Increased Gene Amplification in L5178Y Mouse Lymphoma Cells with Hydroxyurea-induced Chromosomal Aberrations

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ABSTRACT

Chromosomal aberrations and dihydrofolate reductase gene amplification are observed in L5178Y mouse lymphoma cells after treatment with hydroxyurea. The types of aberrations include polyploidy, endoreduplication, chromosome fragmentation, and the presence of extrachromosomal DNA. Hydroxyurea-treated cells analyzed by cell sorting showed a subpopulation of cells with increased DNA and increased dihydrofolate reductase. This subpopulation shows a high incidence of chromosome aberrations and an increased frequency of dihydrofolate reductase gene amplification. Hydroxyurea-treated cells with the normal amount of DNA and dihydrofolate reductase have few aberrations and a low frequency of dihydrofolate reductase gene amplification. We propose that hydroxyurea treatment causes overreplication of DNA and that recombination of overreplicated DNA can lead to chromosome aberrations and gene amplification.

INTRODUCTION

Previous studies in our laboratory have examined the process of gene amplification in cultured mammalian cells. The frequency of cells resistant to MTX3 can be increased by 10-fold or more by pretreatment of cells with agents such as HU, UV light, and different carcinogens (1, 2). These agents which enhance DHFR amplification share the feature that they inhibit DNA synthesis. Mariani and Schimke (3) showed that, if DNA synthesis was inhibited during the second hour of S phase in synchronized CHO cells, DNA synthesized before the HU block was rereplicated once the HU was removed. This overreplication resulted in an increase in the number of DHFR genes and led to enhanced resistance to MTX1. HU is a specific inhibitor of ribonucleotide reductase, inhibits DNA synthesis (4), and is known to induce chromosome aberrations in vivo and in vitro (4–6). The present studies examine the relationship between HU-induced chromosome aberrations and the frequency of DHFR gene amplification. Our results show that the highest frequency of MTX resistance and gene amplification occurs in the cell population with many types of chromosome aberrations including polyploidy, chromosome fragmentation, and the presence of extrachromosomal DNA. We propose that the generation of chromosomal aberrations is a consequence of recombination events subsequent to overreplication of DNA.

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3 The abbreviations used are: MTX, methotrexate; HU, hydroxyurea; BrdUrd, 5-bromodeoxyuridine; SCE, sister chromatid exchange; F-MTX, fluorescein-conjugated methotrexate; DHFR, dihydrofolate reductase; FACS, fluorescence-activated cell sorter; HBSS, Hanks' buffered saline solution; CHO, Chinese hamster ovary; cDNA, complementary DNA.

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MATERIALS AND METHODS

Cell Culture and HU Treatment. TheJsens cells used in these experiments are L5178Y mouse lymphoblastoid cells; the C3 subclone of Jsens has been previously isolated by Dolnick et al. (7). C3 cells are highly resistant to MTX and contain 800 to 1000 copies of the mouse DHFR gene localized in a homogeneously staining region on chromosome 2. Both these cell lines have stable karyotypes with 40 to 42 chromosomes. Jsens and C3 cells were maintained in RPMI medium supplemented with 10% horse serum, 100 units of penicillin, and 100 µg of streptomycin per ml. The medium used for C3 cells also contained 200 µM MTX. The cells were grown in suspension at 37°C in an atmosphere of 5% CO2 and passage every 3 days.

Exponentially growing cultures of Jsens and C3 cells at concentrations of 5 × 106 cells/ml were treated with a freshly made HU stock (Sigma). One or 100 ml stocks of HU were dissolved in medium and added to the cultures in 100- to 500-µl aliquots. Control and treated cultures were incubated for 6 h at 37°C. HU was removed by centrifuging the cultures at 1000 × g for 5 min to pellet the cells. The old medium was removed, and the cell pellet was resuspended in prewarmed HBSS. The cells were centrifuged and washed one more time in HBSS. The cells were then placed into prewarmed medium and incubated at 37°C.

