Different Susceptibilities of Postmitotic Checkpoint-proficient and -deficient Balb/3T3 Cells to ICRF-193, a Catalytic Inhibitor of DNA Topoisomerase II

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Two distinct types of Balb/3T3 cells were isolated which exhibit either 4 *N* DNA or both 4 *N* and 8 *N* DNA after exposure to colcemid for 48 h. They were found to differ with respect to the postmitotic checkpoint, but not the mitotic checkpoint. Firstly, the checkpoint-proficient and -deficient cells exhibited the same accumulation and subsequent decrease in the number of mitotic cells following exposure to microtubule inhibitors. Secondly, after exit from abnormal mitosis in the presence of ICRF (Imperial Cancer Research Fund)-193, the checkpoint-proficient cells were arrested in the next cycle G1, while the checkpoint-deficient cells progressed into S and G2 phase. When either mitotic or asynchronous cells were exposed to ICRF-193, the checkpoint-proficient cells proved more sensitive to the cytotoxic effect of this agent than the checkpoint-deficient cells. The different susceptibilities of the two types of cells to ICRF-193 were not caused by variation in topoisomerase (topo) II function since both the biochemical activity of this enzyme and chromosome segregation were inhibited by similar concentrations of ICRF-193 in both checkpoint-proficient and -deficient cells. We propose that the inhibition of chromosome segregation by ICRF-193 is monitored by the next G1 checkpoint, resulting in an irreversible G1 block in the case of postmitotic checkpoint-proficient cells. As the checkpoint-deficient cells can escape this G1 block, these cells have an increased survival capacity. In summary, ICRF-193 may prove to be a very useful drug for examination of the postmitotic checkpoint.

Key words: Drug sensitivity — Postmitotic checkpoint — Topoisomerase II inhibitor — ICRF-193 — Balb/3T3 checkpoint mutant

Cellular susceptibility to various anti-tumor agents is influenced by many factors. For the elucidation of these different resistance mechanisms, the isolation and characterization of drug-resistant cell lines have proven useful. Concerning topoisomerase (topo) inhibitors, reduced or mutated topo I or II has been found to confer drug resistance to the respective agents.[1, 2] Decreased retention of drugs related to multidrug resistance or multidrug resistance protein also renders the cells resistant.[1–4] Apoptosis-resistant cells demonstrate other mechanisms of resistance.[5] These mutants do not display alterations in either the activity or quantity of topo II, with similar rates of drug efflux being seen in both apoptosis-sensitive and -resistant cell types.

Alteration in cell cycle checkpoints, as proposed by Hartwell and Weinert,[6, 7] may also be involved in drug resistance. Several checkpoints occur within the cell, these acting as safeguards to ensure the correct order of cell cycle progression. These checkpoints are also thought to give cells extra time to respond to drug-induced stresses leading to damaged or unreplicated DNA. Checkpoint mutants have been mostly isolated and characterized in yeast,[7] although a significant number of mammalian cell strains have been identified which display defective checkpoint pathways. Loss of p53, which is increased in response to DNA damage and inhibits DNA synthesis, results in defects in both the G1 and G2 checkpoints.[8, 9] p21, a downstream target of p53, also mediates the G1 arrest induced by DNA damage, this event being associated with inhibition of cyclin E/CDK2 or cyclin D/CDK4.[10, 11] The spindle assembly checkpoint, another type of checkpoint, works in M phase. This checkpoint monitors for proper chromosome attachment to spindle microtubules and for mechanical tension at the kinetochores,[12] and is defective in certain human tumor cells.[13, 14]

There are many reports in the literature which indicate that checkpoint controls in both the G1 and G2 phases influence drug susceptibility. For example, Chinese hamster ovary (CHO) cells having a defect in the DNA replication checkpoint still accumulate protein and cyclin B along with total protein, even when DNA synthesis is inhibited by hydroxyurea or aphidicolin, and the resulting aberrant growth leads to cell death.[15] Furthermore, there have been conflicting reports about the relationship between defects in the p53-associated G1 checkpoint and susceptibility to the lethal effects of ionizing radiation or antitumor drugs.[16–20] On the one hand, defects in p53, through mutational means or otherwise, confer increased sensitivity to radiation and taxol.[18, 19] In contrast, human

lymphoma cells with mutated p53 demonstrate increased resistance to DNA damaging agents such as γ-irradiation,[17, 20] while in other cases no apparent effect on sensitivity to radiation has been observed upon inactivation of the p53 pathway.[16]

ICRF (Imperial Cancer Research Fund)-193, a catalytic inhibitor of topo II,[21–24] allows cell cycle progression without chromosome segregation, leading to the accumulation of multiploid cells.[22, 24] This topo II inhibitor delays cell cycle transition from the S to M and from the M to G1 phases in HeLa S3 cells.[22, 25, 26] Since ICRF-193 does not cause DNA strand breaks, unlike other topo II inhibitors such as etoposide or 4′-(9-acridinylamino)methanesulfon-*m*-aniside (*m*-AMSA),[27] delay of progression through S and G2 phases might be due to incomplete DNA replication and/or impairment of the decatenation of replicated daughter molecules rather than DNA damage.[26, 28–31] MST-16, a compound related to ICRF-193 has found clinical application, notably for treatment of acute leukemia.[32] Since ICRF-193 is most effective against cells in M phase,[26] it is an intriguing possibility that the mitotic checkpoint affects the cytotoxicity of this drug. In order to test the involvement of the mitotic checkpoint in ICRF-induced cytotoxicity, we attempted to isolate mitotic checkpoint-proficient and -deficient cell strains from Balb/3T3 cells, since it has been reported that these mouse cells contain mixed populations of mitotic checkpoint-proficient and -deficient cells.[33] We subcloned these two cell types from Balb/3T3 cells as reported,[33] and characterized the checkpoint in greater detail. Unexpectedly, i.e. different from the previous report,[33] we found that the two cell types that were isolated differed with respect to the post-mitotic checkpoint, but not the mitotic checkpoint.

