Genetic Transformation of Murine Bone Marrow Cells to Methotrexate Resistance

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Genetic transformation of murine bone marrow stem cells to methotrexate resistance was achieved using a modified calcium phosphate-DNA coprecipitation procedure. Bone marrow cells were transformed by DNA derived from methotrexate-resistant mouse 3T6 cells. In vivo selection of drug-resistant bone marrow cells resulted from thrice weekly injections of methotrexate (MTX) for a period of 6–8 wk. Following selection, dihydrofolate reductase activity encoded by the donor DNA species was easily detectable in extracts of recipient mouse spleens. In addition, selection of methotrexate-resistant cells was indicated by the persistence of spleen colony-forming units (CFU-S) in drug-treated animals. Also, changes in ratios of mixed syngeneic bone marrow cells derived from CBA and CBA/T6T6 mice resulted from initial treatment of either cell type with 3T6 DNA. These results confirm and extend the observations of Cline and coworkers that normal bone marrow cells can be genetically transformed to methotrexate resistance.

A NOVEL IDEA for reducing the toxicity of various anticancer drugs to bone marrow cells is to induce drug resistance in these cells. The experiments of Cline and coworkers suggested that methotrexate (MTX) resistant murine bone marrow cells could be selected for in vivo following injection of cells treated in vitro with DNA from a MTX-resistant mouse cell line (3T6). MTX-resistant bone marrow cells were routinely observed in different mice in these experiments, despite the expected low frequencies of transformation to MTX resistance (usually less than 10⁻⁵ with cell lines in tissue culture). The induction of resistance to MTX in the majority of mice, together with the presumably small numbers of pluripotent stem cells injected, suggested that the efficacy of transformation for these cells was higher than for most established cell lines. The generality of transformation of bone marrow cells to MTX resistance has been further investigated, and an improved transformation procedure has been developed.

MATERIALS AND METHODS

Cells

Bone marrow cells were obtained from femurs of 6-wk-old female CBA and CBA/H-T6T6 mice (Jackson Laboratory, Bar Harbor, ME) by flushing the marrow cavity with ice-cold McCoy's 5A medium supplemented with 10% fetal calf serum (FCS, Gibco, Grand Island, NY) and 1 U/ml heparin. Donor mice were treated with 4 mg/kg vinblastine 3 days prior to sacrifice to increase the number of marrow hemopoietic stem cells. Chinese hamster ovary (CHO) wild type and MTX-resistant A29 cells (obtained from Dr. R. Kellems) were grown in Dulbecco's modification of Eagle's medium (DMEM) containing 10% FCS and 3 × normal nonessential amino acids. A29 cells (Pro 3 MTXRII2) possess an increased affinity for MTX and are resistant to MTX. Mouse embryo 3T6 fibroblast cells (obtained from Dr. R. Kellems) were grown in Dulbecco's MEM with 10% FCS and 4 × 10⁻⁶ M MTX. These cells contain high levels of mutant DHFR, which has reduced affinity for MTX compared to wild type enzyme. A29 cells (Pro 3 MTXRII2) possess an increased activity of mutant form of CHO dihydrofolate reductase (DHFR) activity, which is more resistant to MTX inhibition than the wild type enzyme. Mouse embryo 3T6 fibroblast cells (obtained from Dr. R. Kellems) were grown in Dulbecco's MEM with 10% FCS and 4 × 10⁻⁶ M MTX. These cells contain high levels of mutant DHFR, which has reduced affinity for MTX compared to wild type enzyme. For bone marrow cell experiments, 2 × 10⁶ cells/ml in 50 ml McCoy's 5A medium containing 10% FCS were treated directly in a 150 ml calcium phosphate coprecipitate with DNA (100 µg) or chromosomes (1–2 cell equivalents). The cells were incubated for 4 hr and then pelleted and resuspended in fresh medium. This suspension also included particles of the micro-precipitate, and in preliminary experiments, injection of this suspen-
tion resulted in the rapid death of recipient mice. Therefore, the concentrated cell suspensions were layered onto a shelf of 9.6% (w/v) sodium metrizoate (Lymphoprep, Accurate Scientific and Chemical Company, New York) and centrifuged at 500 g for 10 min. Under these conditions, the microprecipitate pellet, leaving the bone marrow cells in a band at the metrizoate-medium interface. The cells were collected, diluted to 6 x 10^5 ml in fresh medium, and 0.3 ml were injected into X-irradiated (875 rad) recipient mice.

