same as that shown for HeLa cells; that is, a most pronounced effect on S phase cells (results not shown).

Age Responses to Alkylating Agents. The age responses of HeLa cells to nitrogen mustard (0.5 μg/ml; 30-min exposure), sulfur mustard (0.5 μg/ml; 10-min exposure), or uracil mustard (7.5 μg/ml; 30-min exposure) are shown in Chart 4. The short exposure time to sulfur mustard follows from the short half-life of this drug in aqueous solution (41). Cells in S are relatively resistant to the lethal action of all 3 agents. As cells pass through G2 to M, they become sensitive to all 3 drugs. Further experiments, in which HeLa cells were synchronized by combined treatment with hydroxyurea and cycloheximide (yielding a surviving cell population mainly in G2; unpublished data), showed that the minimum in survival seen between 21 and 22 hr after collection in Chart 4 is mainly due to the sensitivity of mitotic cells to these 3 agents, and that cells in the early part of G2 are still relatively resistant (results not shown). Chart 4 also shows that cells at the G1/S transition are relatively sensitive to nitrogen and uracil mustards but not to sulfur mustard.

"The term “G1/S transition” used throughout the paper and in Table 3, refers to populations of cells distributed in the late part of G1 and the early part of S phase, and does not imply the definition of specific border regions (similarly, "S/G2 transition").
These results are in accord with the previously reported age responses of Chinese hamster V79 cells (Ref. 38; K. Sakamoto and M. M. Elkind, personal communication) (allowing for the short G1 phase of Chinese hamster cells compared with that of HeLa cells) and of mouse L-cells (57, 58) to bifunctional alkylating agents. There are similarities between the age response to alkylating agents and to X-rays, but the resistance extends to later times in the cycle in the case of the alkylating agents (see also Refs. 38 and 58). Greater similarities are observed between the age response to inhibitors of RNA synthesis and to X-rays (see below).

Age Responses to Inhibitors of DNA Synthesis. The age response of HeLa cells exposed for 3-hr periods to either hydroxyurea (10 mM) or pederine (0.1 μg/ml) are shown in Chart 5. Both of these drugs are known to inhibit DNA synthesis of cultured mammalian cells (Refs. 31, 45, 52, 53; pederine seems to block both DNA and protein synthesis without affecting RNA synthesis; see Ref. 10). The minimum in cell survival corresponds to the peak in the rate of DNA synthesis. The age response to hydroxyurea seen in Chart 5 is in excellent agreement with that described previously for HeLa cells (31) and is similar to the well-known age response of Chinese hamster cells to hydroxyurea (52, 53). However, the concentration of hydroxyurea required to kill S phase HeLa cells (10 mM) is 4 times that required to elicit a similar effect on Chinese hamster cells (2.5 mM; for instance, see Ref. 53).

Age Responses to Inhibitors of RNA Synthesis. The age response of HeLa cells exposed either to actinomycin D (0.14 μg/ml) for 30 min (the special washing procedure used for this drug has been already described in "Materials and Methods") or to nogalamycin (1 μg/ml) for 3 hr is shown in Chart 6. Both of these drugs are known to inhibit DNA-dependent RNA synthesis (8, 49). For ease of comparison, included in Chart 6 is the age response of HeLa cells to a single dose of 400-rad X-radiation. It is clear that the age response of HeLa cells to either of these drugs is similar to that of these cells to X-radiation. The cells are most sensitive at the G1/S transition, while cells in early G1 and in the latter part of S are relatively resistant to these agents. It has previously been shown that the age response of Chinese hamster cells to actinomycin D is similar to the X-ray age response of Chinese hamster cells and, allowing for a short G1 phase, the age responses of HeLa and Chinese hamster cells to actinomycin D or X-rays are similar (19). (While the age response of mouse L-cells to X-rays differs from that of HeLa or Chinese hamster cells, it has recently been reported that the age response of mouse L-cells to actinomycin D is also X-ray-like; see Ref. 3.) The age response of Chinese hamster cells to nogalamycin is also X-ray-like, as shown in Chart 7. The similarity of the age responses to X-radiation and to inhibitors of RNA synthesis is of interest and may be related to the fact that damage produced by actinomycin D is capable of interacting with X-ray damage at least through part of the mitotic cycle (19).