Here, we report that cells which underwent abnormal mitosis were monitored by the postmitotic checkpoint, and that cellular susceptibility to ICRF-193 was influenced by this checkpoint.

MATERIALS AND METHODS

Drugs ICRF-193 was kindly provided by Zenyaku Kogyo Co., Ltd. (Tokyo). TN-16 was purchased from Wako Pure Chemical Industries, Ltd. (Osaka). These agents were dissolved in dimethyl sulfoxide (DMSO), the final concentrations of which did not exceed 0.1% in the culture medium.
Cell culture Balb/3T3 cells were seeded and grown in Iscove's modified Dulbecco's medium (GIBCO-BRL, Grand Island, NY), containing 10% calf serum along with penicillin and streptomycin, under a humidified atmosphere of 5% CO_2 in air.
Isolation of checkpoint-proficient and -deficient Balb/3T3 strains Balb/3T3 cells were subcloned by two different approaches in order to acquire strains with varying checkpoint potentials. Firstly, Balb/3T3 cells were seeded

into 96-well plates (Costar, Cambridge, MA) at a concentration of one cell per four wells. After two weeks in culture, independent clones were isolated. Alternatively, cells were seeded at 20 cells per 100 mm dish (Falcon, Franklin Lakes, NJ) and surviving colonies were isolated using cloning cylinders. To determine the propensity of the independent clones to mediate a checkpoint response, cells were exposed to colcemid (190 n*M*) for 48 h, fixed with chilled 70% ethanol, and kept on ice until the DNA content of the cells was estimated by flow cytometry. Checkpoint-proficient and -deficient strains were distinguished on the basis of having either 4 *N* DNA or 4 *N* and 8 *N* DNA, respectively. Stocks of these two different strains were stored in liquid nitrogen to obviate the risk of alteration in phenotype as a result of prolonged cell culture.
Flow cytometric analysis of DNA content Fixed cells were washed once with phosphate-buffered saline (PBS) and subsequently incubated with the same buffer containing 500 μg/ml RNase A (Sigma-Aldrich, Tokyo) and 10 μg/ml propidium iodide (PI) (Sigma-Aldrich) for 1 h at 37°C, followed by overnight incubation at 4°C. After centrifugation, cells were resuspended in PBS containing PI (5 μg/ml) and the DNA content was examined using a FACScan flow cytometer (Biosciences, San Jose, CA). A minimum of 10 000 cells per sample were analyzed.
M phase synchronization Cells were seeded at $4–5×10^6$ cells/140 mm dish. The following day, the medium was changed so as to remove cell debris. TN-16, a reversible mitotic inhibitor,[26] was then added to a final concentration of 0.3 μ*M*, with the cells being cultured for another 4 h subsequent to this addition. Floating and rounded mitotic cells were then collected by gentle pipetting and washed with culture medium.
Cell cycle analysis DNA synthesis was assessed in terms of incorporation of bromodeoxyuridine (BrdU) (Boehringer Mannheim, Tokyo) into replicating DNA.[34] After being synchronized at M or G1 phase, cells were treated with ICRF-193. Upon removal of ICRF-193, these cells were incubated for various time periods in the presence of 10 μ*M* BrdU, before final fixation with 70% chilled ethanol. After this, cells were lysed with 0.04% pepsin/0.1 *N* HCl for 30 min at 37°C, and then washed with PBS containing 0.5% Tween 20 and 0.5% bovine serum albumin (PBS-TB). The DNA was denatured by incubation with 2 *N* HCl for 10 min at 37°C. After neutralization with 2 volumes of 0.1 *M* $Na_2B_4O_7$, this DNA was stained with 0.2 ml of fluorescein isothiocyanate (FITC)-conjugated anti-BrdU monoclonal antibody (Boehringer Mannheim) (1:20 in PBS-TB) for 60 min at room temperature. The samples were then treated with 500 μg/ml RNase for 30 min at 37°C, and 10 μg/ml PI in PBS-TB for 30 min at room temperature, and resuspended in PBS-TB containing 5 μg/ml PI.
Colony formation Five hundred mitotic or asynchronous

cells per 100 mm dish were treated with ICRF-193 either for 1 h or for a long term, respectively. Treated cells were washed with culture medium, and then seeded into the 100 mm dishes, cultured for approximately 10 days, fixed with chilled methanol and stained with Giemsa solution. Only colonies of more than 50 cells were scored. The surviving fractions of the drug-treated cells were expressed as a percentage of the number of colonies produced after treatment with DMSO alone.