Early experiments utilizing the above protocol with calcium phosphate coprecipitates with CHO or A29 DNA were unsuccessful (see Results). Modifications designed for cells propagated in suspension were tested using genetic transformation of CCRF-CEM cells to MTX resistance as a model system. The optimal protocol involved treating cells at about 10^6/ml with an equal volume of calcium phosphate coprecipitate with either 3T6 or L5178Y DNA. After 15 min, the cells were pelleted and resuspended in 5 volumes of fresh medium (~10^7 cells/ml). Following an overnight incubation, the cells were collected and separated from the precipitates on a sodium metrizoate shelf, as described above. These cells were then incubated in fresh medium at 2 x 10^5/ml for 30 hr, and MTX was added at 10^-6 M for 72 hr. Finally, the cells were diluted with 1.5 volumes of 0.2% agar in RPMI + 20% horse serum and plated in culture tubes. Attempts to genetically transform murine 416B cells involved an identical protocol, except that 416B cells were exposed to 3 x 10^-7 M MTX for 168 hr before plating in agar.

Successful adaption of the above modified protocol for genetic transformation of bone marrow cells involved treating 10^8 cells in 6 ml of McCoy's 5A medium containing 10% FCS with 6 ml of a calcium phosphate coprecipitate with either 3T6 or L5178Y DNA. After 15 min, the cells were diluted into 60 ml fresh medium and incubated for a further 4-5 hr at 37°C. Isolation of DNA-treated cells and subsequent injection into recipient irradiated CBA mice was as described above. In experiments involving mixing CBA and CBA/T6T6 cells, 0.2 ml of L5178Y DNA-treated cells was mixed with 0.1 ml of 3T6-treated cells prior to injection. The MTX treatment regimen was initiated 4 days after injection of donor marrow cells into recipient mice. All injections were administered intraperitoneally on a Monday/Wednesday/Friday schedule.

**CFU-S Assays**

The number of spleen colony-forming cells (CFU-S) in bone marrow of experimental animals was determined by injecting 5 x 10^5 or 10^6 cells into previously irradiated (900 rad) CBA mice. Spleen colonies were scored after 10-12 days.

**DHFR Assays**

DHFR activity of mouse spleens was assayed using crude extracts prepared as follows. Spleens were minced in 50 ml Tris, pH 7.5, 2.5 mM MgCl2, 150 mM KCl, and 100 μg/ml 3 mM dithiothreitol (0.2 ml/spleen). The extract was sonicated on ice with 4 15-sec bursts, using a Branson sonicator at setting 5. The sonicate was then centrifuged at 15,000 rpm for 30 min at 4°C in a Sorvall refrigerated centrifuge, and the supernatant was collected and assayed directly. DHFR activity was measured in a Gilford spectrophotometer at 37°C. The assay mix, in 1 ml, included 100 mM Tris, pH 7.5, 150 mM KCl, 100 μg/ml NADPH, 20 μM FH2, plus the extract and drug solution. The oxidation of NADPH was followed at 340 nm in 4 different assays simultaneously. Spleen extracts from experimental animals were usually assayed in the presence and absence of MTX (3 x 10^-7 M) in simultaneous readings recorded on the same chart. DHFR activities were estimated from the slopes of the ODmax versus time plots. One unit of enzyme activity is defined as 1 nmole of NADPH oxidized in 1 hr.

**Hematocrit Measurements**

Hematocrits were measured using blood drawn from the retroorbital sinuses of live mice using heparinized capillary tubes.

