Induction of mutant lymphocytes in cyclophosphamid- and chlorambucil-treated patients

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Monitoring patients treated with single antineoplastic agents is aiding our understanding of what hazard these drugs pose in vivo. In this study, the frequency of mutant 6-thioguanine-resistant (TG8) peripheral blood lymphocytes was monitored before treatment and for ≤35 weeks after treatment of patients with cyclophosphamide (CP) or chlorambucil (CAB). The mean mutant frequency before treatment for six multiple sclerosis patients treated with high-dose CP was 2.53 × 10−5 and increased after treatment to 4.61 × 10−5 (P = 0.08, paired t-test). Using each patient as their own control, there were significant increases (each at P < 0.04) detectable within 2–4 weeks in four of the multiple sclerosis patients treated with CP. There was no increase in an untreated control monitored over the same period. In a patient receiving five sequential CP treatments at 1 month intervals, there were cumulative increases in the frequency of mutant cells. The mutant frequency increased from 0.31 × 10−5 before treatment to 3.64 × 10−5 after the final treatment and had decreased to 0.53 × 10−5 at 35 weeks after treatment. In one of two CAB-treated patients with indolent non-Hodgkin’s lymphoma, there was a significant increase in mutant frequency (P < 0.03) after treatment. Freshly isolated peripheral blood lymphocytes treated with 4-hydroperoxy-CP in vitro demonstrate a dose-dependent increase in mutant frequency. The increment in mutant frequency observed in vivo is of the order expected from the in vitro experiments. Although this study demonstrates that single or multiple doses of a single antineoplastic agent are mutagenic in vivo for some patients, further studies are needed to determine the extent and mechanism of the inter-individual variations in mutagenic response.

Introduction

Cytotoxic alkylating agents are used clinically for treatment of neoplastic diseases, as immunosuppressive agents in the treatment of inflammatory connective tissue diseases and in experimental therapies for multiple sclerosis (MS; Carter et al., 1988). The genetic hazard posed by the use of alkylating agents has been recognized since at least the 1960s (Conen and Lansky, 1961) and the occurrence of secondary malignancies following treatment with alkylating agents is well documented. Treatment-related secondary leukemias have been reported following treatment for Hodgkin’s disease (Tucker et al., 1988), breast cancer (Curtis et al., 1990) and ovarian cancer (Kaldor et al., 1990). Acute myelogenous leukemia is the most common malignancy caused by alkylating agents in treated cancer patients (for review see Shulman, 1993). Secondary leukemias have also been reported for patients treated with antineoplastic drugs or radiation for non-neoplastic diseases (for review see Grunwald and Rosner, 1979). The types of mutation responsible for treatment-related malignancies are not yet known (Povirk and Shuler, 1994).

Manufacturing workers, nurses, pharmacists and other healthcare workers are potentially exposed to antineoplastic alkylating agents. Evidence is inconclusive as to whether this exposure is at a level that poses a genetic hazard to these individuals. There are conflicting reports as to whether mutagenic activity is increased in the urine of healthcare workers who handle cytotoxic drugs (Falck et al., 1979; Staiano et al., 1981; Gibson et al., 1984; Venitt et al., 1984; Krepsinsky et al., 1990), although significant mutagenic activity has been detected in the urine of patients treated with cytotoxic drugs (Eder et al., 1986; Peters et al., 1987; Krepsinsky et al., 1990).

Significant increases in the frequency of 6-thioguanine-resistant (TG8) mutant lymphocytes have been observed in cancer patients treated with cytotoxic drugs (Dempsey et al., 1985), in nurses and pharmacists working with cytotoxic drugs (Chrysostomou et al., 1984) and in workers involved in the manufacture of cyclophosphamide (Huttner et al., 1990). A review of occupational exposure to cytotoxic drugs suggests that, while there is evidence of potential hazardous exposure via skin absorption, potential confounders need to be considered before a conclusion about the real hazard can be drawn (Sorsa and Anderson, 1996).