Assay for clamp formation between topo II and DNA
Mitotic cells were incubated with TN-16 plus or minus ICRF-193, lysed in ice-cold lysis buffer [50 mM Tris-HCl (pH 7.4), 350 mM NaCl, 0.1% (v/v) Nonidet P-40, 5 mM EDTA, 50 mM NaF, 1 mM phenylmethanesulfonyl fluoride, 2 μg/ml leupeptin and 2 μg/ml aprotinin] containing ICRF-193 while standing for 1 h on ice, and then centrifuged at 15 000 rpm for 15 min at 4°C. Half a volume of 3× sodium dodecyl sulfate (SDS) sample buffer was added to the extract supernatant. The supernatants were then subjected to electrophoresis on 8% polyacrylamide gel and electroblotted onto Immobilon-P membranes (Millipore, Bedford, MA). Subsequently, membranes were incubated with anti-topo II monoclonal antibody (4E12), treated with horseradish peroxidase (HRP)-conjugated F(ab′)$_2$ fragment of sheep anti-mouse IgG, and visualized by enhanced chemiluminescence (ECL) using the ECL western blot detection system from Amersham Pharmacia Biotech (Tokyo).

RESULTS

Isolation of checkpoint-proficient and -deficient cell strains from Balb/3T3 cells DNA damage often results in G2 arrest, during which cells repair the damage and progress through into M phase.[7] In both *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, inactivation of topo II leads to mortality of mitotic cells, and nocodazole, an inhibitor of tubulin assembly, protects against lethality due to the inability of topo II *per se* to prevent exit from M phase.[35] In order to test the involvement of the M phase checkpoint in ICRF-induced cytotoxicity, we attempted to isolate mitotic checkpoint-proficient and -deficient cell strains from Balb/3T3 cells, since it has been reported that these mouse cells contain mixed populations of checkpoint-proficient and -deficient cells.[33] We isolated a large number of independent colonies from Balb/3T3 cells, without using any selection procedure, and individually tested for the presence of the spindle assembly checkpoint. Upon exposure to colcemid for at least one generation, checkpoint-proficient cells were arrested with 4 N DNA content, while the checkpoint-deficient cells escaped the M phase block and continued to progress through the cell cycle, resulting in cells with 8 N DNA content (Fig. 1). These results are consistent with a

previous report[33] indicating that Balb/3T3 cells contain distinct types of populations with respect to the checkpoint. We selected randomly three checkpoint-proficient (BD-2, B-1L and B-12L) and three checkpoint-deficient (BD6-5, BD-12 and B-8L) cell strains for the following experiments. All of the chosen strains were tested as to whether polyploid cells were induced in the presence of
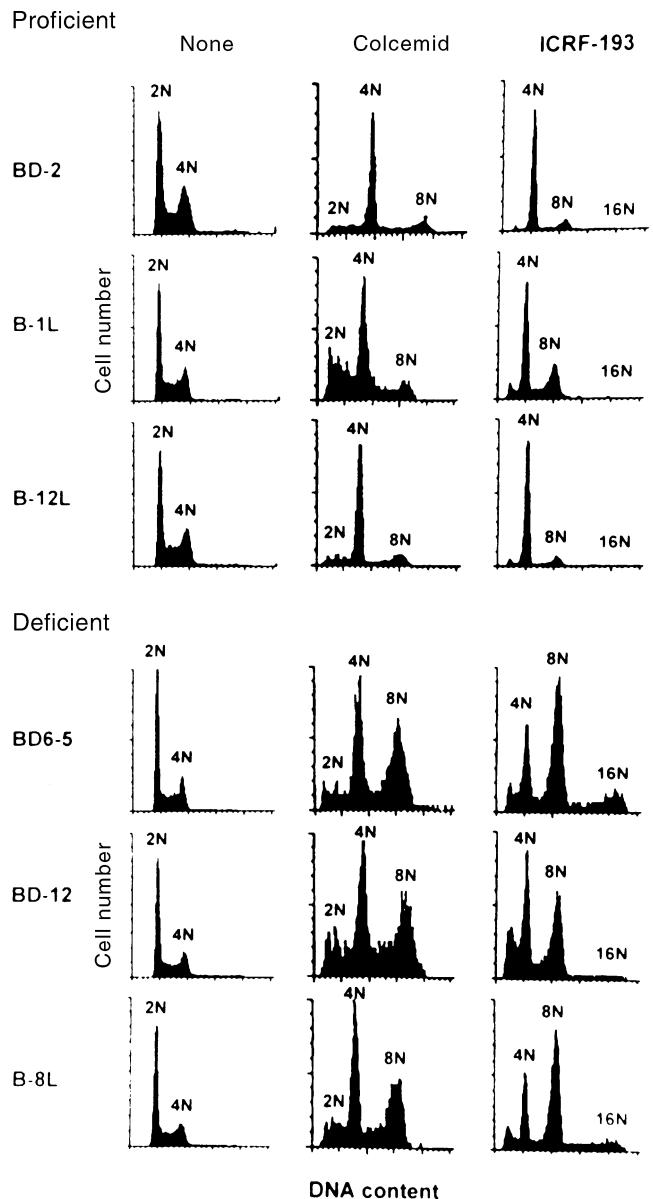

Fig. 1. Flow cytometric analysis of checkpoint-proficient and -deficient clones, isolated from Balb/3T3 cells, following drug treatment. Each cell strain was exposed to 190 nM colcemid and 3 μM ICRF-193 for 48 h. DNA content was analyzed through the use of a FACScan.