**Chromosome Analysis**

Analysis of chromosomes from bone marrow cells of CBA and CBA/H-T6T6 mice was based on the method of Ford.13 Mice were injected intraperitoneally with 4 mg/kg colcemid 70-90 min before sacrifice. Bone marrow cells were flushed from the femurs of freshly killed mice with McCoy's 5A medium, 15% FCS, and 1 U/ml heparin. Cells were swollen in 75 mM KCl for 30-35 min at 37°C and fixed in ice-cold methanol/glacial acetic acid (3:1) for 1 hr (fixative changed 3 times). Chromosome spreads were prepared by dropping the cell suspension onto microscopic slides from a height of 2-5 feet. Final chromosome spreads were stained with Trypsin-Wright's stain.14 Spreads of 50-100 chromosomes were scored for each analysis.

**RESULTS**

**Establishment of Methotrexate Regimen for CBA Mice**

An appropriate maximally tolerated dose regimen for MTX was first established using CBA mice. This was done by monitoring hematocrits and CFU-S levels in live mice as an indication of the hemopoietic suppression induced by various doses of MTX. Mice were initially X-irradiated with 875 rad and injected on the following day with 2 x 10^5 bone marrow cells from vinblastine-treated CBA donors. Injections were initiated 4 days later, and hematocrits were monitored after 3 and 6 wk. Control experiments indicated that blood sampling (up to 0.1 ml) from these mice, even on a weekly basis, had no effect on hematocrits and CFU-S values. Table 1 shows the change in hematocrits and CFU-S values with different dose schedules. The schedule chosen for selection of transformed bone marrow cells involved treatment with 2 mg/kg in the first week following irradiation, 6 mg/kg in the second week, and 12 mg/kg in the third and subsequent weeks (2/6/12 regimen). The 0.5/2/4 regimen was similar to that employed by Cline and coworkers in their experiments. However, in our hands, this regimen had no detectable hematologic toxicity.

**Failure to Transform Bone Marrow Cells to Methotrexate Resistance Using A29 DNA and Chromosomes**

Initial attempts to genetically transform bone marrow cells to MTX resistance involved the use of calcium phosphate coprecipitates with A29 DNA or chromosomes. Chinese hamster ovary A29 cells have been shown to overproduce a mutant species of DHFR that is more resistant to MTX inhibition than the wild
type enzyme. DNA and chromosomes prepared from A29 cells can transform mouse LTK cells to MTX resistance with efficiency in excess of 10^5. We have routinely used genetic transformation of LTK cells as a positive control in our experiments. Despite successful transformation of these cells to MTX resistance using A29 DNA or chromosomes, we were initially unable to find any evidence for transformed MTX-resistant CBA bone marrow cells using similar protocols (see Materials and Methods). This conclusion was based on hematocrit and CFU-S measurements.

Transformation of Human Lymphoblast Cells in Suspension Culture to Methotrexate Resistance

Following our failure to transform bone marrow cells to MTX resistance with A29 DNA or chromosomes, we decided to examine in more detail the gene transfer procedure. In particular, we wished to optimize the genetic transformation of cells grown in suspension culture, a procedure that we and others have observed to be less efficient than transformation of cells attached to a substrate. We observed that only about 20% of a freshly isolated bone marrow cell suspension adheres to the surface of tissue culture flasks in the 4-hr period employed for exposure to calcium phosphate-DNA coprecipitates. Most spleen colony-forming cells are detachable in the suspension during this periods. Attempts were made to genetically transform human lymphoblast CCRF-CEM suspension cells to MTX resistance using A29 DNA or chromosomes. The standard transformation protocol used previously failed to yield MTX-resistant CCRF-CEM. However, a modified protocol, which involved increasing the cell number and the volume of calcium phosphate microprecipitate by a factor of 5 (see Materials and Methods), led to the isolation, in 5 of 6 plates, of colonies of MTX-resistant CCRF-CEM cells induced by A29 chromosomes. The DHFR activity associated with one of the resulting cell lines was then characterized to test for expression of the mutant A29 DHFR species. The specific activity of DHFR in the putatively transformed CCRF-CEM was found to be elevated about 36-fold compared to wild type CCRF-CEM cells (Table 2). This correlated with the observed survival of the transformed cells at 10^5 M and 10^-4 M MTX, despite selection in 10^-6 M MTX (data not shown). Titration of equal amounts of enzyme activity with different doses of MTX revealed that DHFR from transformed CCRF-CEM cells was more resistant to MTX inhibition than wild type CCRF-CEM enzyme and was similar in resistance to A29 DHFR (Fig. 1). These results strongly suggest that the MTX-resistant CCRF-CEM cells were genetically transformed by donor A29 chromosomes using the modified transformation protocol.

