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The cytokinetic and cytotoxic effects of ICRF-159 and ICRF-187 *in vitro* and ICRF-187 in human bone marrow *in vivo*

Richard H. Wheeler,[1] Daniel J. Clauw,[1] Ronald B. Natale[1, 2] and Raymond W. Ruddon[3]

[1] *Department of Internal Medicine, Division of Hematology/Oncology, University of Michigan, Ann Arbor, Michigan, U.S.A.*

[2] *Veterans Administration Medical Center, Ann Arbor, Michigan, U.S.A.*

[3] *Department of Pharmacology, University of Michigan, Ann Arbor, Michigan, U.S.A.*

Key words: cytokinetic effects, cytotoxicity, ICRF-159/187

Summary

The cytotoxic and cytokinetic effects of ICRF-159 and its d-enantiomer ICRF-187 have been examined *in vitro*. The effects of both agents were identical. Cytotoxicity is dependent on both the drug concentration and the duration of drug exposure. Drug exposure for twice the cell cycle time is necessary for maximum effect. Cytotoxicity is also dependent upon the rate of cell proliferation. A rapidly growing cell population is more sensitive to brief drug exposure than a slowly growing population.

The cytokinetic effects were studied using flow cytometry, determination of [^{3}H]-thymidine incorporation and mitotic index. ICRF-159/187 appears to act only during the G_2 phase of the cell cycle. There is no detectable delay in cell passage through the G_1/S boundary or in transit through S phase. Inhibition of DNA synthesis occurs only after the G_2 block prevents subsequent entry of cells in S phase. A fraction of the cells, depending upon drug concentration, undergo further DNA synthesis without cell division, resulting in a tetrapoid cell population.

The cytokinetic effects were determined in the bone marrow of patients receiving ICRF-187. All dose-rates produced G_2/M accumulation in the marrow with depletion of S phase cells. One patient was given a single injection of 1.0 gm/M^2. G_2/M accumulation was observed 24 h after treatment, with recovery to a pretreatment DNA cycle distribution 24 h later.

These studies suggest that a continuous drug infusion, or intermittent infusions timed to allow the normal cell population to recover, may produce superior clinical activity with this agent. A Phase I study of such an intermittent schedule is indicated.

Introduction

ICRF-159 (razoxane), an EDTA derivative developed at the Imperial Cancer Research Fund, has shown antineoplastic activity in animal tumor models (1) as well as in clinical studies (2). A synergistic antineoplastic effect has been demonstrated in animal tumor models for combinations of ICRF-159 plus irradiation, adriamycin, 5-fluorouracil, cisplatinum, cyclophosphamide, or hexamethylmelamine (3–6). Methotrexate resistant L5187Y cells are more sensitive to ICRF-159 than a methotrexate sensitive line (7).

Previous studies of the cellular effect of ICRF-159 have produced somewhat conflicting results. Studies by Hellman and co-workers have suggested

Address for reprints: Richard H. Wheeler, M.D., Comprehensive Cancer Center, University of Alabama in Birmingham, University Station, Birmingham, AL 35294, U.S.A.

a site of action in the G_2 phase of the cell cycle with production of G_2 arrest (8–10). Other investigators have demonstrated inhibition of RNA, protein and DNA synthesis and have suggested that ICRF-159 acts as an alkylating agent (11). G_2 arrest can be a non-specific effect of cell treatment with a number of antineoplastic agents with different mechanisms of action. Arrest in the G_2 phase has been demonstrated with alkylating agents, antitumor antibiotics, and protein synthesis inhibitors (12–14). Adriamycin has been shown to produce G_2 arrest *in vitro* and cell recovery with subsequent progression through the cell cycle occurs only after brief exposure to low drug concentrations (15). Exposure to high drug concentrations or long exposure times result in irreversible arrest. Bleomycin, on the other hand, produces a G_2 block that is reversible, and release of the block results in a synchronized cell population (16). This action of bleomycin has been demonstrated in human tumors *in vivo* (17) and has been successfully incorporated into clinical treatment regimens (18). Tobey has shown that various agents stop progression in different points in the G_2 phase and has suggested that the G_2 phase may contain a surveillance mechanism for DNA damage (19). Demonstration of G_2 arrest, therefore, does not automatically imply a G_2 phase specific site of action.

ICRF-159 is a racemic mixture. Because of poor water solubility, clinical trials were conducted with an oral formulation. However, recognition of variable bioavailability led to isolation and formulation of the more soluble d-enantomier ICRF-187 (20, 21). This parenteral agent has completed Phase I trials and is currently undergoing Phase II testing. This study was undertaken to further define the cytokinetic effects of ICRF-187, compare the *in vitro* effects of ICRF-159 and ICRF-187, determine the cytotoxic and cytokinetic effects of variation in drug concentration and exposure time, and assess the cytokinetic effect of ICRF-187 on the bone marrow of patients entered into Phase II trials utilizing continuous infusion, daily × 3 and daily × 5 treatment schedules.

Materials and methods

Burkitt lymphoma cells (P_3J) were maintained in continuous culture at 37°C in RPMI-1640 medium containing 10% fetal bovine serum, penicillin 100 μg/ml and streptomycin 100 μg/ml. Cells in the log phase of growth for 24 h were diluted with fresh culture medium to a concentration of $2.5-3.0 \times 10^5$ cells/ml just prior to each experiment. At this initial cell concentration, untreated cultures remained in the log phase of growth for 36 h with a doubling time of 13–15 h. After 72 h of growth, the cultures reached a growth plateau at a density of $2.2 \pm .2 \times 10^6$ cells/ml. Cell viability determined by erythrocin B dye exclusion, was in excess of 95% at the cell densities used in these experiments.

The small cell lung cancer line NCl-H69C, obtained from Dr. John Minna, The National Cancer Institute, was maintained in RPMI-1640 containing 15% fetal calf serum with penicillin and streptomycin. Cells in the log phase of growth have a doubling time of 40–44 h and maintain a dye exclusion viability in excess of 85%.