In some experiments, Jsens cells were grown in soft agar so that surviving cells could form individual colonies. When cells were grown without MTX, 200 to 500 cells were suspended in 5 ml of medium containing 0.4% agar (SeaPlaque). The cultures were then placed over a 2-ml underlayer of medium containing 0.9% agar in a 100-mm Petri dish. Individual colonies were visible in 4 to 6 days. For MTX selections, MTX stocks were prepared as described by Brown et al. (1). One × 105 to 5 × 106 cells were suspended in medium containing 0.9% agar and 10 µM MTX. These cells were placed over a 5-ml underlayer of medium containing 0.9% agar and 10 µM MTX in a 100-mm Petri dish. Individual colonies were visible in 2.5 to 3 wk. At 1-wk intervals after cells were plated out, the plates were briefly cooled until the agar became solid, and 10 ml of fresh medium containing 0.4% agar and 10 µM MTX were carefully pipetted over the cells. The number of surviving colonies was counted using a dissecting microscope. Individual clones were removed by suction from a pipet, and clones were expanded by continuous growth in suspension.

Determination of DNA Synthesis. Either 6 h after the addition of HU or 6 h after the removal of HU, the number of Jsens and C3 cells in each culture was determined by using a hemocytometer. The cultures in 100- to 500-µl aliquots. Control and treated cultures were counted using a dissecting microscope. Individual clones were removed by suction from a pipet, and clones were expanded by continuous growth in suspension.

Chromosome Preparations. To observe SCEs, cells were grown for 24 h (2 cell cycles in normal cells) in the presence of 10 µM BrdUrd. Two h before cell fixation, Cordanidin (2 × 107 M final concentration) was added. Chromosome fixations and fluorescence plus Giemsa staining were as described by Hill and Wolff (8), except that the cells were swollen in 0.075 M KCl for 10 min. SCEs were analyzed in 50 metaphases for each point, and the data were expressed as the mean number of SCEs per cell. Each determination was done in triplicate.
GENE AMPLIFICATION AND CHROMOSOMAL ABERRATIONS

Fluorescein MTX, DNA Fluorochrome Staining, and Use of the FACS. For F-MTX staining, Jsens control and HU-treated cells were placed in medium with 5 μM F-MTX supplemented with 30 μM each of glycine, hypoxanthine, and thymidine 24 h before FACS analysis. Before sorting, the medium containing F-MTX was removed, and the cells were resuspended in medium lacking F-MTX. F-MTX purification and cell sorting on the FACS II were as described by Johnston et al. (9). Distribution of fluorescence for a cell population was determined from at least 10,000 cells.

Jsens cells were sorted into 2 populations; the bright population in the control cells was the cells with F-MTX fluorescence of 90 units or higher. The bright population of the HU-treated cells was all cells that had F-MTX fluorescence greater than or equal to the control bright population. The control dull population was those cells with F-MTX fluorescence less than 90 units; the HU dull population also had F-MTX fluorescence of less than 90 units. F-MTX dull and F-MTX bright populations were sorted from each sample and placed into medium. For chromosome analysis, sorted cells were incubated for 8 h in the presence of 2 x 10^{-7} M Colcemid, and chromosome preparations were made as above. Cells from sorted populations were also placed in soft agar with and without MTX to determine the number of viable cells and the frequency of MTX resistance.

Determination of the amount of DNA in viable cells was previously described by Amt-Jovin and Jovin (10). The amount of DNA per cell was measured by staining Jsens cells with 5 μM Hoechst 33342 for 1 h at 37°C prior to FACS analysis. A UV laser (355 nm) was used to analyze the Hoechst-stained cells. Jsens cells were sorted into 2 populations; the control bright population was those cells with Hoechst fluorescence of 110 units or higher. The bright population in the HU-treated cells included all cells with fluorescence greater than or equal to the control bright population. The control dull population was those cells with less than 110 units of Hoechst fluorescence; the HU dull population was also those cells with Hoechst fluorescence of 110 units or less. Control and HU-treated cells were sorted into Hoechst dull and bright populations, and these cells were analyzed for chromosome abnormalities and growth in soft agar with and without MTX as described above.

Assessment of DHFR Gene Amplification. Individual clones isolated after growth in soft agar were expanded by growing cells in suspension in the presence of 10 nm MTX. DNA from these clones was isolated and purified as previously described (1). One μg of DNA from each clone was bound to a nitrocellulose filter using the slot hybridization technique (1). As a standard, increasing amounts of Jsens DNA were also applied to each filter. Filters were prepared in duplicate so samples could be hybridized to both 32P-labeled DHFR cDNA (11) or 32P-labeled mouse α-fetoprotein cDNA (12). Nick translations and hybridization conditions were as described in Brown et al. (13). Filters were autoradiographed, and the degree of hybridization was estimated by scanning exposed X-ray film with a Quick-Scan densitometer (Helena Laboratories).