| Table 1. Effect of Methotrexate Treatment Schedules on Hematologic Parameters |
|-------------------------|-------------------------|-------------------------|-------------------------|
| Dose Schedule of MTX (mg/kg) | Week 1 | Week 2 | Weeks 3-6 |
| Week 1 | Week 2 | Weeks 3-6 |
| hematocrit (Week 3) (%) | hematocrit (Week 6) (%) | CFU-S/10^6 Cells (Week 6) |
| 0 | 0 | 0 | 49 | 51 | 20 ± 3 |
| 0.5 | 2 | 4 | 51 | 50 | 29 ± 2 |
| 1 | 4 | 8 | 51 | 46 | 4 ± 2 |
| 2 | 6 | 12 | 48 | 27 | 1 ± 1 |
| 3 | 8 | 16 | 39 | ND* | ND |
| 4 | 10 | 20 | 36 | ND | ND |

Results are means of at least three independent measurements (± SD for CFU-S measurements). Standard deviations for hematocrits were all within 15% of the means. The dose schedules involved Monday/Wednesday/Friday (p.m.) injections, and hematocrits and CFU-S levels were assayed on Thursdays (p.m.), as described in Materials and Methods.

*ND, not determined due to death of mice.

Following the successful transformation of suspension-cultured CCRF-CEM cells, we attempted to genetically transform the biopotential hemopoietic cell line, 416B with A29 chromosomes. The cell line may be closely related to murine hemopoietic stem cell by virtue of a virally induced block in differentiation, and therefore may provide a good in vitro model for proliferating stem cells. Despite the low plating efficiency (0.5%) for 416B cells in soft agar, we were able to effectively screen 3.2 x 10^5 cells (equivalent to 6.4 x 10^6 cells plated) for genetic transformation to MTX resistance by A29 chromosomes. However, no colonies were isolated after exposure to 3 x 10^-7 M MTX, suggesting a transformation efficiency with A29 chromosomes of less than 3 x 10^-6. Other experiments aimed at genetically transforming freshly isolated bone marrow cells to MTX resistance, as judged by the appearance of MTX-resistant granulocyte-macrophage colonies (CFU-C) in methylcellulose (plating efficiency of 0.1%), were also unsuccessful, having effectively screened 8 x 10^4 cells.

| Table 2. Comparative DHFR Activities of Hamster and Human Cell Lines |
|-------------------------|-------------------------|-------------------------|
| Cell Line | DHFR Activity (U/mg Protein) |
| CHO | 98 |
| A29 (CHO MTXx10^6) | 4,832 |
| CCRF-CEM | 80 |
| CCRF-CEM MTX | 2,912 |

Enzyme activities are expressed in units. One unit is defined as 1 nmole of NADPH oxidized per hour. The activities are means for 3 estimations, with standard deviations of less than 20%.
Transformation of Bone Marrow Cells to Methotrexate Resistance With 3T6 or pHG DNA

The choice of donor A29 genetic material for previous bone marrow transformation experiments was based on the observation that mutant A29 DHFR genes can efficiently induce easily selectable transformed MTX-resistant mouse cells. The previously successful genetic transformation of mouse bone marrow cells by Cline and coworkers involved treatment of marrow cells with DNA from 3T6 cells. Recent studies have revealed that 3T6 cells produce a mutant DHFR activity over 100-fold resistant to MTX inhibition compared to the wild type enzyme activity. This represented a proportionally greater MTX resistance than was found for the DHFR activities of A29 cells compared to wild type CHO cells (15-fold; Fig. 1). Studies with the 3T6 subline, induced in this laboratory to grow in 2 mM MTX, revealed an even greater relative resistance (10,000-fold) of 3T6 DHFR activities to MTX (Fig. 2). The mutant enzyme activity in this case titrated over a relatively narrow dose range of MTX ($10^{-5}$–$10^{-4}$ M) compared with the previously characterized 3T6 activity ($10^{-3}$–$10^{-5}$ M).