ICRF-193, since this agent inhibits chromosome segregation and causes polyploid cells to appear.[26] As shown in Fig. 1, the checkpoint-proficient cells displayed 4 *N* DNA content, while the checkpoint-deficient cell strains had not only 4 *N* DNA, but also 8 *N* DNA content.

Differences in the postmitotic checkpoint displayed by these cell lines An earlier report suggested that p53 had a part to play in regulating the mitotic checkpoint.[36] However, recent studies have revealed that p53 is not involved in the mitotic checkpoint, but rather in the postmitotic checkpoint.[37, 38] We addressed whether the checkpoint-proficient and -deficient cells have distinct mitotic checkpoints. Firstly, the kinetics of the mitotic index was examined after exposure of cells to nocodazole. Both types of cells undergoing mitosis increased after 12 h and then decreased by 24 h, indicating that all of the cell strains exhibit a transient mitotic arrest, with subsequent progression into the next G1 phase without chromosome segregation having occurred, as observed previously (data not shown).[39] The data also imply that the mitotic check-point does not differ between the checkpoint-proficient and -deficient cells.

To ascertain whether cells are arrested in the next cycle G1 after exit from mitosis, flow cytometric analysis of DNA, labeled with BrdU and stained with PI, was performed to monitor progression into the S phase (Fig. 2). Mitotic cells of all checkpoint-proficient and -deficient cells, accumulated by exposure to TN-16, progressed into G1 phase after removal of the agent. The progression from M to G1 phase at 1 h was also confirmed by degradation of cyclin B which normally occurs concomitant with decrease of cdc2 kinase activity, the key enzyme (data not shown). Next, mitotic cells, accumulated by exposure to TN-16, were treated with or without ICRF-193, with subsequent addition of BrdU and incubation for 24 h. Without ICRF-193, the enriched mitotic cell population was shown to exhibit cells with 2 *N* and 4 *N* DNA content which were BrdU-positive. In the presence of ICRF-193, mitotic cells could traverse into the next G1 phase in spite of chromosome missegregation, resulting in the appearance of cells
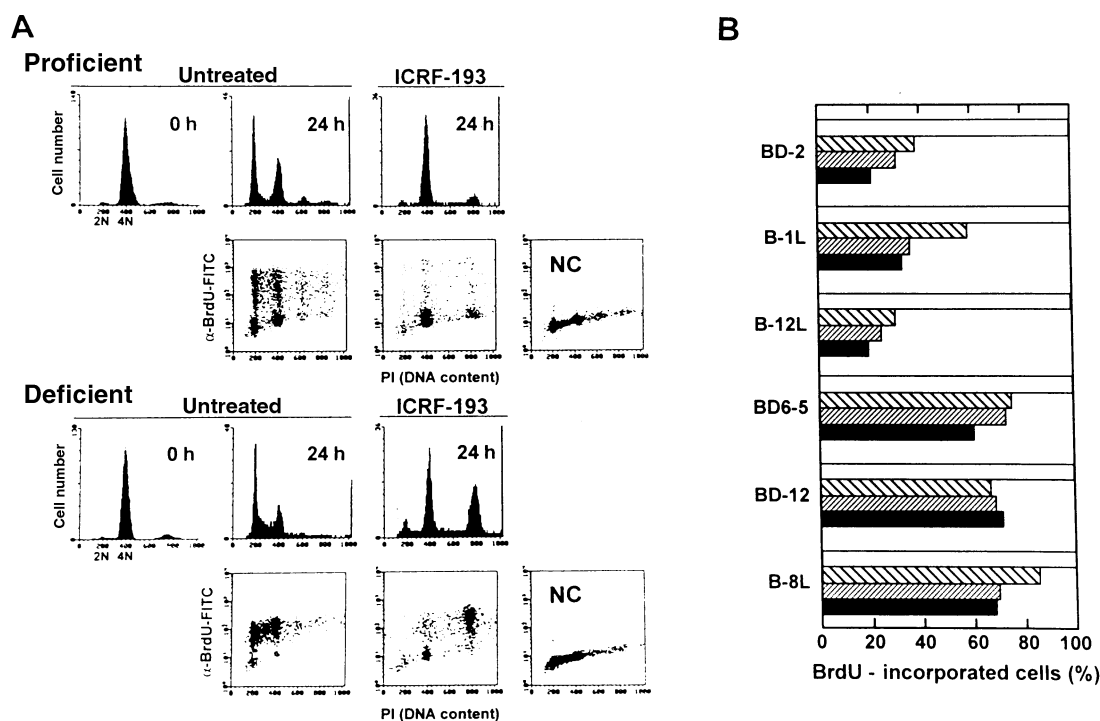

Fig. 2.   Flow cytometric analysis of cells synchronized at M phase, and treated with ICRF-193. The cells were synchronized at mitosis by TN-16. After removal of TN-16 and treatment with or without 3 *μM* ICRF-193 (A) and various concentrations of ICRF-193 (B) for 1 h, the cells were cultured for 24 h in the presence of BrdU. Cells were fixed and stained with FITC-conjugated anti-BrdU monoclonal antibody and PI. Cells progress into G1 phase at 1 h after removal of TN-16. (A) The *x*-axis corresponds to relative DNA content and the *y*-axis represents BrdU-incorporated cells. NC represents a negative control cell population cultured without BrdU. (B) ICRF-193 concentrations are 0 (☐), 1 (▨), 3 (▩) and 10 *μM* (■). One hundred percent represents the proportion of BrdU-incorporated cells in the absence of ICRF-193.