RESULTS

Transient Inhibition of DNA Synthesis in L5178Y Cells. The experimental protocol in which HU was used to block DNA synthesis in actively growing Jsens and C3 cells is shown in Chart 1. After 6 h of incubation with 1 and 10 mM HU, [3H]-thymidine incorporation was reduced to less than 5% of control (control, 5 x 10^{3} counts/cell; HU, 1.8 x 10^{-3} counts/cell). Six h after removal of HU, [3H]-thymidine incorporation returned to 40 to 80% of the control (control, 4.1 x 10^{3} counts/cell; HU, 16 to 34 x 10^{3} counts/cell). These results show that HU at these concentrations is a strong inhibitor of DNA synthesis, but DNA synthesis resumed rapidly once HU is removed. These data are consistent with the transient HU inhibition studies of Brown et al. (1) and Mariani and Schimke (3).

HU Enhancement of Sister Chromatid Exchange Occurring during S-Phase. The presence of SCEs is considered a manifestation of S-phase-specific DNA damage. Detection of SCEs requires that BrdUrd be incorporated into DNA for 2 cell cycles, and we have used 2 protocols to study SCEs. In the first protocol, BrdUrd was added to the medium either directly after the HU treatment (early BrdUrd label) or BrdUrd was added after the cells were allowed to recover from the treatment for 6 h (late BrdUrd label). The number of SCEs per cell was determined.

An increase in the number of SCEs produced by HU is detected when the BrdUrd was added directly after the HU treatment (Table 1). A 1 mM HU treatment gave a 4-fold increase in SCEs in Jsens cells, while the increase was 2-fold in C3 cells. Ten mM HU induced fewer SCEs than 1 mM HU in both the C3 and Jsens cell lines. In each case, the increase in number of SCEs induced by 10 mM HU was half that induced by a 1 mM HU treatment. Fig. 1, A and D, shows examples of metaphases with many SCEs induced by the HU treatment.

If BrdUrd was added 6 h after removing HU from the culture, the number of SCEs was not significantly different from the control. The Jsens cells showed no effect with either a 1 mM or a 10 mM treatment, while the C3 cells increased by only 2 SCEs per cell at 10 mM HU; neither value is significantly different from the control using Student’s t test (P > 0.05). These results show that HU induces a large increase in the number of SCEs at the time of treatment, but that once the HU is removed, the number of SCEs detected rapidly diminishes. The events that generate SCEs are completed within 6 h after HU removal, a time when S phase is still continuing in many cells.

Many metaphases present 24 h after the HU treatment was fragmented and had other chromosome aberrations. These cells did not complete an S phase or underwent only a single S phase in the presence of BrdUrd, as indicated by the lack of differentially...
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labelled chromatids. Thus, it was impossible to detect SCEs in such cells. To determine whether highly fragmented chromosomes had a high frequency of SCEs, Jsens cells were treated with BrdUrd for 12 h before 1 mw HU was added to the cultures, and following removal of HU, the cells were cultured for 24 h in medium containing BrdUrd. Under these conditions, fragmented metaphases had differentially labeled chromatids when stained by the fluorescence plus Giemsa technique; i.e., such cells had undergone an S phase prior to HU block and had completed the S phase in which HU had transiently inhibited DNA synthesis. In most of the metaphases with fragmented chromosomes, a very high number of SCEs was evident; an example is shown in Fig. 1F. We interpret this finding to indicate that fragmentation of chromosomes as induced by HU is a lethal event; i.e., cells can complete the S phase in which HU-induced fragmentation has occurred, but they cannot progress through an S phase subsequent to chromosome fragmentation. The decrease in number of SCEs observed with 10 mw HU compared with 1 mw HU as detected with BrdUrd labeling in the first 6 h after removal of HU (Table 1) is probably explained by the fact that 10 mw HU results in a greater number of cells with fragmented chromosomes; hence, they would not progress through a second S phase to allow detection of SCEs.

Chromosomal Aberrations Produced by Transient Inhibition of DNA Synthesis. A feature of both the Jsens and C3 cell lines is that the karyotype is stable under normal growth conditions. Forty to 42 chromosomes are seen in both cell types; aneuploids and polyploid cells are rare (Table 2). Yet after cells were treated with HU for 6 h and fixed 24 h after HU removal, and metaphases were stained with the fluorescence plus Giemsa technique, a wide range of chromosomal aberrations was observed. Features such as polyploidy (Fig. 1A), endoreduplication (Fig. 1D), chromatid breaks, translocations, andacentric fragments (Fig. 1C), and dicentrics (Fig. 1E) were observed commonly. Metaphases with intact chromosomes and varying amounts of extrachromosomal DNA were also seen frequently (Fig. 1B). Staining with Hoechst 33258 verified that the extra-chromosomal material was DNA. The same spectrum of chromosomal aberrations was seen when cells were treated only with HU, i.e., no BrdUrd. Thus the chromosomal aberrations observed are the consequence of HU treatment.