ICRF-159 (preservative-free, furnished by the National Cancer Institute) was dissolved in 0.1 N HCl immediately prior to use. ICRF-187, also obtained from the National Cancer Institute, was dissolved in distilled water. Cytotoxicity was determined using a soft agar colony-forming assay. At indicated times aliquots were removed from replicate control and drug treated cultures, centrifuged then resuspended in medium containing 15% fetal bovine serum and 0.3% agar. One ml, containing 5×10 cells was pipetted over 1.0 ml base layers containing 0.5% agar in 35 mm Petri dishes. The number of colonies per plate (>30 cells/colony) were hand counted, using an inverted microscope, 5–7 days after plating. Percent survival was calculated as the number of colonies/plate for the drug treated cultures divided by the number of colonies/plate for the controls. Cloning efficiency averaged $50 \pm 5\%$ for the untreated P_3J cultures, and $10 \pm 2\%$ for H69C. Each cloning assay was performed in triplicate. Each experiment was repeated at least once for both agents.

Thymidine incorporation was measured after the cultures were incubated with [^{3}H]-thymidine (Me-

thyl-^{3}H-thymidine, 20 Ci/mM, New England Nuclear, Boston, Mass), 0.5 μCi/ml, for 30 min. The cells were immediately centrifuged and washed with 2 volumes of iced 0.9% NaCl. The nucleic acid was precipitated by addition of 4 ml iced 5% perchloric acid, and the sample was centrifuged. After two further washes with iced perchloric acid, the pellet was resuspended in 1 ml 5% perchloric acid and incubated at 90°C for 30 min. [^{3}H]-thymidine content of the supernatant was determined by liquid scintillation counting in a Packard Tricarb Scintillation Counter and the DNA content was assayed by the diphenylamine method of Burton (22).

Mitotic index was determined by counting the number of mitoses/1000 cells using Wright-stained preparations. Cell counts were done with a Model F Coulter Counter.

Flow cytometry was performed using a Coulter TPS-1 Cell Sorter. Cells were prepared for fluorometric analysis of DNA content by incubation for 10 min in 0.05 mg/ml propidium iodine in 0.1% sodium citrate buffer at 0°C (23). $1.2-2.0\times10^4$ cells were analyzed per sample at a peak channel setting at 10^4 cells.

Patient samples

Approval of this investigation was obtained from the University of Michigan Committee to Review Grants from Clinical Research and Investigation Involving Human Beings. Written informed consent was obtained from all participating subjects.

Bone marrow aspirates were obtained from the posterior iliac crest using a Jamshidi needle. Two to 3 ml samples were withdrawn, anticoagulated with EDTA and divided into two aliquots for analysis by flow cytometry. The mononuclear cell fraction was separated by centrifugation in a Ficol/Hypaque gradient (Histopaque-1077, Sigma Chemical Co., St. Louis, Mo.), and stained with propidium iodide. The bone marrow sample cell count was determined on a Model F Coulter Counter after gradient separation of mononuclear cells.

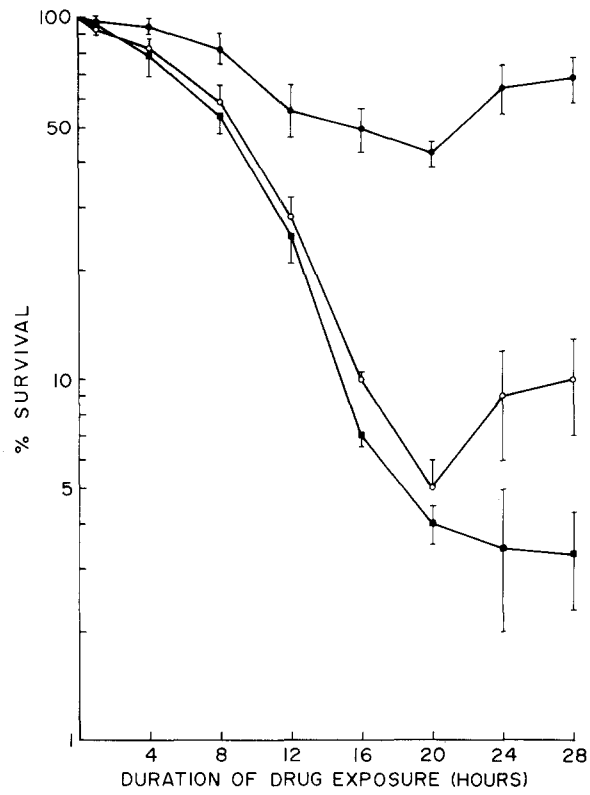

Fig. 1. The effects of drug concentration and exposure duration on ICRF-187 cytotoxicity. P_3J cells were exposed to ICRF-187 at the concentrations shown. At the indicated times aliquots were removed and cell kill was determined by the soft agar cloning assay.
●, 1 μg/ml; ○, 3 μg/ml; ■, 10 μg/ml.

Results

The experiments using P_3J cells shown in Figs. 1, 4, 8 and 9 were performed using ICRF-159 and ICRF-187. The cytotoxic and cytokinetic effects observed were identical for both agents. Dissolving ICRF-187 in 0.1 N HCl did not alter the drug effect. Therefore the results obtained with either agent have been used interchangeably.

The effects of a timed exposure to 1 μg/ml, 3 μg/ml or 10 μg/ml of ICRF-187 is shown in Fig. 1. A 28-h exposure to 10 μg/ml was required for maximum cytotoxicity. A drug concentration of 40 μg/ml produced the same effect as is shown for 10 μg/ml. The cytotoxicity of 3 and 10 μg/ml was the same for exposure times of 12 h or less. Exposure to 3 μg/ml for 16 h or longer produced less cytotoxi-

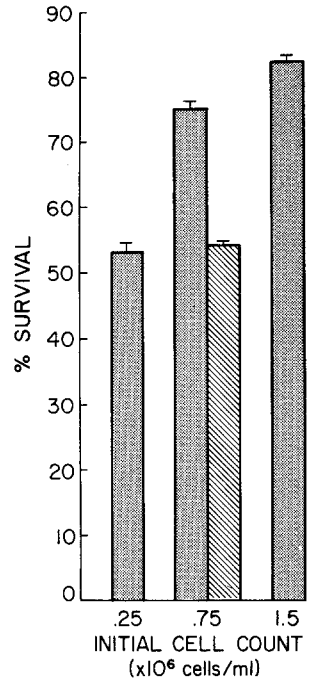

Fig. 2. The effect of cell proliferation rate on ICRF-159 cytotoxicity. P_3J cells at the initial cell densities shown, were incubated with 20 μg/ml ICRF-159 for 14 h (stippled bars) or 21 h (hashed bar).