Age Responses to Inhibitors of Protein Synthesis. The age response of HeLa cells exposed either to puromycin, cycloheximide, or streptovitacin A at a concentration of 100 μg/ml for a period of 3 hr in each case is shown in Chart 8. These 3 drugs are known to inhibit protein synthesis (6, 7, 14, 23, 59). All 3 drugs elicit an age response characterized by a maximal sensitivity of cells at the G1/S transition, cells in other parts of the cycle being relatively resistant. No age response of Chinese hamster cells to these inhibitors of protein synthesis is shown because these cells have such a short G1 phase and hence, following synchronization with hydroxyurea, only the brief ascending limbs of the age responses as cells enter S were observed. However, these unpublished results are in accord with the age responses of HeLa cells shown above.

Age Response to Phleomycin. The age response of HeLa cells to this drug, which has been reported to prevent cell division and to inhibit RNA synthesis, possibly in an independent way (29), was also obtained (5.0 μg/ml, 3-hr exposure). The results are not shown, as they are similar to those already reported for HeLa cells by other authors (16). The cells exhibit greater sensitivity in G1, which is the phase in which they are also most sensitive in terms of arrest in progression (29), and possibly also in mid-G1, as observed in the case of vinblastine (36).

Effect of Drug Treatment on Progression of Cells to Mitosis. Since it has been shown that HeLa cells given a single moderate dose of X-radiation may undergo any of a number of morphologically distinct modes of death (28), it was of interest to investigate the effect of interphase treatment with a number of drugs on the progression of cells to mitosis. In particular, since actinomycin D is in many ways "radiomimetic" (see, for example, the age response to actinomycin D in Chart 6 above and, for a discussion of the subject, Ref. 19), it was of interest to...
see whether this drug is radiomimetic in respect to the mode of cell death. Accordingly, synchronous populations of HeLa cells were exposed to a drug for discrete intervals of time at stages in the mitotic cycle in which the cells are most sensitive in each case. The drugs, chosen to represent the different categories listed in Table 1, were: colchicine, actinomycin D, hydroxyurea, hydroxylamine, and sulfur mustard (similar observations for vincristine and vincristine have already been published; see Ref. 36). Following drug treatment, the total number of cells in selected fields and the percentage of cells rounded in mitosis were monitored by repeated microscopic observation of representative fields (37). The results are shown in Charts 9 and 10. The duration and the time in the mitotic cycle of drug exposures are given in the chart legends. As expected from previous work with vinblastine and vincristine (36), following treatment with colchicine in the S phase, the cells proceed at the normal rate to round up at mitosis and there accumulate in the rounded state (Chart 9) with no increase in total cell number (Chart 10). Following treatment with actinomycin D at the G1/S transition, with hydroxyurea or hydroxylamine in the S phase, or with sulfur mustard in early G1, the cells remain constant in number up to 26 hr after collection of mitotic cells but fail to round up at mitosis. Further observations by size analysis (data not presented) indicate that the cell sizes continue to increase for at least another day before the cells undergo lysis at some later time (a similar observation has already been reported for Chinese hamster cells exposed to sulfur mustard; see Ref. 40). None of these 4 drugs induces either mitotic death or interphase cell lysis within 48 hr, and the cells do not divide. The cells might undergo an extended interphase death. In this respect, the lethal action of actinomycin D on HeLa cells differs from that of X-radiation. Following a dose of X-rays which, in terms of surviving fraction is comparable with that of actinomycin D in this study, the majority of cells undergo mitotic death in the 2nd and subsequent generations following treatment (28).

**Dose-Survival Curves for Asynchronous Cell Populations.** In addition to the age-response studies, dose-survival curves for HeLa cells treated with each of the various agents listed in Table 1 were determined. The results are summarized in Table 2 which includes the values of $D_{10}$ (here defined as the dose required to reduce the surviving fraction to a value of 0.1, independently from the shape of the curve), $D_5$ (a parameter describing the final slope; see Ref. 2), and $n$ (extrapolation number; see Ref. 2). Representative results are illustrated by 6 survival curves shown in Charts 11 and 12. With the exception of inhibitors of DNA synthesis (hydroxyurea and pederine), and of nitrogen mustard (when the data are corrected for cell multiplicity), in each case a "type C" (1, 2) dose-survival curve was obtained; that is, on a semilog plot, a shoulder precedes an exponential component of the survival curve. It is of interest that such a variety of chemical lethal agents should elicit dose-survival curves similar in form to those produced by radiation. Both pederine (Chart 11) and hydroxyurea yield survival curves typified by a plateau at a surviving fraction of about 0.45, a result consistent with the hypothesis that essentially only S phase cells are killed by these 2 agents under the conditions of these experiments.