A summary of chromosomal aberrations seen in Jsens and C3 cells 24 h after the HU was removed is shown in Table 2. Chromosome aberrations of all types were increased in both C3 and Jsens cells. Twenty to 30% of all metaphases were abnormal after the 1 and 10 mw HU treatments. There were some differences in the frequency of aberrations induced in C3 and Jsens cells. The Jsens cells had more fragmented metaphases than did the C3 cells, while C3 cells had a larger number of metaphases with extrachromosomal DNA than did the Jsens cells.

An important observation is that the majority of chromosome aberrations appeared in metaphases seen 24 h after the HU treatment. If metaphases were examined 12 or 2 h after a 10 mw HU treatment, 92 to 95% of the metaphases were normal. Thus the time for observation is critical. Cells with aberrant chromosomes either fail to divide multiple times (hence not detected at mitosis), or the aberrations are resolved, since few chromosome aberrations are seen 72 h after HU treatment.

HU Effects on Cell Survival and MTX Resistance. Since many chromosomal abnormalities were present after HU treatment, it was important to determine the long-term viability of treated cells. Although Jsens and C3 cells normally grow in suspension, they can form colonies from single cells when grown in 0.4% soft agar. Eighteen h after HU treatment, Jsens cells were counted and placed into soft agar to determine relative plating efficiencies. At the same time, cells were placed in soft agar containing 10 mw MTX to determine the number of MTX-resistant colonies that arose after the HU pretreatment.

Treatment with 1 or 10 mw HU reduced the plating efficiency of the Jsens cells to 28% of the control. When HU-treated cells were tested for resistance to 10 mw MTX, a 4.8- to 6.5-fold increase in resistant colonies was observed (Table 3). The increase in MTX-resistant colonies was dependent on when cells were plated into MTX. If the cells were allowed to recover from the HU treatment 72 h before they were plated into soft agar, the relative plating efficiency without MTX increased so that 73% of the cells formed colonies in soft agar. When these same cells are selected in 10 mw MTX, the number of resistant colonies from a 10 mw HU treatment was only 1.7-fold higher than from control cells. These results are consistent with the studies of Brown et al. (1), which showed that the frequency of MTX resistance was most enhanced if cells were plated into MTX 15 h after the removal of HU.

MTX resistance can occur by several mechanisms including altered transport of MTX into cells (14), altered affinity of DHFR for MTX (15, 16), and amplification of the DHFR gene (17). Soft agar clones resistant to 10 mw MTX from control, 1 mw HU, and 10 mw HU-treated cells were expanded by growing them in suspension, and DNA from each clone was analyzed to determine if the DHFR gene had been amplified. The analysis was done by a modified dot hybridization technique in which 1 μg of DNA from each clone was hybridized to 32P nick-translated cDNA to DHFR. At the same time, 1 μg of DNA from each clone was hybridized to 32P nick-translated α-fetoprotein. Previous studies
by Brown et al. (1) showed that α-fetoprotein does not amplify under MTX selection; therefore it acts as an internal standard to measure the amount of DNA present.

The comparison between DHFR cDNA hybridization and α-fetoprotein cDNA hybridization in the 12 clones studied is shown in Fig. 2. The amount of hybridization was quantitated by densitometry, and the degree of DHFR amplification was determined by comparing the ratio of DHFR to α-fetoprotein hybridization; clones showing a ratio of 1.5 or greater were considered to have amplified DHFR genes (Fig. 2). With this technique, all 8 of the clones pretreated with HU showed increased DHFR gene copy number relative to the control. None of the 4 MTX-resistant clones from control cells had amplified the DHFR gene. These results in lymphoma cells show that HU-pretreated cells have a higher frequency of DHFR gene amplification to account for the increased amount of DNA per cell and the increased cell size are transient properties of the cell population. If cells are fixed 24 h after the removal of HU and examined under the microscope, many of these cells have a diameter of more than 2 times control cells. The increased amount of DNA per cell and the increased cell size are transient properties of the cell population. If cells are fixed 24 h after the removal of HU and examined under the microscope, many of these cells have a diameter of more than 2 times control cells.