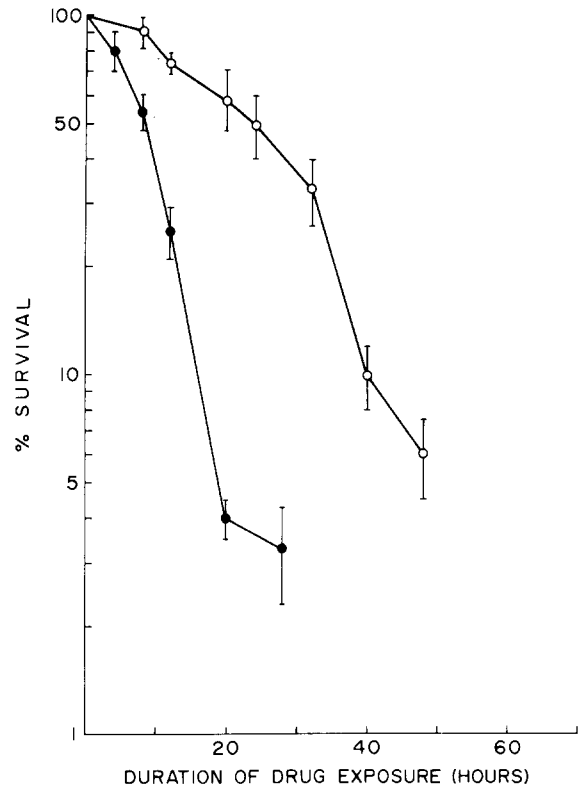

Fig. 3. Comparison of exposure time dependent cytotoxicity of ICRF-187 in P_3J and H69C cells. Cultures were incubated with 20 μg/ml ICRF-187, and aliquots were removed for soft agar cloning assay at the times indicated. Medium and drug was replaced at 24 h in the H69C cultures.
●, P_3J; ○, H69C.

city than the 10 μg/ml drug concentration. By thirty hours, the cloning efficiency in cultures exposed to 3 μg/ml began to increase as the cells recovered from drug exposure, or possibly as cells resistant to this concentration continued to proliferate.

Some of the cytotoxicity difference between 10 μg/ml and 1 μg/ml could result from drug breakdown with loss of effect at the lower concentration. Cells were therefore exposed to these two drug concentrations for a total of 24 h, however, every 8 h the cultures were centrifuged and resuspended in fresh, drug-containing medium. Renewing the drug concentration every 8 h increased the cytotoxicity of 1 μg/ml from 51 ± 2% to 30 ± 4% colony survival. This suggests that drug breakdown may have occurred during a single 24-h exposure. The cytotoxicity produced by 10 μg/ml was not increased by drug replacement. Under both exposure conditions the cytotoxicity of 10 μg/ml was 5 ± 2% colony survival. Thus, sufficient drug apparently remained after a single addition of 10 μg/ml to maintain the cytotoxic effects.

These results demonstrate that maximum cell kill is achieved by a continuous drug exposure. However, with a phase specific agent, a given duration of drug exposure should produce greater cell kill in cells with a short cycle time. The effect of Burkitt cell proliferation rate on ICRF-159 cytotoxicity is shown in Fig. 2. The doubling time of untreated log phase P_3J cultures was 13–15 h, and during a 14-h incubation period, the viable cell count of untreated cultures with an initial cell concentration of 0.25×10^6 cell/ml increased 107%, compared to a 53% increase with an initial inoculum of 0.75×10^6 cells/ml and 19% increase with an initial inoculum of 1.5×10^6 cells/ml. Cultures with an initial concentration of 0.75×10^6 cells/ml doubled in cell count after 19–20 h of growth. The cytotoxic effects of a 14-h exposure to 20 μg/ml ICRF-159 decreased as the initial cell inoculum increased, however, when the drug exposure time was extended to 21 h to encompass the longer doubling time of cultures plated at 0.75×10^6 cells/ml, the cytotoxicity produced was equal to that observed with the faster

growing cell population. Since the cell cultures with higher cell counts were at a lower initial pH (7.0 compared to 7.4 ± 0.1), the effect of culture pH on the cytotoxicity of ICRF-159 was also investigated. Variation in pH from 6.9 to 7.9 had no effect on the cytotoxicity of a 12-h exposure to 20 μg/ml.

Similar results are seen when two cell lines with different cycle times are compared. Figure 3 shows the effect of a 20 μg/ml exposure on the Burkitt cells, and on a small cell lung cancer line NCl-H69C which has a log phase doubling time of 40 h. Twenty μg/ml produced maximum cytotoxicity in both cell lines. For all durations of exposure there is a preferential killing of the Burkitt line.

In the clinical treatment of solid tumors, sensitive normal tissues, particularly the bone marrow in the case of ICRF-187, would be represented by the more rapidly proliferating cells. An intermittant drug administration which allowed normal tissue recovery would be a potential alternative to continuous drug administration. This alternative was examined *in vitro*. Burkitt cells were exposed to 20 μg/ml of ICRF-187 for 8 h (a concentration × time exposure that produced 50% cell kill), centrifuged, then resuspended in either drug containing medium for an additional 8 h, or drug-free medium. Aliquots of cells incubated in the drug-free medium were removed at 4-h intervals, then re-exposed to ICRF-187 for a second 8-h duration and cytotoxicity assayed. As shown in Fig. 4, an 8-h drug exposure reduced cell survival 50%, and a 16-h continuous exposure produced 80% cell kill. As the drug-free incubation was lengthened from 4 to 20 h, the cell population gradually recovered from the initial drug effect. With a 12- to 16-h delay between the two drug exposures, cytotoxicity was equal to a single 8-h exposure.