Studies of the frequencies of mutant cells from patients exposed to multiple cytotoxic drugs are difficult to interpret because the effect of each individual drug cannot be ascertained. Although studies of exposure to a single drug are more straightforward to evaluate, there are relatively few clinical situations where therapy with a single cytotoxic drug, rather than a combination of drugs, is indicated. Patients with severe sub-acute erythematous and systemic lupus erythematosus (SLE) are treated with cyclophosphamide (CP). Dawisha et al. (1994) found that treatment with CP did not correlate as strongly with mutant frequency as did total duration of active SLE disease. In a single-patient study, a significantly elevated frequency of TG8 mutant cells has been observed (Wood et al., 1994). Some patients with indolent non-Hodgkin’s lymphomas are treated with chlorambucil (CAB) as a single agent and some MS patients have been treated with CP as a single agent during the chronic progressive stages of the disease (Carter et al., 1988). CP requires activation to exert its activity (Benedict et al., 1977). It undergoes metabolism, primarily in the liver, to 4-hydroxy-CP, which spontaneously transforms to aldophosphamide and then to phosphoramidate mustard and acrolein (Shulman, 1993). The initial metabolite of CP, 4-hydroxy-CP, is mutagenic and may be spontaneously transformed to the phosphoramidate mustard metabolite intracellularly.

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(Sanderson et al., 1991). It is the phosphorylase mustard metabolite that is suggested to be the mutagenic metabolite in bladder cancer (Khan et al., 1998). Ammenheuser and co-workers (1988), using an autoradiographic assay for TG<sup>R</sup> lymphocytes, observed statistically significant increases in mutant frequency 2 weeks after treatment in five of six MS patients treated with CP. However, these increases were transient and mutant frequencies returned to normal by 4 weeks after treatment. Twelve patients with connective tissue diseases who had received long-term daily CP had higher frequencies of TG<sup>R</sup> lymphocytes detectable by T-lymphocyte cloning than normal controls (Palmer et al., 1988). One study found that cancer patients receiving CP had higher SCE frequencies than control untreated cancer patients (Jacobson-Kram et al., 1993). Similarly, higher mutant frequencies have been found in pediatric cancer patients treated with CP (Sawada et al., 1998). In contrast, elevated TG<sup>R</sup> frequencies were not detected in CAB-treated patients (Palmer et al., 1986). Also, no significant increase in chromosome aberrations were detected in CP-treated MS patients after treatment (Ammenheuser et al., 1991).

The current study used each patient as their own control, which was important because pre-treatment mutant frequency was expected to vary between individuals with MS. TG<sup>R</sup> in T-lymphocytes is probably an indication that the cell has been activated in vivo, so it is not surprising that the frequency of TG<sup>R</sup> lymphocytes in patients with chronic progressive disease appears to correlate with clinical progression of the disease (Sriram, 1994). CD45RO<sup>+</sup> cells have a higher mutant frequency than CD45RA<sup>+</sup> cells, suggesting that some subgroups of T-lymphocytes are activated in vivo and undergo cellular division which may influence TG<sup>R</sup> frequency (Baars et al., 1995).

In this study we prospectively measured the frequency of TG<sup>R</sup> lymphocytes before treatment and for ~35 weeks after treatment for six patients treated with single high doses of CP, one patient who received five successive monthly treatments of lower dose CP and two patients treated with monthly CAB. The induction of TG<sup>R</sup> cells in vivo was compared with the response of human cells to exposure to these two drugs in vitro. 4-Hydroperoxy-CP (4HCP), the synthetic precursor of 4-hydroxy-CP, was used for the in vitro studies.

### Materials and methods

**Patients and controls**

Peripheral blood (50 ml) was collected in heparin coated tubes and lymphocytes were isolated by Ficoll gradient centrifugation. Patients were recruited for this study from the Neurology and Hematology/Medical Oncology clinics of Oregon Health Sciences University and Good Samaritan Hospital in Portland, OR, USA, from a group of patients who had not previously been treated with cytotoxic drugs and whose physicians had recommended therapy with CP or CAB. Patients were Caucasian-Americans aged 22–58 years. Four patients (numbers 12, 14, 41 and 46) had chronic progressive MS, with no individual history of malignancy; the other two patients (numbers 44 and 55) had indolent non-Hodgkin’s lymphoma. The study was approved by the respective Institutional Review Boards and informed consent was obtained from all participating individuals.

Studies of in vitro exposure used the lymphoblastoid cell line WIL2-NS or freshly isolated lymphocytes from a 30 year old female with no known illness. WIL2-NS cells were actively cycling and maintained in continuous culture. Freshly isolated lymphocytes were in G<sub>0</sub> when harvested, but were stimulated into cycling by exposure to phytohemagglutinin before mutation assays.