Analysis of Hydroxyurea-treated Cells Using the FACS. Studies by Mariani and Schimke (3) showed that if HU were added during S phase to synchronously growing CHO cells, overreplication of DNA occurred when HU was removed from the culture. We were interested in studying whether J sera cells had an increased cellular DNA content following treatment with HU. The amount of DNA was measured by staining cells with the fluorochrome Hoechst 33342 and sorting them with the FACS 24 h after HU was removed. The data from these experiments are displayed as a 2-dimensional contour plot of cell distribution in which cell size is displayed along the Y axis and DNA content on the X axis. A series of such distributions for control cells and cells treated with 10 mM HU is shown in Chart 2. In control cells, integration analysis indicated that 15.8% of the cells showed a fluorescence intensity of 110 units or greater (Chart 2A). Twenty-four h after the removal of cells from 10 mM HU, 35.2% of the cells show fluorescence greater than 110 units (Chart 2B). The cells that show higher Hoechst fluorescence also have a larger amount of forward scatter during the sorting, which indicates increased cell size. If cells are fixed 24 h after the removal of HU and examined under the microscope, many of these cells have a diameter of more than 2 times control cells. The increased amount of DNA per cell and the increased cell size are transient properties of the cell population. If cells are allowed to recover for 72 h prior to FACS analysis, the population is very similar to the control with respect to cell size and DNA content (Chart 2C).

J sera cells treated with 10 mM HU were also analyzed 24 h after the removal of HU to determine if individual cells in the population had an increased amount of DHFR enzyme. This was measured by staining cells with a fluorescein conjugate of MTX (F-MTX) and measuring the number of highly fluorescent cells on the FACS. A number of studies from this laboratory (9, 18) have shown that the fluorescence intensity of cells stained with F-MTX is a function of DHFR content.

Contour plots for cells stained with F-MTX are shown in Chart 2, D to F. The brightest cells in the control population, those with

**Table 3**

<table>
<thead>
<tr>
<th>Unsorted J sera cells</th>
<th>Plating efficiency in soft agar (%)</th>
<th>Frequency of survivors in 10 mm methotrexate ($10^{-5}$)</th>
<th>Fold increase over control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>71.0 ± 8.9</td>
<td>0.92 ± 0.1</td>
<td>1.0</td>
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<tr>
<td>1 mM HU</td>
<td>20.5 ± 4.5</td>
<td>5.93 ± 0.9</td>
<td>6.5</td>
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<tr>
<td>10 mM HU</td>
<td>19.5 ± 2.8</td>
<td>4.44 ± 0.5</td>
<td>4.8</td>
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<tr>
<td>10 mM HU (72 h after HU)</td>
<td>52.0 ± 5.3</td>
<td>1.57 ± 0.3</td>
<td>1.7</td>
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</table>

<table>
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<tr>
<th>Cell-sorted populations</th>
<th>Plating efficiency in soft agar (%)</th>
<th>Frequency of survivors in 10 mm methotrexate ($10^{-5}$)</th>
<th>Fold increase over control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, F-MTX dull</td>
<td>13.7</td>
<td>0.66</td>
<td>1.0</td>
</tr>
<tr>
<td>Control, F-MTX bright</td>
<td>3.2</td>
<td>3.30</td>
<td>5.0</td>
</tr>
<tr>
<td>10 mM HU, F-MTX dull</td>
<td>10.5</td>
<td>2.86</td>
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<td>10 mM HU, F-MTX bright</td>
<td>0.8</td>
<td>5.69</td>
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<td>Control, Hoechst dull</td>
<td>33.2</td>
<td>0.48</td>
<td>1.0</td>
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<tr>
<td>Control, Hoechst bright</td>
<td>31.2</td>
<td>0.10</td>
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<td>10 mM HU, Hoechst dull</td>
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<td>0.32</td>
<td>0.7</td>
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<tr>
<td>10 mM HU, Hoechst bright</td>
<td>10.4</td>
<td>1.54</td>
<td>3.2</td>
</tr>
</tbody>
</table>