The same drug exposure conditions were used with the H69C cell line except that a 20-h exposure time was used. Twenty hours was selected to produce a 50% cell kill. The results are also displayed in Fig. 4. The time to recovery from the first drug exposure is only slightly delayed compared to the more rapidly proliferating P_3J cell line. This suggests that recovery from drug-induced damage is not cycle time dependent, and is not a result of cell growth during the drug-free incubation.

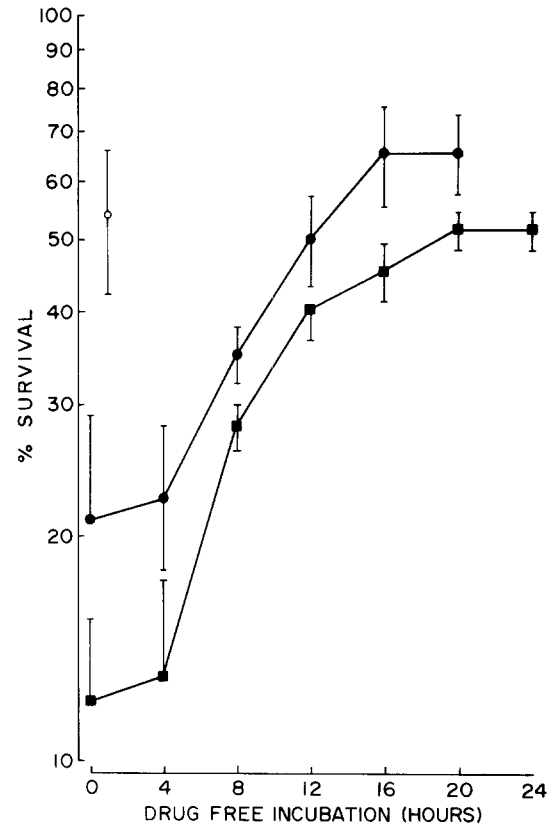

Fig. 4. Time course of recovery from an initial drug exposure. P_3J cells were exposed to 20 μg/ml ICRF-187 for 8 h then placed in drug-free medium for 0 to 24 h prior to a second 8-h exposure. H69C cells were exposed to 20 μg/ml for 20 h during each drug incubation. Cytotoxicity was determined following the second drug exposure.
● P_3J; ■ H69C; ○, a single drug exposure: 8 h for P_3J, 20 h for H69C.

The cytokinetic effect of ICRF-159

The effect of 20 μg/ml of ICRF-159 on thymidine uptake, mitotic index, and DNA content per cell are shown in Fig. 5. The mitotic index began to fall between 60 and 90 min of drug exposure; however, thymidine incorporation was not affected until exposure time exceeded 4 h. There was a progressive increase in the DNA content per cell. For comparison, the effects of nitrogen mustard (0.5 μg/ml) and colchicine (1.0 μg/ml) on thymidine incorporation and DNA content per cell are shown in Fig. 6. This was the lowest nitrogen mustard concentration that produced maximum cell kill in this culture system

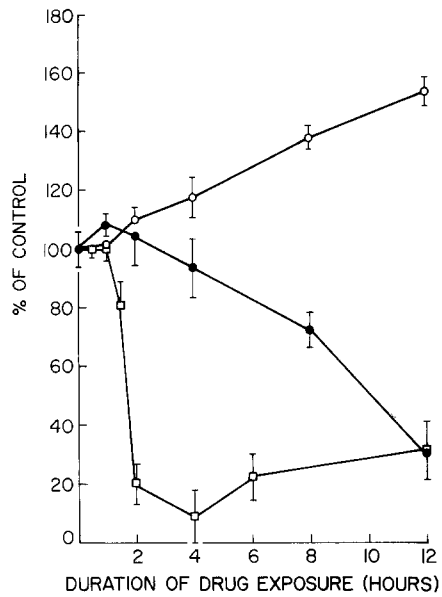

Fig. 5. The cytokinetic effects of ICRF-159 in P_3 J cells. Simultaneous control and drug treated cells (20 μg/ml) were assayed for mitotic index, thymidine incorporation and cell content of DNA. Percent of control was calculated by dividing the results from the drug treated cultues by the results from concomitant controls. ○, cell content of DNA; ●, thymidine incorporation; □, mitotic index.

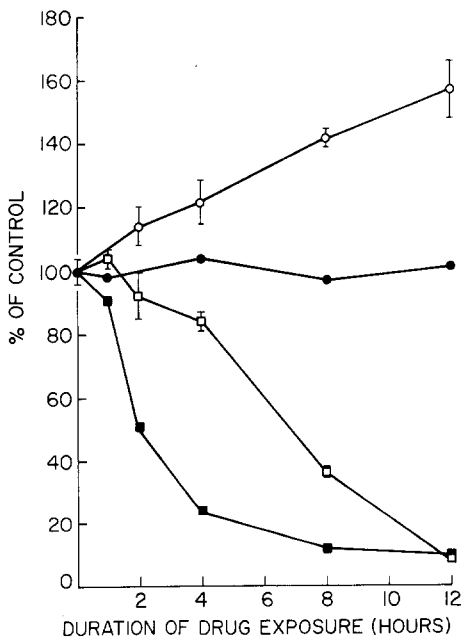

Fig. 6. The cytokinetic effects of colchicine and nitrogen mustard in P_3 J cells.
○, cell content of DNA, colchicine; ● cell content of DNA, nitrogen mustard; □, thymidine incorporation, colchicine; ■, thymidine incorporation, nitrogen mustard.