with 4 *N* DNA content. The checkpoint-deficient cells progressed further into S and G2 phases while the checkpoint-proficient cells stayed in G1. Data from the above-mentioned BrdU labeling assay showed that the checkpoint-deficient cells traversed into S phase, while the checkpoint-proficient cells were arrested in G1 phase (Fig. 2A). Inhibition of progression into S phase by ICRF-193 was compared in the checkpoint-proficient and -deficient cells (Fig. 2B). The S phase progression of the checkpoint-proficient cells was much more inhibited than that of the checkpoint-deficient cells. Thus, both types of cells differ in the postmitotic checkpoint, but not with regard to the mitotic checkpoint. Taken together, we conclude that the two cell types differ in respect to the postmitotic checkpoint.

Susceptibility of postmitotic checkpoint-proficient and -deficient cell strains to ICRF-193 and CPT-11 Since ICRF-193 kills mitotic cells most efficiently,[26] the susceptibility of the two cell types to this agent, when in the mitotic phase, was compared. Three postmitotic checkpoint-proficient and -deficient cell strains were first synchronized in M phase, exposed to various concentrations of the topo II inhibitor for 1 h, and then assayed for colony formation. As shown in Fig. 3A, the checkpoint-proficient cells were more sensitive to the cytotoxic action of ICRF-
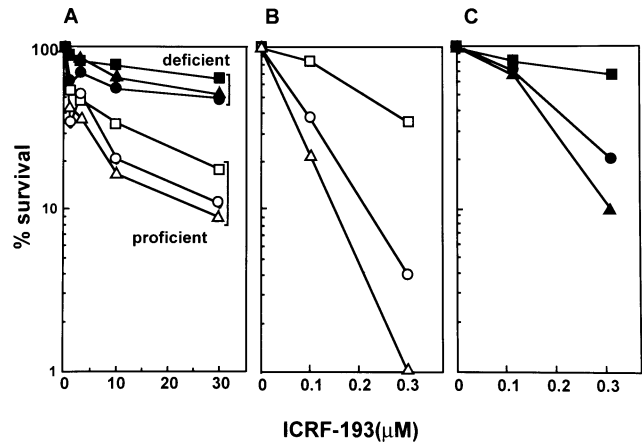

Fig. 3.   Susceptibility of postmitotic checkpoint-proficient and -deficient cells to ICRF-193. (A) Mitotic cells, obtained by using TN-16, were exposed to various concentrations of ICRF-193 for 1 h, washed with medium, and then seeded for assessment of colony formation as described in "Materials and Methods." B-1L(○), BD-2 (□), B-12L (△), BD-12 (●), BD6-5 (■) and B-8L (▲). The data shown represent the means from 3 independent experiments. Non-synchronized (B) B-1L, a checkpoint-proficient line, and (C) B-8L, a checkpoint-deficient line, were exposed to either 0.1 or 0.3 *μM* ICRF-193 for 1(□ , ■), 2 (○ , ●) or 3 (△ , ▲) days, respectively. Culture medium containing ICRF-193 was changed at one-day intervals.
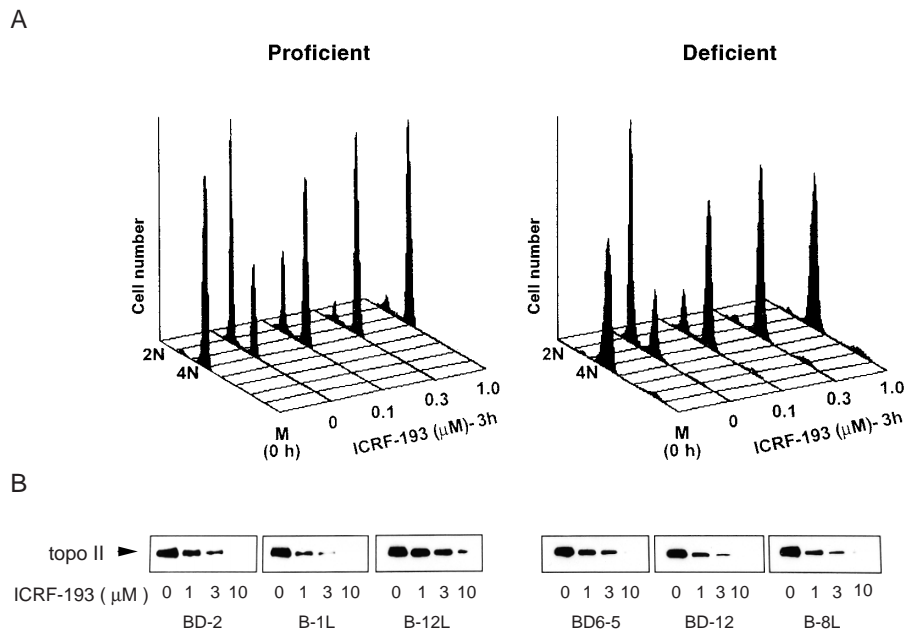

Fig. 4.   Dose dependence of ICRF-193 with respect to inhibition of chromosome segregation (A) and clamp formation (B) in postmitotic checkpoint-proficient and -deficient cells. (A) Typical data for B-12L, a postmitotic checkpoint-proficient line and BD-12, a checkpoint-deficient line. Mitotic cells, obtained by using TN-16, were exposed to various concentrations of ICRF-193 for 3 h, with DNA contents subsequently being analyzed using a FACScan. (B) Clamp formation between topo II and DNA as a function of ICRF-193 concentration. Inhibition of topo II activity by ICRF-193 was examined by clamp formation assay as described in "Materials and Methods."