The presence of one or more mutant DHFR genes in 3T6 cells might explain the success of the bone marrow cell transformation experiments of Cline and coworkers. Therefore, we subsequently used 3T6 DNA in attempts to transform bone marrow cells to MTX resistance. For these experiments, we utilized mixtures of syngeneic CBA and CBA/T6T6 bone marrow cells, which are easily distinguishable by chromosomal analysis. Mixtures of cells treated with calcium phosphate coprecipitates with 3T6 DNA and L5178Y DNA in ratios of 1:2 were injected into irradiated recipient CBA mice (Table 3). Hematocrits were measured 43 days after injection, and the mice were sacrificed between days 46 and 58. About half of the mice were treated with colcemid 70–90 min before sacrifice, and bone marrow cells from these mice were used for chromosomal analysis. Bone marrow cells from the remaining mice were used for CFU-S assays. The spleens were removed from every experimental mouse and utilized for DHFR assays. Enzyme assays for DHFR were performed in the presence or absence of $3 \times 10^{-7}$ M MTX for animals injected with 3T6 DNA-treated marrow cells. This dose is completely inhibitory to wild type mouse DHFR enzyme, while it does not inhibit the activity from 3T6 cells (Fig. 2).

A summary of results of all of the analyses performed on mice injected with 3T6-treated bone marrow cells is provided in Table 3. In every case, animals transformed with 3T6 DNA displayed elevated hema-
tocrits compared to control animals injected with untreated or L5178Y DNA-treated cells only. This increase was striking for animal 3T6-3, which displayed a hematocrit in the range of uninjected controls. In spleens derived from all but one animal (3T6-9), DHFR activities characteristic of those encoded by 3T6 genes were detected, indicating that these genes were being expressed in the spleens of positive animals. Chromosomal analysis of bone marrow cells revealed a significantly increased relative proliferation of 3T6 DNA-treated marrow cells compared to L5178Y-treated cells in all animals except 3T6-8. Finally, spleen colony-forming units derived from marrow cells of the 3T6 series mice were detected in all cases, except from mice 3T6-9 and 3T6-10.

Therefore, at least two positive results suggesting the presence of transformed MTX-resistant bone marrow cells were obtained for all animals, apart from mice 3T6-9 and 3T6-10. These mice displayed slightly elevated hematocrits, for reasons that are presently unclear. The results indicate that treatment of mouse bone marrow cells with calcium phosphate coprecipitates and 3T6 DNA, using a modified protocol, gives rise to transformed MTX-resistant cells at high efficiency.

**DISCUSSION**

These results confirm the observations of Cline and coworkers\(^1\) that MTX-resistant mouse bone marrow cells can be efficiently produced in vivo by transformation with calcium phosphate–3T6 DNA coprecipitates in vitro. Several improvements in the protocol used by Cline and coworkers have been implemented including: (A) exposure of more concentrated cell suspensions to increased volumes of calcium phosphate–DNA coprecipitates; (B) separation of these coprecipitates from bone marrow cells prior to injection into recipient mice; and (C) increasing the doses of MTX used for in vivo selection of MTX-resistant transformed bone marrow cells.

These improvements were shown to increase the efficiencies of the genetic transformation, bone marrow cell administration, and bone marrow cell selection steps in the protocol. As a result, genetically transformed MTX-resistant bone marrow cells were detected in 9/11 mice that had received 3T6-treated marrow cells (Table 3). However, no genetically transformed cells were detected in mice injected with A29 DNA-treated marrow cells or with L5178Y DNA. The high level of resistance of the mutant DHFR activity encoded by 3T6 DNA may have permitted proliferation of 3T6 DNA-transformed cells in vivo in conditions where A29 DNA-transformed cells were unable to proliferate. In addition, in view of the markedly altered 3T6 R400 DHFR gene, it is likely that transfection with only a single 3T6 gene would impart MTX resistance to the recipient marrow stem cell.