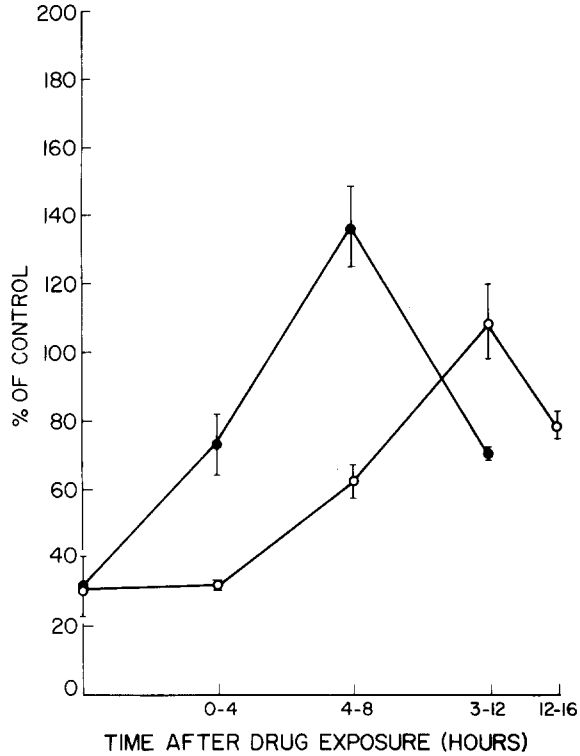

Fig. 7. Changes in mitotic index and thymidine incorporation following exposure to ICRF-159. P_3 J cells were incubated with 20 μg/ml ICRF-159 for 14 h, then placed in drug-free medium. Aliquots were removed at the times indicated for determination of mitotic index and thymidine incorporation.
○, thymidine incorporation; ●, mitotic index.

(data not shown). The effect of colchicine on thymidine incorporation and cell content of DNA was quite similar to that of ICRF-159. In contrast, nitrogen mustard caused a more rapid drop in thymidine incorporation without increase in DNA content per cell.

To investigate the potential use of ICRF-159 as a synchronizing agent, thymidine incorporation and mitotic index were studied following drug removal (Fig. 7). A peak in mitotic accumulation occurred 4 to 8 h following removal of ICRF-159. A peak in DNA synthesis was seen 8 to 12 h following drug removal. These results show that following ICRF-159 exposure, cells move through the subsequent division cycle in a partially synchronized wave.

The effect of a continuous exposure of ICRF-187 at concentrations of 20 μg/ml, 5 μg/ml, and 1 μg/ml

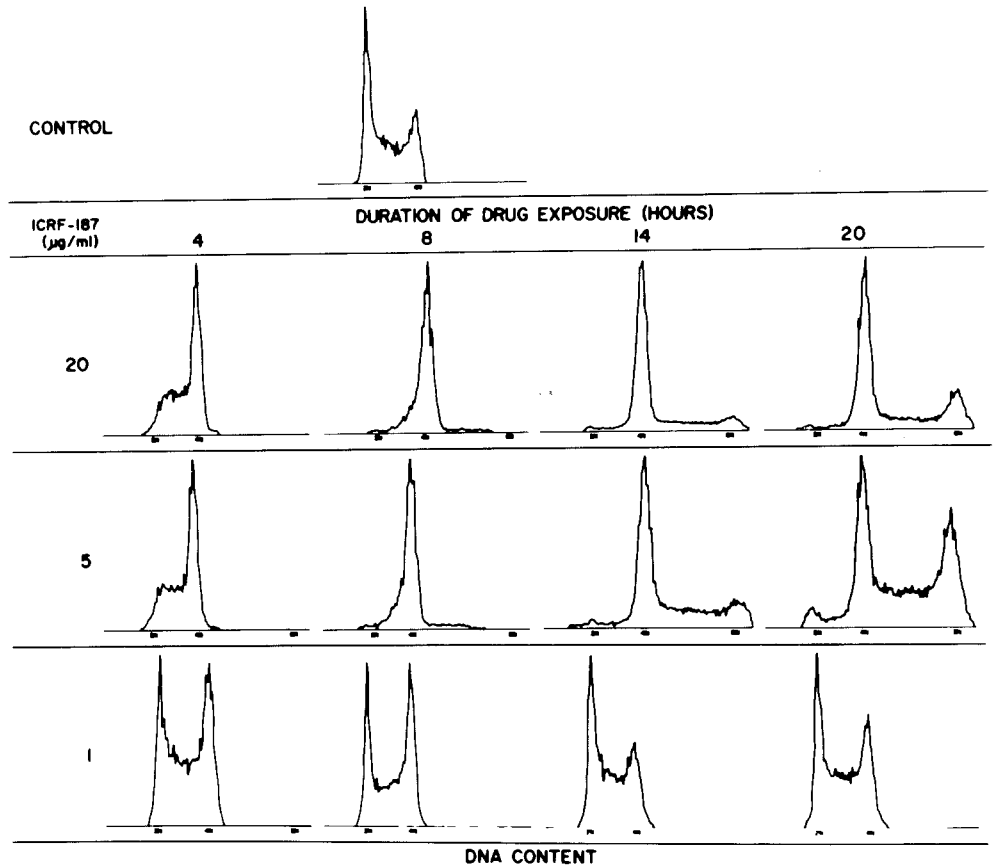

Fig. 8. Changes in cell cycle distribution during a continuous exposure to ICRF-187. P_3J cells were incubated with ICRF-187 at the concentrations shown. Aliquots were removed for flow cytometry at the indicated times.

on cell cycle distribution was determined by flow cytometry. As shown in Fig. 8, the two higher drug concentrations rsulted in maximum cell accumulation with a G_2 DNA content, whereas 1 μg/ml produced a partial effect. There was no inhibition of cell progression through the G_1/S boundary or through the DNA synthetic phase. There was, however, an accumulation of cells with an 8N DNA content suggesting that a portion of the cells were capable of subsequent DNA synthesis, but that cytokinesis was prevented. This effect was most pronounced at the 5 μg/ml drug concentration.

To further evaluate the possible use of the ICRF-159 as a synchronizing agent, cells were exposed to 20 μg/ml and 5 μg/ml for 14 h, then resuspended in a drug-free medium. Flow cytometry was performed at sequential time points thereafter. Figure 9 shows the results of this experiment. After removal from the drug-containing medium, the cells proceeded through G_1 into S in a partially synchronized wave.