193 than were the checkpoint-deficient cells. To examine drug susceptibility under asynchronous conditions, checkpoint-proficient and -deficient cells were exposed to ICRF-193 for 1, 2 or 3 days with the drug being changed at one-day intervals (ICRF-193 is inactivated within a period of 24 h). Fig. 3, B and C show data pertaining to B-1L (checkpoint-proficient) and B-8L (checkpoint-deficient) cells, respectively. B-1L cells displayed an increased sensitivity to ICRF-193, compared with B-8L. BD-2 and B-12L cells also proved more sensitive to the topo II inhibitor than BD6-5 and BD-12 cells (data not shown). CPT-11, a topo I inhibitor, specifically targets cells in S phase. This agent, used as a control drug to compare with ICRF-193 treatment, was found to inhibit the growth of both cell types at approximately equivalent concentrations (data not shown).

Comparison of the inhibition of chromosome segregation and topo II by ICRF-193 between checkpoint-proficient and -deficient cell strains ICRF-193 inhibits chromosome segregation through inactivation of topo II,

but does not inhibit other mitotic events, so that polyploid cells can result.[26] To examine whether chromosome segregation is affected by ICRF-193 in a differential manner between postmitotic checkpoint-proficient and -deficient cell strains, ratios of cells with 4 N and 2 N content were estimated (cells which progress into G1 phase without chromosome segregation contain 4 N DNA content). Mitotic cells from the checkpoint-proficient and -deficient lines were cultured in the presence of various concentrations of ICRF-193 for 3 h, with the DNA content of the cells subsequently being analyzed by flow cytometry. ICRF-193 inhibited the conversion of 4 N to 2 N cells in a dose-dependent manner (Fig. 4A). ICRF-193 (0.3 μM) inhibited chromosome segregation completely in both strains. ICRF-193 inhibited segregation in the other checkpoint-proficient and -deficient cells at similar concentrations (data not shown).

We previously clarified that ICRF-193 is a non-cleavable complex-forming type of topo II inhibitor.[21–23] This drug inhibits the release of topo II from DNA, after one
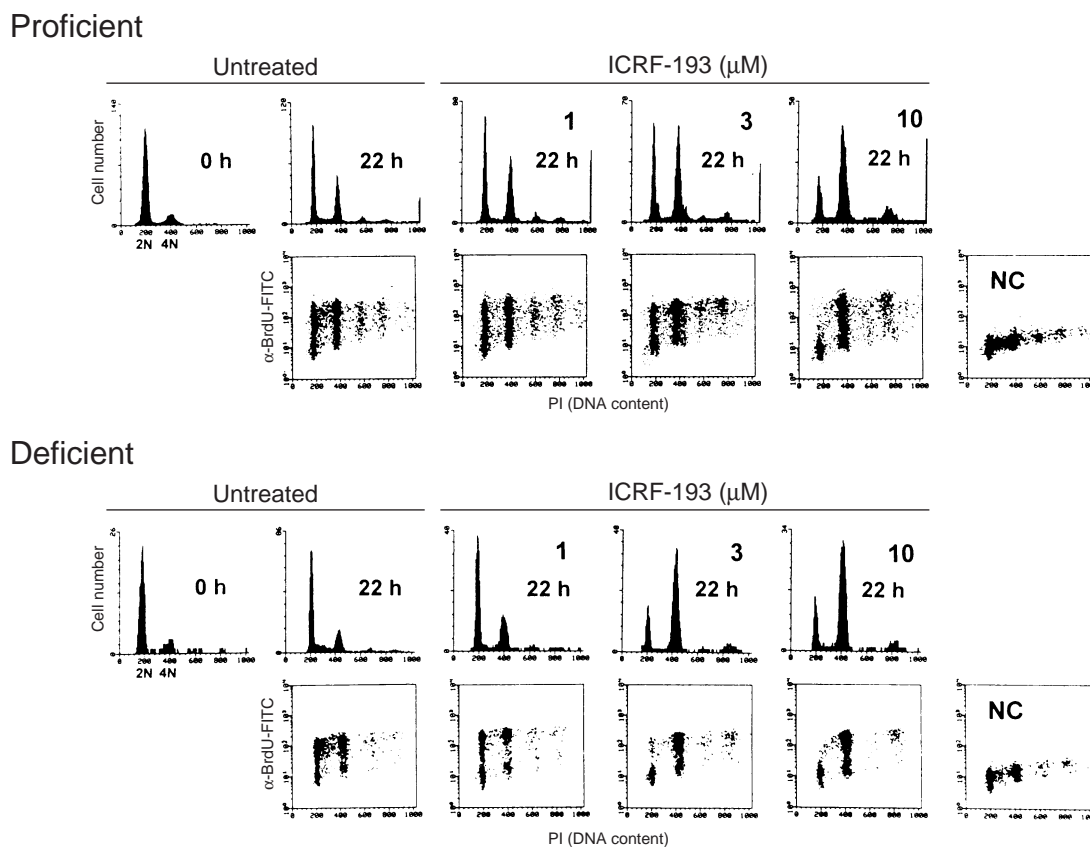