* Mean ± SE.
and have an increased amount of DNA per cell. Hoechst fluorescence were also bright for Hoechst fluorescence, control recover from HU for 72 h before FACS analysis, the pattern is larger cell size are transient; thus if the cells are allowed to than 90 units (Chart 2E). In addition to having more DHFR, these of the population (Chart 2D). Twenty-four n after the removal of populations Sorted Using the FACS. By analysis with the larger than normal cells, have increased DHFR enzyme content, Greater than 50% of the cells bright for F-MTX fluorescence and HU-treated cells were stained with both F-MTX and Hoechst. activated cell sorter. Chart 2, A to C, shows cells stained for 1 h with Hoechst 33342 to determine DNA content per cell. A, control cells; 15.8% of the population has a fluorescence intensity of 110 units or greater. In B, cells are pretreated with 10 mM HU and analyzed 24 h after HU removal; 35.5% of the population has fluorescence intensity of 110 units or greater. In C, cells are pretreated with 10 mM HU and analyzed 72 h after HU removal; only 11% of the population has a fluorescence intensity of 110 units or greater. Chart 2, D to F, shows cells stained for 24 h with F-MTX to determine DHFR enzyme content. D, control cells; 8.3% of the population has a fluorescence intensity of 90 units or greater. In E, cells are pretreated with 10 mM HU and analyzed 24 h after the removal of HU; 51.6% of the population has a fluorescence intensity of 90 units or greater. In F, cells are treated with 10 mM HU and analyzed 72 h after HU removal; only 4% of the cells have a fluorescence intensity of 90 units or greater. Contour plots are based on 10,000 cells; each line represents 20% of the population.

F-MTX fluorescence greater than 90 units, make up only 8.3% of the population (Chart 2D). Twenty-four h after the removal of HU, 51.6% of the cells have a fluorescence intensity of greater than 90 units (Chart 2E). In addition to having more DHFR, these cells are also larger in size. This increase of DHFR per cell and the larger cell size are transient; thus if the cells are allowed to recover from HU for 72 h before FACS analysis, the pattern is similar to untreated cells (Chart 2F).

In order to show that the HU-treated cells with bright F-MTX fluorescence were also bright for Hoechst fluorescence, control and HU-treated cells were stained with both F-MTX and Hoechst. Greater than 50% of the cells bright for F-MTX fluorescence were also bright for Hoechst fluorescence (data not shown). From these data, we conclude that, after cells are treated with HU, a subpopulation of cells is generated in which the cells are larger than normal cells, have increased DHFR enzyme content, and have an increased amount of DNA per cell.

Chromosome Abnormalities and MTX Resistance in Cell Populations Sorted Using the FACS. By analysis with the FACS, we have shown that a subpopulation of cells with high fluorescence for F-MTX (Chart 2, D to F) and Hoechst 33242 (Chart 2, A to C) appeared 24 h after HU treatments. These subpopulations were analyzed for the frequency of chromosomal abnormalities and increased resistance to MTX. Control and HU-treated cells were stained separately with F-MTX and Hoechst and sorted into bright and dull subpopulations. The cells were then treated with Colcemid, and metaphase spreads were examined. The types of abnormalities observed are summarized in Table 4.

When sorting cells for variable staining with F-MTX, metaphases observed in the control dull population were normal. Among the control bright cells, only 2% of the metaphases were abnormal. In contrast, in cells treated with 10 mM HU, chromosome aberrations were common. Four % of the treated dull cells were abnormal, while 28% of the bright cells had chromosome alterations. Many of the latter were polyplid, had extrachromosomal DNA, and/or were fragmented.

When the same cells were stained with Hoechst 33342 and sorted into dull and bright subpopulations, the metaphases in the control dull population were normal, while 5% of the metaphases in the control bright population were abnormal. In the 10 mM HU-treated cells, the dull population had few aberrant metaphases; 8% of the metaphases were abnormal. The greatest number of chromosome aberrations was seen in the HU-treated bright population. Fifty-eight % of these metaphases were abnormal, and many types of chromosome aberrations were seen (Table 4).

These studies show that the subpopulations of large cells produced by HU treatment, which has more DHFR and more DNA per cell, also has a high frequency of cells with chromosome aberrations. To determine whether there were viable cells in this subpopulation and if these cells display increased resistance to MTX select, experiments were performed on control cells and on cells sorted 24h after HU treatment (Table 3). Cells sorted by the FACS had a much lower viability than unsorted cells. In cells selected with MTX, only 13.7% of the control dull cells formed colonies in soft agar. Both control bright cells and the 10 mM HU bright cells had low plating efficiencies compared to the control dull cells and the 10 mM HU dull cells. However, all populations did produce viable colonies. When these same F-MTX dull and bright populations were selected in soft agar containing 10 mM MTX, almost 9 times as many colonies resistant to MTX occurred in the 10 mM HU bright cells than from the control dull cells. An intermediate increase in MTX resistance was seen in the control bright cells and the 10 mM HU dull cells (Table 3). In a separate experiment, control and 10 mM HU-treated cells were stained with Hoechst 33342 and sorted 24 h later. An increased frequency of MTX resistance was seen only in the Hoechst bright subpopulation.