To investigate the potential relationship of these *in vitro* data to clinical therapy, the cytokinetic effects of ICRF-187 on the bone marrow of patients receiving the agent were determined. Figure 10 shows sequential cytofluorographs on a patient who received 1.5 gm/M^2 ICRF-187 for three successive days. The baseline marrow shows a standard pretherapy DNA distribution pattern. Twenty-four hours after the first dose G_2/M accumulation and S phase depletion is evident. Twenty-four hours following the second dose, G_2/M accumulation persists with some cells in the 4N–8N range. Cells with a 4N–8N DNA content are still evident 24 h after the third dose. Seventy-two hours after the third dose few S or G_2/M cells remain in the

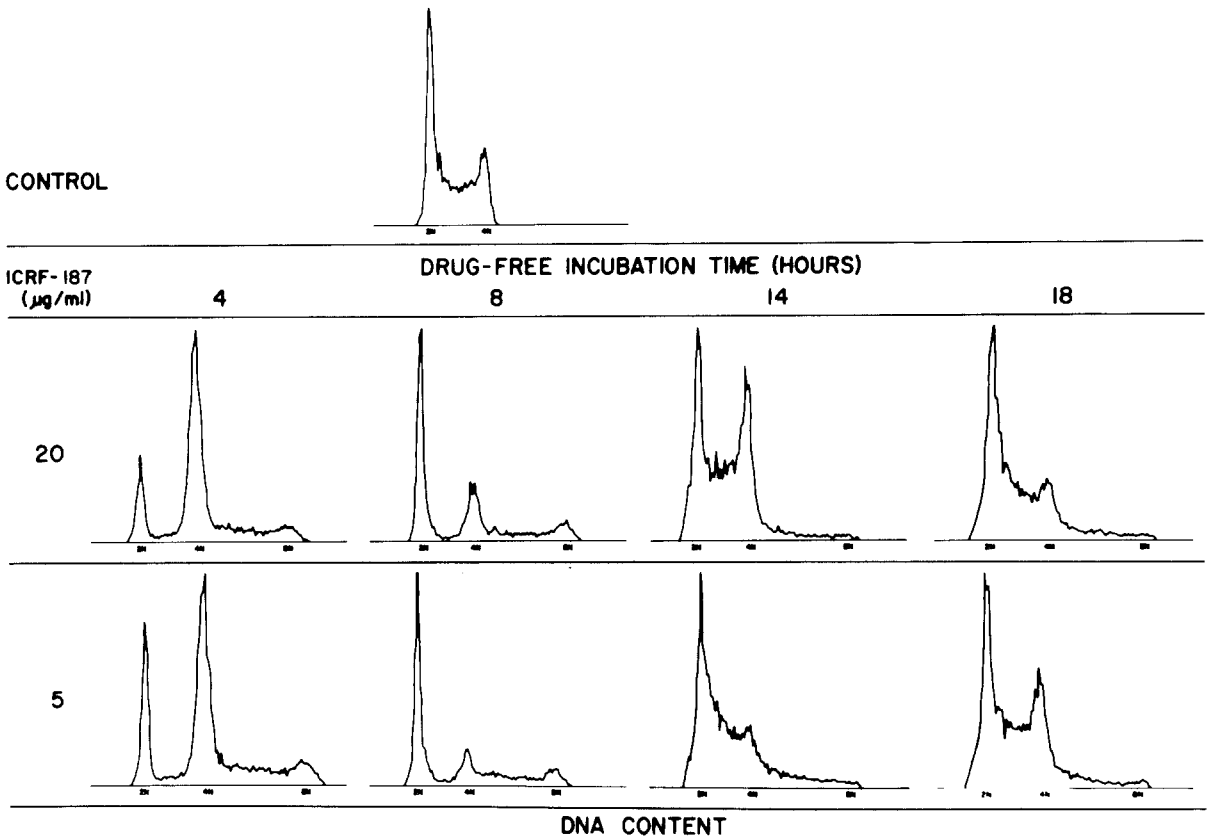

Fig. 9. Changes in cell cycle distribution following exposure to ICRF-187. P_3J cells were exposed to 5 or 20 μg/ml ICRF-187 for 14 h, then placed in drug-free medium. Aliquots were removed for flow cytometry at the times indicated.

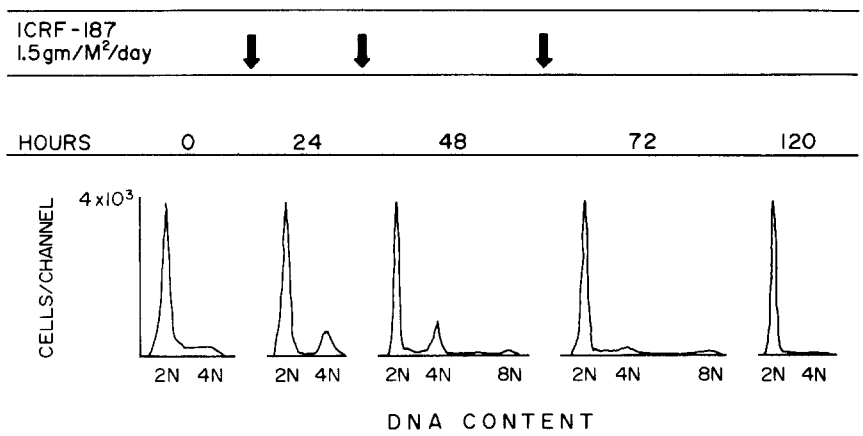

Fig. 10. Effect of ICRF-187 on the bone marrow cell cycle distribution during a daily × 3 treatment course. Bone marrow samples were obtained at the times indicated and processed for flow cytometry. Arrows signify ICRF-187 administration.