Fig. 5.   Flow cytometric analysis of cells synchronized at G1 and treated with ICRF-193. The cells were synchronized at mitosis by TN-16. Two hours after removal of TN-16, B-12L and B-8L cells were exposed to various concentrations of ICRF-193 for 1 h, and then cultured for 22 h in the presence of BrdU. The cells were fixed and stained with FITC-conjugated anti-BrdU monoclonal antibody and PI.
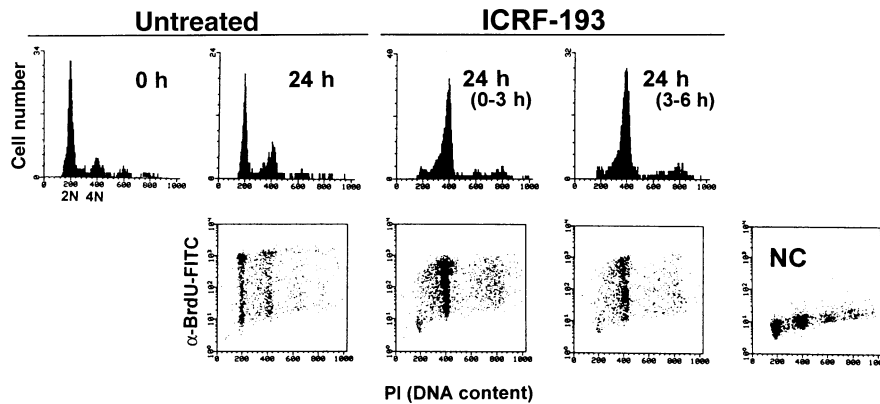
Fig. 6. Flow cytometric analysis of cells synchronized at G1(G0) and treated with ICRF-193. BD-2 cells were synchronized at G1(G0) phase by 0.25% serum starvation, released into complete medium, and then treated with 3 μM ICRF-193 during 0–3 h or 3–6 h. After having been washed, the cells were cultured in the presence of BrdU. Twenty-four hours after release from G1 arrest, the cells were fixed and stained with FITC-conjugated anti-BrdU monoclonal antibody and PI.

round of the catalytic cycle, through inhibition of the ATPase activity of the enzyme, and stabilizes it in a protein-closed clamp which cannot be dissociated with 1 M NaCl.[23] Since it is difficult to test directly whether or not ICRF-193 inhibits topo II activity *in situ* within cells, we examined the amount of topo II in protein-closed clamps stabilized by ICRF-193. It was found that intracellular topo II formed a salt-stable complex with DNA in the presence of ICRF-193.[40] Mitotic cells of both types, collected by exposure to TN-16, were treated with various concentrations of ICRF-193 for 1 h. Then the topo II was extracted with 350 mM NaCl and the extracted amount of topo II was examined. As shown in Fig. 4B, the level of topo II extractable by NaCl was reduced by ICRF-193 in a dose-dependent manner. There was no significant difference observed between checkpoint-proficient and -deficient cells.

Progression of G1 cells exposed to ICRF-193 into S phase The checkpoint-proficient cells were arrested in the next G1 phase, following exit from M phase, upon treatment with ICRF-193 (Fig. 2). We then examined whether G1 arrest is evident in G1 cells exposed to ICRF-193 (Fig. 5). The checkpoint-proficient (B-12L) and checkpoint-deficient (B-8L) cells were synchronized with TN-16 at the mitotic phase. Two hours after removal of the inhibitor, the cells in G1 phase were treated with or without ICRF-193 for 1 h, and then cultured for 22 h in the presence of BrdU. During this time, the checkpoint-proficient and -deficient cells progressed into the next G1 phase, following through to the G2 and M phases. ICRF-193 delayed cell cycle progression, as shown previously.[22, 26] At 10 μM ICRF-193, the majority of both cell types were G2/M phase cells. BrdU analysis also showed that both types of cells progressed into S and G2/M phase in the

presence of ICRF-193. In addition, ICRF-193 did not cause G1 arrest in the other checkpoint-proficient and -deficient cells (data not shown).

In order to confirm that exposure of G1(G0) cells to ICRF-193 does not result in a G1 block, BD-2, checkpoint-proficient cells were synchronized at G1(G0) phase by serum starvation, and released into complete medium with or without ICRF-193 for 3 h, followed by addition of BrdU (Fig. 6). By 24 h, most of the cells had reentered the G1 phase. When the cells were treated with 3 μM ICRF-193 for 3 h immediately after addition of serum, progression through the cell cycle was reduced, with subsequent arrest in G2/M phase at 24 h. A similar result was obtained following ICRF-193 treatment during 3–6 h after release of the G1 block. The other cell strains also did not exhibit an ICRF-193-induced G1 arrest (data not shown). Taken together, these results indicate that ICRF-193 does not cause G1 block, even in the checkpoint-proficient cells, when the cells in G1 are treated with this agent.