From the series of cells sorted with F-MTX, 6 MTX-resistant colonies from the control cells (3 dull and 3 bright) and 6 MTX-resistant colonies from 10 mM HU-treated cells (3 dull and 3 bright) were expanded by growing them in suspension; metaphases and DHFR gene copy number for each colony were analyzed. No gross chromosomal aberrations were seen in any of the clones, although polyplid cells were more common than...
in the parent population. Analysis of the DHFR gene copy number showed that none of the control dull or HU dull colonies had amplified the DHFR gene. One of the 3 control bright clones and 2 of the 3 HU bright clones had amplified the DHFR gene. Because of the small number of colonies examined, we can made no conclusions concerning the relative frequency of gene amplification among the various cell populations.

**DISCUSSION**

Previous studies by Mariani and Schimke (3) examined effects of HU on synchronously growing CHO cells that contained 50 copies of the DHFR gene. When DNA synthesis was blocked by HU in the second hour of S phase, a large number of surviving cells could form colonies in medium containing a 100-fold increase in MTX concentration. Few control cells or cells treated with HU in other parts of the cell cycle could survive this MTX selection. In these same experiments, Mariani and Schimke found that the 10% of the genome replicated before the addition of HU in the second hour of S phase was rereplicated after HU was removed. These studies provided biochemical evidence that part of the genome is rereplicated after DNA synthesis is transiently inhibited.

Our present studies have examined the effects of HU on lymphoma cells to determine if DNA synthesis inhibition increases the frequency of gene amplification and results in overreplication of DNA. Our approach has been to use the FACS to measure DNA content by measuring the amount of Hoechst dye bound intracellularly. The data presented here show that Jsens cells that survive DNA synthesis inhibition by HU have a high rate of resistance to MTX due to an increased DNA content of DHFR gene amplification. HU-pretreated cells stained with Hoechst show a significant portion of the population with increased fluorescence, indicating increased DNA content. When metaphase chromosomes of HU-treated cells with high Hoechst fluorescence were examined, many of these showed extra chromosomes or small, extrachromosomal DNA. The data from these studies and from Mariani and Schimke (3) demonstrate an increased amount of DNA by 2 independent experimental approaches. Studies by Johnston et al. (2) show in Chinese hamster ovary cells that critical parameters in demonstrating an increase in DNA content as measured by Hoechst staining are the length of HU exposure as well as the population density of the exposed cells.

The results from our laboratory are consistent with the idea that at least some forms of DNA synthesis inhibition result in uncoordinated DNA synthesis. Mariani and Schimke (3) interpreted their results to indicate that rereplication occurred in the time frame of a single cell cycle, defined as the time from M to M. An alternative (not mutually exclusive) hypothesis is that treatment with HU results in a partial dissociation between S and M phases, such that some cells progress into the period of chromosome condensation, i.e., into M at a time when they are in the first portion of a second S phase. Either hypothesis results in cells with additional DNA per cell. After HU treatment, many of the surviving cells have more DHFR enzyme and more DNA per cell. If these cells are placed under drug selection, a high proportion of cells with extra functional copies of the DHFR gene will survive MTX selection and form a resistant colony. Other overreplicated DNA presented after HU treatment will be highly unstable and be lost in the absence of a specific selection.

An important aspect of these studies is the extent of cell death induced by HU treatment. If Jsens cells are examined for viability by trypan blue exclusion directly after HU is removed, few cells are dead (data not shown). However, only 30% of these cells are able to form colonies in soft agar. When HU-treated cells are sorted through the FACS, the cell viability as measured by colony formation diminishes to 10% or less. A similar, but less extensive decrease in viability after sorting was reported by Mariani and Schmike in Chinese hamster ovary cells (3). The reason for such a reduced viability is unknown, but we speculate that a combination of mechanical fragility of cells and reduced viability as a result of numerous chromosome aberrations is involved. The dyes used in FACS sorting are somewhat toxic and may further lower plating efficiencies.