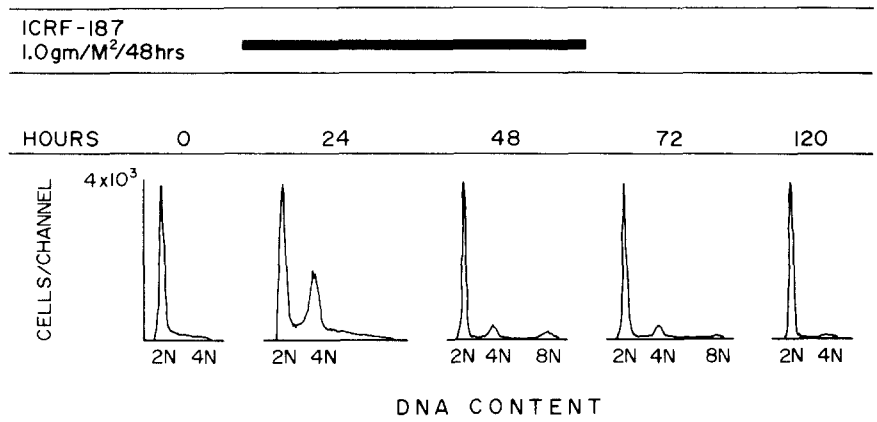

Fig. 11. Effect of ICRF-187 on the bone marrow cell cycle distribution during a continuous 48-h treatment course.

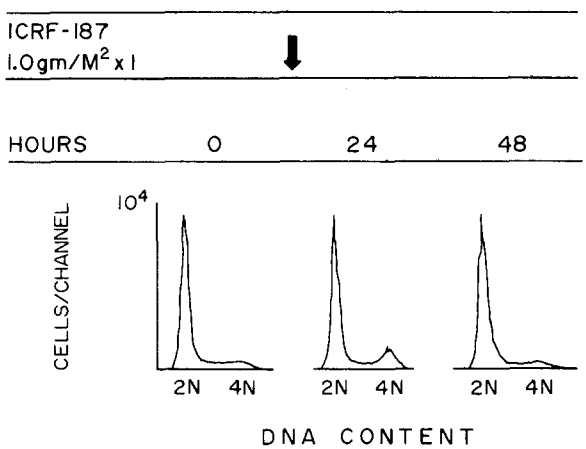

Fig. 12. Changes in bone marrow cell cycle distribution following a single dose of ICRF-187.

marrow. The cytokinetic effects of a 48-h continuous infusion are shown in Fig. 11. The effects are almost identical to the results seen in the previous patient with G_2/M accumulation, 4N–8N cells and few S, G_2/M cells at 120 h. Identical DNA distribution patterns were seen in two patients who received 1.25 gm/M^2 ICRF-187 daily for five consecutive days and in one additional patient at both the continuous infusion and daily × 3 dose rates.

To assess the time to recovery from a single dose of ICRF-187, one patient was given an injection of 1.0 gm/M^2. As shown in Fig. 12, G_2/M accumulation is evident 24 h after the dose. Twenty-four hours later the bone marrow has regained a pretherapy DNA distribution.

Discussion

The cytotoxicity of ICRF-159/187 is clearly related to both drug concentration and duration of exposure. There is minimal inhibitory effect with exposure times less than one-half the cycle time, even with high drug concentrations. Maximum drug effect requires an exposure time at least twice the cell cycle time. Hellman & Field (9) found similar results using HEp/2 cells assayed by the colony forming technique and suggested that the agent must act in a brief portion of the cell cycle. Hallowes *et al.* (8) found exposure-time dependent variation in ICRF-159 cytotoxicity in three different cell lines. In contrast to our findings, the agent had no effect on cell proliferation during the initial 24-h exposure, but produced progressive cell-kill during a subsequent 48-h incubation.

Part of the difference in cytotoxicity observed *in vitro* for various drug concentrations is due to disappearance of the active compound from the medium with a consequent decrease in the duration of exposure. Dawson has shown that the breakdown of ICRF-159 is a pH-dependent hydrolysis (24). The half-life of the drug is approximately 12 h at pH 7.0. Replacing a 1 μg/ml concentration with fresh drug every 8 h significantly increased the cytotoxicity. Clear concentration differences remain however, as evidenced by the fact that the addition of fresh drug at a concentration of 1 μg/ml every 8 h for 24 h produces less cytotoxicity than an identical 24-h exposure to 10 μg/ml. Since the peak

plasma level achieved in man following a 3 gm/M^2 dose is less than 5 μg/ml (25), the duration of drug exposure becomes crucial for attaining an antineoplastic effect. Creaven *et al.* (26) have shown that the bone marrow suppression that occurs with the clinical use of ICRF-159 is highly schedule-dependent. Part of the schedule dependency, however, may be due to poor oral absorption of high doses with decreased bioavailability (20).

The magnitude of observed drug effect is also greatly dependent upon cell proliferation. As shown by the experiments illustrated in Figs. 2 and 3, slowly growing cell populations are less sensitive to an equal concentration × time exposure than rapidly growing cells. Equal cytotoxicity can be achieved when exposure time is increased. This proliferation dependence has been demonstrated in other *in vivo* and *in vitro* systems (8, 27, 28). This suggests that the slowly growing tumors encountered clinically could be effectively treated only by a long duration of drug exposure.

Our studies support the contention that ICRF-159/187 is a phase specific agent having a site of action in G_2 phase of the cycle (8–10, 27, 28). Figures 5 and 8 show that ICRF-159 produces a G_2 arrest in treated cells with a gradual accumulation of cells in this phase of the cell cycle. The mitotic index begins to decline within 60 to 90 min of drug addition (Fig. 5). Since the duration of G_2 in this cell line is 1.5 h, the site of action must be in the G_2 phase in order to produce this rapid drop in mitotic index. If the drug produces an immediate effect when added to the cell cultures, then the site of activity would be in early G_2 (19). However, Dawson has shown that ICRF-159 enters cells slowly by diffusion, and maximum intracellular concentration is not attained for 2 to 3 h (24). Therefore, the assumption of immediate action may not be warranted, and the drug effect may be occurring later in G_2. Others have reported an increase in mitotic index or the percent of prophase cells with ICRF-159 exposure (10, 29). We did not observe an increase in mitotic index, and the mitotic cells present were not arrested in prophase.