DISCUSSION

Although it has been previously reported that Balb/3T3 cells are a mixed population with respect to mitotic checkpoint capability,[33] we found that these cells differ in the postmitotic, not mitotic, checkpoint. The postmitotic checkpoint-proficient and -deficient cells that were isolated by our group did not differ in the accumulation and subsequent reduction of mitotic cells in the presence of nocodazole, while the two cell types displayed different responses in terms of next cycle G1 arrest after exit from abnormal mitosis. Namely, checkpoint-deficient cells can progress into S phase, whereas the checkpoint-proficient cells can not traverse into S phase (Fig. 2). Dewald *et al.*[33]

concluded that Balb/3T3 cells are heterogeneous concerning the mitotic checkpoint because in the presence of colcemid, "stringent" checkpoint cells arrested with 4 *N* DNA in the mitotic phase while "relaxed" checkpoint cells became tetraploid. However, these investigators did not confirm by microscopic observation in which phase the cells with 4 *N* DNA were blocked.

In addition to the DNA damage-induced first cycle G1 arrest, p53 functions to arrest cells in second cycle G1 which have progressed without undergoing cell division, this phenomenon being caused by exposure to inhibitors of microtubule formation.[37–39] pRb- or p21-deficient cells also undergo re-replication after exit from abnormal mitosis.[37, 38, 41, 42] Here, we show that the postmitotic checkpoint operates on those cells that have progressed into the next G1 phase without having undergone chromosome segregation. While the general signal for the postmitotic checkpoint to occur appears to be similar following exposure to either ICRF-193 or microtubule inhibitors, as both classes of agents inhibit chromosome segregation and result in the formation of polyploid cells, the particular mechanism of inhibition is distinct between these two drug types. Since p53 and p21 are involved in the postmitotic checkpoint in the case of inhibitors of microtubule formation,[41, 42] we examined whether p53 and p21 are related to postmitotic G1 arrest induced by ICRF-193, using human p53 or p21-deficient cells, and found that these genes are indeed involved in the postmitotic checkpoint (data not shown).

In the present study, we have shown that postmitotic checkpoint-proficient cells are more sensitive to the cytotoxic action of ICRF-193 than checkpoint-deficient cells, when either mitotic or asynchronous cells are exposed to the drug. In contrast, both cell types showed the same sensitivity to an apoptosis-inducing topo I inhibitor, CPT-11 (data not shown). Thus, the difference between checkpoint-proficient and -deficient cells regarding ICRF sensitivity is not due to variation in the induction of apoptosis. Indeed, ICRF-193-induced inhibition of cell growth was related not to apoptosis, but rather to cell cycle arrest, as induction of apoptosis was never more than 20% (data not shown). The variations in sensitivity to ICRF-193 indicated above do not appear to be modulated by the amount of topo II or the degree of inhibition of topo II activity in the checkpoint-deficient and -proficient cells.

ICRF-193 is a specific topo II inhibitor.[21–23] This drug inhibits the later stage of DNA replication,[28] and delays the cell cycle progression from S to G2 and, subsequently, M phase.[22, 26, 29, 31] During the cell cycle, ICRF-193 causes cell cycle arrests at two phases, G2 and second cycle G1,[22] of which the former arrest is transient and the latter is irreversible. Thus it may be concluded that the irreversible G1 arrest corresponds to the ICRF-193-induced growth inhibition. Since ICRF-193 is mostly effective against mitotic cells, we had speculated that chromosome missegregation through reduced topo II function is the main cause for the cytotoxic action of ICRF-193.[26] This concept may still hold true, but the present study has modified slightly the rationale behind this idea and indicates that the postmitotic checkpoint recognizes the abnormal cells passing through M phase, stopping the cell cycle progression. An inability to segregate chromosomes is not in itself a signal for cell death, as the checkpoint-deficient cells were shown to be capable of escaping the G1 block and of growing. As proposed by Hartwell and Weinert,[6, 7] checkpoints are thought to act as safeguards to ensure the correct order of cell cycle progression, and to give cells extra time to respond to drug-induced stress. The transient nature of the ICRF-193-induced G2, but not G1 arrest is suited to acting as a checkpoint control, while the G1 arrest is either prolonged or irreversible, and rather resembles that seen in senescence in human fibroblasts.[43]

In summary, we have isolated postmitotic checkpoint-proficient and -deficient Balb/3T3 cells and demonstrated differences in the susceptibilities of these two cell types to ICRF-193. This was not caused by variation in the response of topo II to ICRF-193, but rather by later events following incorrect chromosome segregation, possibly involving the G1 checkpoint. In order to study this type of postmitotic checkpoint, ICRF-193 is superior to microtubule inhibitors, as the former agent inhibits chromosome segregation, but not other mitotic events, while the latter stops all mitotic events.[38] Therefore, short-term exposure of mitotic cells to ICRF-193 is effective in producing cells with evidence of chromosome missegregation, while continuous exposure to inhibitors of microtubule is needed for the same event to occur. For this reason, ICRF-193, a catalytic inhibitor of topo II, is a very useful drug for analysis of the mechanisms surrounding the postmitotic checkpoint.

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