**Chemicals**

CP (Cytosar) was obtained from Bristol-Myers-Squibb and CAB for patient therapy from Burroughs Wellcome. 4HCP and CAB for in vitro experiments were obtained from Nova Pharmaceutical and Sigma, respectively. 4HCP was dissolved in distilled water and CAB in ether; they were used within 30 min. Control cells were exposed to the appropriate volume of water or ether.

**Table I: Plating efficiency and frequency of TG<sup>R</sup> mutant lymphoblasts in a continuous population of WIL2-NS cells sampled over 1 year**

<table>
<thead>
<tr>
<th></th>
<th>Plating efficiency</th>
<th>Mutant frequency (× 10&lt;sup&gt;–6&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.65</td>
<td>0.58&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Range</td>
<td>0.30–0.95</td>
<td>0.14–0.91</td>
</tr>
</tbody>
</table>

Samples were assayed as detailed in Materials and methods.

**Mutation assay procedure**

Mutation assays selecting for TG<sup>R</sup> cells were performed as previously described (Morley et al., 1985) except that cells were cultured in RPMI with 10% fetal bovine serum (Hazelton) and 20% HL-1 medium (Ventrex). Medium was further supplemented with 20% LAK cell medium (a gift of Dr T.J.Eberline) or 20% Lymphocult-T (Biostet Diagnostics) as a source of interleukin-2. For each sample, mutation assays were performed on freshly isolated lymphocytes and then repeated on batched cryopreserved lymphocytes from the same blood sample. The in vitro dose–response experiments were performed as described in Sanderson et al. (1991). Briefly, cells were resuspended at 0.5 × 10<sup>5</sup>/ml, exposed to the drug for 1 h in RPMI/5% fetal bovine serum, washed six times and cultured for 10 days (to allow expression of induced mutations) before assaying mutant frequency. Statistical significance was determined by pooled or paired t-tests, as appropriate for the data.

### Results

Experiments in this study were conducted over a period of 1 year. To monitor the reproducibility and reliability of the HPRT assay, a continuous cell line (WIL2-NS) was maintained and assayed over the same period. As shown in Table I, there was no significant change in mutant frequency for WIL2-NS over the period of these experiments.

The TG<sup>R</sup> mutant cell frequency was determined for six patients treated with high-dose (4.5–7.5 g) intravenous CP administered over 9–15 days. Mutant frequency was determined for lymphocytes drawn immediately before treatment and periodically in the months after treatment. Each patient served as his or her own control and the mean of multiple determinations of mutant frequency before treatment was compared with the mean mutant frequency for the first post-treatment samples obtained (at 2–4 weeks for all but one patient; see Table II). The mean mutant frequency for the six patients was 2.53 × 10<sup>–5</sup> before treatment and increased to 4.61 × 10<sup>–5</sup> after treatment (P = 0.076; paired t-test). When the pre- and post-treatment mutant frequencies for individual patients were compared, there were significant increases in the mutant cell frequency following high-dose CP for four of the six patients (Figure 1). The increases were significant for patients 12 (P = 0.001), 41 (P = 0.029), 42 (P = 0.004) and 46 (P = 0.034). Mutant frequencies in lymphocytes obtained from patients ≤30 weeks after high-dose CP had returned to pre-treatment levels by the last sample obtained, with the exception of one patient (patient 42).

One patient with MS was treated with five intravenous doses of CP (500 mg each) at 1 month intervals. In this patient, mutant frequency gradually increased with each cumulative dose, reaching 10 times the pre-treatment level (P < 0.01), and then declined over the 35 weeks after treatment (Figure 2).

Two patients with indolent non-Hodgkin’s lymphoma were treated monthly with 64 mg CAB (16 mg orally daily for 4 days) and their lymphocyte mutant frequency determined before the first, second, third and fourth monthly treatments.
Table II. Frequency of TG\textsuperscript{R} mutant lymphocytes in peripheral blood samples from six patients before and at various times after an acute dose of CP