**DISCUSSION**

Several conclusions may be drawn from the results of these experiments.

Two established mammalian cell lines, when exposed to a wide variety of drugs representing various chemical species known to have different biochemical action, exhibit well-defined, distinctive age responses through the generation cycle, as they do to ionizing and ultraviolet radiation. In the instance of 4 drugs (vinblastine, vincristine, sulfur mustard, and actinomycin D), these fluctuations in survival have been shown already (by us and others; Refs. 19, 36, 38) to be characterized by changes in both slope and shoulder width of the survival curve. This may well be the case for the majority of agents. In general, the concentrations of the drugs required to produce a lethal effect are remarkably similar for both cell lines. The chief exceptions to this generalization are demecolcine and griseofulvin, to the lethal action of which HeLa cells are relatively resistant as compared with Chinese hamster cells. The main difference between the age responses of the 2 cell lines are those due to the diminutive G1 phase of Chinese hamster V79 cells.

The age-response pattern (Table 3) appears to be characteristic for a particular group of drugs, in spite of the fact that, as stated above, the classification has been made on traditional, rather arbitrary grounds. (The results of the age-response studies summarized in Table 3 include the work of other authors to be found in the literature to date.) All the mitotic poisons, chemical mutagens, and the inhibitors of DNA synthesis tested exert their chief lethal action on S phase cells. In the case of inhibitors of DNA synthesis, it is likely that only S phase cells are lethally sensitive, as suggested by the dose-survival curves for asynchronous cell populations (Table 2 and Chart 11). Of course, it does not follow that the biochemical mechanisms leading to the lethal results are the same for these 3 classes of agent. While it is reasonable to suppose that the lethal effect of inhibitors of DNA synthesis on S phase cells is causally related to the inhibition of DNA synthesis, and similarly that the lethal effect of chemical mutagens is causally related to their interaction with DNA, these assumptions have not been proved, and in the case of the mitotic poisons it is quite clear that their lethal effect on S phase cells may not be related to DNA synthesis per se. We have shown that treatment of HeLa cells in the S phase with either vinblastine, vincristine, or colchicine under the conditions described above (when more than 90% of the cells are killed) has no effect whatsoever on the rate of incorporation of thymidine-14C by the cells (unpublished results).
The progression to mitosis of cells so treated is also unaffected (see Chart 11 for colchicine, and Ref. 36 for vinblastine and vincristine). The hypothesis that mitotic poisons act by binding to an organelle synthesized during the S phase, functional at mitosis (for example, the centrioles which are synthesized during the S phase), has been proposed (36). It is also possible that lethal action and mitotic inhibition are entirely unrelated.

Exactly how inhibition of DNA synthesis results in a lethal effect is not known. In the case of hydroxyurea and other DNA synthesis inhibitors (not examined in this study but which would be expected to elicit an age response similar to that of hydroxyurea or pederine), it has been proposed that a state of unbalanced growth ensues which may be similar to the thymineless death observed in bacteria (50, 53). However, a recent report has indicated that fluorodeoxyuridine (in contrast to hydroxyurea and pederine) is equally effective in G1, S, and G2, mitotic cells being the only ones to show differential resistance (35). Therefore, other factors may be playing a role in the determination of cellular response to this group of agents. Alternatively, fluorodeoxyuridine may undergo a metabolic conversion to a derivative which has an action other than that of an inhibitor of DNA synthesis.

The lethal action of hydroxyurea and pederine differs from that of other agents because of the extended threshold of the concentration-survival curve in the resistant regions. Cells in the sensitive region of the cycle (S phase) are killed by exposure to an appropriate concent-
Concentration of the drug, whereas cells in resistant regions (e.g., G1 phase) are not lethally affected at all even by relatively higher concentrations (unpublished data). This characteristic is reflected also in the shape of the survival curve for asynchronous populations (Chart 11), which shows a terminal plateau.

Regarding the chemical mutagens, the mutagenic action of nitrosoureas may be due to deamination of DNA bases resulting in the induction of base-pair replacement (24) or to the unwinding of the deoxyribose phosphate backbone resulting in chain damage (32) (or both). Hydroxylamine is believed to interact preferentially with the cytosine bases of DNA (24) and may also cause chain scission in DNA (9). It seems reasonable to suppose that, for such damage to be extensive, the DNA molecules might be most susceptible to these agents at the moment and point of replication in the S phase at which the strands are uncoiled.