We are currently attempting to isolate the cDNA for DHFR from 3T6 cells for future gene transfer experiments. It should be possible, using the transformation procedure developed, to transfer almost any cloned gene to mouse bone marrow cells without impairing the proliferation capacity of these cells in vivo. Genes coding for nonselectable markers may

**Table 3. Analysis of CBA Mice Injected With Calcium Phosphate–DNA Treated Bone Marrow Cells and Treated With MTX for 46–58 Days**

<table>
<thead>
<tr>
<th>(A) DNA Treatment (CBA)</th>
<th>Percent Hematocrit</th>
<th>Percent 3T6 DHFR Activity</th>
<th>Final Percent CBA (Marrow)</th>
<th>CFU-S (per 10^6 Cells, ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>45–53</td>
<td>0 (33)</td>
<td>30 (± 4)</td>
<td>None</td>
</tr>
<tr>
<td>3T6</td>
<td>Mouse no. 1</td>
<td>34.5 39 60</td>
<td>Mouse no. 6</td>
<td>30 18 48</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>35.5 33 48</td>
<td>7 39.5 28 40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>50 78 93</td>
<td>8 39 26 13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>39.5 45 30 (± 17)</td>
<td>9 32.5 0 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>39 36 14 (± 3)</td>
<td>10 35 14 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L5178Y</td>
<td>24 0 33</td>
<td>L5178Y 23.5 0 24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>0 10</td>
<td>28 4 34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>29.5</td>
<td>4 1 (± 2)</td>
<td>28 0 25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>0 10</td>
<td>26 0 32</td>
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</table>

Hematocrits were measured after 42 days with blood drawn from the retroorbital sinus of donor mice. DHFR activities, measured as rates of change of 340 nm absorption, are expressed as percentage activity ratios in the presence or absence of 3 x 10^{-5} M MTX (from one analysis only). Activity of DHFR in the presence of MTX was considered to be 100%. The ratio of CBA or CBA/H-T6T6 bone marrow cells was calculated from at least 100 chromosome spreads for each analysis. This ratio is presented in the table as the percent of CBA chromosomes found of the total. CFU-S levels for each individual mouse are expressed as means for 3–6 separate analyses.
potentially be transferred to bone marrow cells with selectable genes,17,18 such as those encoding DHFR and thymidine kinase.19 Alternative gene transfer procedures, such as cell-to-cell fusion and liposome-mediated gene transfer, are more likely to impair the normal functioning of these cells.

The transfer of drug-resistant genes to normal bone marrow cells permits increased hematologic tolerance to intensive drug treatment schedules, as demonstrated in these studies. This tolerance may improve therapeutic ratios for treatment of a variety of tumors with common anticancer agents, and experiments are planned to assess this possibility using model tumors in mice. A major drawback of the present method for induction of MTX resistance in bone marrow cells is that a relatively long selection time is necessary to amplify MTX-resistant cells to detectable levels. Further improvements in transformation efficiencies are therefore desirable. However, it is likely that an appreciable number of bone marrow cells initially express donor DHFR genes shortly after gene transfer, as demonstrated in other systems.20 This initial transient expression may explain the elevated hematocrits observed for mice 3T6-9 and 3T6-10 (see Table 3) in the absence of detectable donor DHFR activity in the spleen. Therefore, intensive chemotherapy may be possible immediately after receiving bone marrow cells transformed with genes concerning drug resistance. This idea is currently being investigated.

The high efficiency of bone marrow cell transformation to MTX resistance observed in the experiments reported supports the conclusion of Cline and coworkers1 that normal murine bone marrow is as transformable as some tissue-cultured cell lines. Therefore, the mouse system provides a good model system with which to study the potential clinical applications of bone marrow cell transformation. Further characterization of this genetic transformation system is necessary prior to adaption of these procedures to humans. The location of the 3T6 R400 DNA in the marrow cells has not yet been determined. One explanation for the transient resistance of mice 9 and 10 to MTX could be that the 3T6 DNA was extrachromosomal and not integrated into host chromosomes.

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