Following the G_2 arrest, a fraction of the cells move into DNA synthesis without cell division. This is best demonstrated in the flow cytometric analysis illustrated in Fig. 8. These cells are again arrested in a second G_2 peak with an 8N DNA content. Other investigators have observed a progressive increase in cell size and an increase in tetraploid cells (8, 30). Since the majority of cells remain arrested in the initial G_2 block, the subpopulation of cells that proceed into a second synthetic phase are presumably more resistant to the drug effect. Taylor & Bleehen (31) demonstrated that EMT6 cells resistant to ICRF-159 are polyploid with twice the usual DNA content and chromosome numbers. Hallowes *et al.* (8) have observed that dead cells as defined by dye exclusion are mononuclear. The demonstration in Fig. 8 of a greater percentage of tetraploid cells with a 5 μg/ml suggests that these cells that achieve tetraploidy are partially drug resistant. In order to kill these cells, the drug must be present throughout the second G_2 phase and this explains why a drug exposure of twice the cycle time is necessary for maximum cytotoxicity. In contrast to the stable polyploidy noted by Taylor & Bleehan, the cultures that recovered from drug treatment in our experiments were made up of cells with normal DNA content. The exposure time used in our experiments, however, may not have been sufficient to produce a stable resistant cell population.

The flow cytometry and thymidine incorporation studies do not show any delay of progression through the G_1/S boundary or in S phase transit. DNA synthesis inhibition has been previously reported (11). However, we observe decreased DNA synthesis only after the arrest in G_2 progression has depleted the cell pool entering G_1 and S. This is in contrast to an equally cytotoxic concentration of nitrogen mustard which produces a rapid inhibition of thymidine incorporation, and similar to the effect seen with colchicine (Fig. 6). We were unable to detect any increase in thymidine incorporation as reported by Creighton & Birnie (11) and by Grieder *et al.* (29). In the latter instance, the thymidine uptake was corrected by the cell count. Since the ICRF-159 produces a rapid decrease in cells passing through mitosis while leaving a S phase undisturbed, an artificial increase in thymidine incorporation would be detected. Hallowes *et al.* (8) also found no effect on the labelling index during the initial phase of drug exposure.

There are clear cytokinetic differences between ICRF-159/187 and other compounds which produce G_2/M arrest *in vitro*. The alkylating agents, with nitrogen mustard as the prototype, are not phase specific, produce DNA synthesis inhibition at a high concentration, and slow or inhibit transit throughout the cell cycle (12, 32). Adriamycin produces a G_2 block that correlates with cytotoxicity (13). However, at high drug concentration, transit throughout the cell cycle is inhibited (15), and S phase cells are most sensitive to the cytotoxic effect (33). Mithramycin and bleomycin produce primarily G_2 arrest but inhibit DNA synthesis at high concentrations (16, 34–36). Protein synthesis inhibitors such as puromycin delay transit through the G_1/S boundary in addition to producing G_2 arrest (14). Colchicine and the periwinkle analogues vincristine and vinblastine produce metaphase arrest, whereas ICRF-159 exposure results in a fall in mitotic index.

As further contrast between the alkylating agents, antitumor antibiotics and ICRF-159, Dawson was unable to demonstrate covalent binding of ICRF-159 to DNA, or any physical interaction between ICRF-159 and intracellular macromolecules (24). Also, Sharpe *et al.* could find no visible evidence of chromosome damage following ICRF-159 exposure (10).

Any conclusions concerning the mechanism of action of ICRF-159/187 must remain highly speculative. As Tobey has summarized, completion of mitosis requires two discrete periods of protein synthesis just prior to mitosis (37). The mRNA necessary for synthesis of these proteins is transcribed early in G_2. ICRF-159/187 could interfere with the early G_2 events or could inhibit synthesis of the proteins necessary for initiation of mitosis (38). The cytokinetic effects of ICRF-159/187 may also result in part from inhibition of microfilament synthesis or function. Gosalvey *et al.* (39) have shown that ICRF-159 will inhibit the capping of surface immunoglobulins, a process dependent upon microfilaments. This inhibition occurs at low drug concentrations and may result from chelation of ferric iron. Cytochalasin B, an inhibitor of microfilament formation, also produces multinucleated cells (31). With ICRF-159/187 additional mechanisms must be active since nuclear division is also inhibited in the majority of cells.

The studies also have implications for clinical therapy with ICRF-159/187. It is clear from Fig. 2 that the drug must be present for at least twice the cell cycle time to produce maximum cell kill. This suggests that a continuous infusion should be more effective than intermittent bolus treatment. However, human tumors in general have considerably longer cell cycle durations than most normal cell populations, and excessive toxicity could result from prolonged infusions. Intermittent drug infusions, with drug-free intervals timed to allow recovery of the normal cells with a short cycle time, may produce a selective cytotoxicity to a slowly growing cell population.

The cytokinetic analysis of the bone marrows of patients receiving ICRF-187 reveal the same effects that were observed *in vitro*. Proliferating cells are initially blocked in G_2/M. A fraction of the cells continue in cycle without cell division and accumulate with an 8N DNA content. A 48-h continuous infusion, and daily 3- or 5-day injections produce marked depletion of the S and G_2/M compartments consistent with the subsequent myelosuppression that developed in these patients. The one patient who received a single injection of ICRF-187 had cytokinetic recovery of the bone marrow 48 h following the dose. Based on these observations, a Phase I study of intermittent ICRF-187 with injections timed to allow bone marrow recovery is indicated.

ICRF-159/187 also has some potential for use as a synchronizing agent in a combination drug regimen. ICRF-159/187 treatment could be followed after an appropriate interval by an S phase specific agent to take advantage of the partial cell cycle synchrony demonstrated in Fig. 7 and 9. An alternative approach would be to administer the S phase specific agent at the end of an ICRF-159 infusion timed to produce bone marrow arrest in G_2. A tumor cell population, having a longer cell cycle time should not have achieved S phase cell depletion during this short infusion period. These potential clinical applications will require further investigation in animal tumor models.

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