Chromosome analysis of HU-treated cells shows many types of chromosome aberrations. The mechanism by which HU induces chromosome aberrations is not clear. HU slows down the rate of DNA chain elongation (19). Even in the presence of high HU concentrations, low levels of DNA synthesis continue (20). DNA synthesized during HU treatment is abnormally small and is ligated into large-molecular-weight DNA at a reduced rate. Studies by Radford et al. (21) showed that the accumulation of low-molecular-weight DNA was correlated with increased cytotoxicity in HU-treated cells. Radford et al. (21) proposed that DNA synthesis inhibition is the key event in HU-induced cytotoxicity; DNA synthesis inhibition leads to chromosome lesions, specifically chromosome breaks which are the ultimate cause of cell death.

Other DNA synthesis inhibitors have effects similar to HU. Rainaldi et al. (22) showed that when aphidicolin, 1-β-D-arabinofuranosylcytosine, and thymidine were used to inhibit DNA synthesis, a large increase in SCEs was seen if synchronized cells were treated in the early part of S phase. Woodcock and Cooper (23) showed that when 9-β-D-arabinofuranosyladenine, 1-β-D-arabinofuranosylcytosine, and cyclohexamide inhibited DNA synthesis, aberrant double replication of chromosomal segments resulted. They proposed that double replication of chromosomal DNA is a general consequence of freezing DNA replication forks and not specific to the type of inhibitor. Studies by Tlsty et al. (2) showed that 3T6 cells pretreated with UV light or N-acetoxy-N-acetylaminofluorene had an increased frequency of MTX resistance and gene amplification. Treatment of Chinese hamster ovary cells with UV light results in chromosomal aberrations similar to those described here. Increases in MTX resistance were seen when cells were pretreated by aphidicolin, and Johnston et al. have shown that treatment of Chinese hamster cells with aphidicolin results in increased DNA per cell. Collectively such studies suggest that DNA synthesis inhibition produces altered DNA replication that can later lead to specific gene amplification under selection conditions, chromosome aberrations, and cell death.

A striking finding of our studies is that the subset of HU-treated cells with additional DNA per cell is the same subset of cells with chromosomal aberrations. Such cells cannot have simply undergone arrest in M phase, followed by endoreduplication in a second S phase, inasmuch as the additional DNA per cell is not a multiple of the 2C content; furthermore the chromosomal aberrations are of multiple types, i.e., fragmented chromosomes, dicentric chromosomes, extrachromosomal DNA, and varying degrees of polyploidy. In addition, we do not believe that the increase in DNA content per cell results from fusion of cells with fragmented chromosomes for 2 reasons. (a) Following resumption of DNA synthesis after HU treatment, DNA per cell as determined by the FACS increases progressively over a 6- to 24-h period. If cell fusion was occurring, the number of cells with increased DNA would have increased, not the amount of

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*Johnston, et al., manuscript in preparation.

*P. C. Brown, unpublished results.

*6P. C. Brown, unpublished results.

*8T. D. Tlsty, unpublished results.

*7Johnston, et al., unpublished observation.
DNA per cell. (b) Coculturing of HU-treated L5178Y C3 cells (which have more than 800 copies of the DHFR gene) and HU-treated CHO DHFR− cells (24) did not result in any (frequency < 7 x 10^-8) CHO cells converting to DHFR+. This is an extremely sensitive method for detecting uptake and function of DNA (25).

Our current hypothesis is that overreplication of DNA is a prior event in which various modes of recombination result in observed chromosomal aberrations. We speculate that DHFR gene amplification and chromosome aberrations result from overreplication of DNA after HU treatments. Thus, to the extent that various agents used in cancer chemotherapy kill cells by chromosome fragmentation, the very same molecular process results in emergence of drug resistance by gene amplification.

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Fig. 1. Chromosomal aberrations induced by hydroxyurea. Metaphases were examined 24 h after the removal of HU, and differentially stained sister chromatids were examined for SCEs. A, polyploid cell with many SCEs. B, metaphase with normal chromosomes and a large amount of small extrachromosomal DNA (arrow). C, metaphase with multiple chromosome gaps and breaks. D, an endoreduplicated metaphase with a large number of SCEs. E, metaphase with a dicentric chromosome (arrow). F, a highly fragmented metaphase (note break at arrow) showing a very high frequency of SCE.
Increased Gene Amplification in L5178Y Mouse Lymphoma Cells with Hydroxyurea-induced Chromosomal Aberrations

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