The age response of cells to alkylating agents appears to be characterized by a relative sensitivity at or around mitosis (all bifunctional alkylating agents in Table 3) and, in the case of nitrogen and uracil mustards, an additional sensitivity at the G1/S transition. It is not known whether the condensed state of the chromosomes at mitosis should make them vulnerable to alkylating agents. However, regarding the 2 instances of sensitivity at the G1/S transition, there is some evidence that these agents interact with DNA at or just before replication (25) and also that they may interact with DNA precursor molecules such as thymidylate (34, 51).

The inhibitors of RNA and protein synthesis characteristically elicit an age response typified by a marked relative sensitivity of cells at the G1/S transition. The reason for this is not known but it is interesting to note that at this point in the cycle the rate of RNA synthesis sharply increases by a factor of 2 in both HeLa cells (47) and in another line of Chinese hamster (CHO) cells (55).

It should be pointed out that this discussion of age-response patterns rests upon the assumption that, when exposure to a given drug is terminated by washing the cells and adding fresh medium, the drug is entirely removed. If a small quantity of a drug were not entirely washed away, or if the drug were retained within an intracellular pool, then when the cells progressed to a key point in the cycle they might still, in effect, be exposed to a residual quantity of the drug. Further studies utilizing labeled chemical agents are necessary to explore this possibility. However, in those cases in which the chemical half-life of the drug is short (for example, sulfur mustard and nitrogen acid), this consideration does not apply. Again, it should be stressed that the attempt to account for the lethal action of a drug in terms of its known biochemical action must take into consideration the fact that frequently the drug concentration required to induce a lethal effect is much greater than that required to produce a given biochemical or cellular response. For example, the concentration of vinblastine, vincristine, or colchicine required to induce a lethal effect following a discrete exposure is 10 times that required to arrest cells in mitosis when the drug is present as the cells reach mitosis (13, 33, 46). Similarly, the concentration of hydroxyurea (10 mM) required to kill S phase HeLa cells is 10 times that required to inhibit DNA synthesis almost totally by HeLa cells under the same growth conditions (45).

An example of the strict dependence of the lethal action of a cytotoxic agent upon the detailed molecular structure of the agent has already been discussed in the case of vinblastine and vincristine (36). The difference between the lethal effectiveness of colchicine and demecolcin described above affords a further sample. The sole difference between the molecular structure of these 2 mitotic poisons is that the acetylamido group of colchicine is replaced by a methylamino group in demecolcin (25). This one alteration in molecular structure results in a marked difference in lethal effectiveness which is particularly pronounced for HeLa cells.

The finding that the various chemical cytotoxic agents elicit such varied patterns of age response should, theoretically, yield important applications in cancer chemotherapy. For example, when a choice of agents to be used in combination is made, that choice should consider the possibility of maximizing the lethal effect by administering agents each of which are most effective on different phases of the cell cycle. If the various agents are given at different times, then the synchronizing action of the first agent administered should be considered [e.g., as in the case of hydroxyurea, which has been demonstrated to synchronize cycling cell populations both in vitro (52, 53) and in vivo (42)]. Of course, such theoretical considerations are difficult to apply if the cycle parameters of the tumor cells in question and of the normal cells of cell renewal systems are unknown. Furthermore, differences may exist between various cell lines and between in vitro and in vivo systems. However, studies underway in our laboratory on mouse lymphoma cells (42) indicate that proliferating tumor cells exhibit radiation and drug age responses similar to those of cultured cells.

Finally, the finding that most of the agents yield dose-survival curves of the sigmoid type (that is, characterized by a shoulder region preceding the exponential component of the survival curve) is potentially of great interest. Interpretation of sigmoidal response in terms of a detailed theoretical model (the target theory in cellular radiation biology) is not yet at hand. Nevertheless, certain generalizations indicating one of the possible directions of future work can be drawn. Although the extrapolation numbers for asynchronous cell populations (Table 2) are sometimes quite small, they are probably higher in the resistant portions of the cycle. The presence of the shoulder in these survival curves suggests (by analogy with radiation studies; see Ref. 22) that cells must accumulate damage for a lethal drug effect, and, therefore, survivors are initially damaged sublethally. Repair of sublethal damage (22) has already been described for sulfur mustard (39) and for ethylmethanesulfonate and methylmethanesulfonate (26), and may well be a general response of cells to cytotoxic drugs. Analogous findings have been reported for tumor cells treated in vivo with
cyclophosphamide (15). Additional data confirming this suggestion will be described in a forthcoming communication.

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