<table>
<thead>
<tr>
<th>Individual</th>
<th>0 week (\times 10^{-5})</th>
<th>2–4 weeks</th>
<th>6–8 weeks</th>
<th>10–12 weeks</th>
<th>14–16 weeks</th>
<th>20–25 weeks</th>
<th>28–30 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 12</td>
<td>1.35 (1.0–1.9)</td>
<td>3.64 (3.1–4.3)</td>
<td>NA</td>
<td>2.30 (1.3–4.3)</td>
<td>NA</td>
<td>0.79 (0.7–0.95)</td>
<td></td>
</tr>
<tr>
<td>Patient 14</td>
<td>3.80 (3.0–4.8)</td>
<td>3.49 (2.1–7.2)</td>
<td>3.26 (1.5–4.8)</td>
<td>7.90 (3.5–17.7)</td>
<td>0.36 (0.1–3.2)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Patient 41</td>
<td>1.17 (0.7–1.9)</td>
<td>4.34 (2.4–7.8)</td>
<td>3.95 (3.3–4.8)</td>
<td>NA</td>
<td>NA</td>
<td>7.26 (4.1–13.0)</td>
<td>1.49 (0.7–3.2)</td>
</tr>
<tr>
<td>Patient 42</td>
<td>2.76 (2.0–3.8)</td>
<td>9.26 (6.8–12.6)</td>
<td>9.37 (4.7–18.7)</td>
<td>NA</td>
<td>3.77 (3.0–4.7)</td>
<td>NA</td>
<td>14.18 (10.3–19.6)</td>
</tr>
<tr>
<td>Patient 43</td>
<td>3.32 (1.7–6.4)</td>
<td>NA</td>
<td>1.44 (0.8–2.6)</td>
<td>2.03 (1.4–2.9)</td>
<td>NA</td>
<td>2.14 (1.6–3.0)</td>
<td>NA</td>
</tr>
<tr>
<td>Patient 46</td>
<td>1.80 (1.0–3.4)</td>
<td>3.86 (2.6–5.7)</td>
<td>0.88 (0.7–1.3)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Control</td>
<td>0.54 (0.2–1.1)</td>
<td>0.23 (0.6–1.0)</td>
<td>0.24\textsuperscript{a}</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Single observation.

NA, sample not available.

The control was an untreated individual with no known illness. Samples were drawn at the times indicated and the lymphocytes isolated and assayed as detailed in Materials and methods. Data are shown as a geometric mean ± sd from two to four assays, except where indicated.

Fig. 1. The frequency of mutant lymphocytes before and after treatment with CP. Patients 12, 14, 41 and 46 were monitored 2 weeks after treatment, patient 42 after 4 weeks and patient 43 after 6.5 weeks. Lymphocytes were isolated and assayed at the \textit{hprt} locus as detailed in Materials and methods. Values are the mean of two to four assays per patient. The increases were significant for patients 12 (\textit{P} = 0.001), 41 (\textit{P} = 0.029), 42 (\textit{P} = 0.004) and 46 (\textit{P} = 0.034).

Fig. 2. Frequency of mutant lymphocytes after successive cyclophosphamide doses. A single dose of CP was given at monthly intervals, as required by the treatment protocol. Treatments 1–4 were low-dose (500 mg) and treatment 5 was high-dose (7 g) CP. Results are the geometric mean ± sd based on two to four assays, performed 2 weeks after each treatment and 35 weeks after the final treatment.

(Figure 3). For one of the two patients, the mutant frequency 8 weeks after treatment was significantly higher (\textit{P} < 0.03) than that before treatment (two treatments).

In \textit{vitro} exposure of freshly isolated lymphocytes to an active metabolite of CP, 4HCP, produced dose-dependent increases in the frequency of TG\textsuperscript{R} mutants in the exposed cells. An equal exposure to 4HCP was two- to four-fold more mutagenic for the freshly isolated lymphocytes than for the lymphoblastoid cell line WIL2-NS (Figure 4). A similar level of \textit{in vitro} exposure to CAB has previously been shown to be mutagenic for the same lymphoblastoid cell line (Sanderson \textit{et al.}, 1991).

Discussion

In this study, the number of TG\textsuperscript{R} mutant T-lymphocytes in the peripheral blood of some treated patients increased after \textit{in vivo} exposure to CP or CAB. Although the number of patients studied was small, each patient was monitored before treatment, thereby allowing them to act as their own control for post-treatment monitoring. We found evidence that CP and CAB induce mutations in the hypoxanthine-guanine phosphoribosyl transferase (\textit{hprt}) gene \textit{in vivo}, manifest as significant increases in TG\textsuperscript{R} mutant frequency in four of six CP-treated patients and one of two CAB-treated patients. Further evidence in support of the \textit{in vivo} mutagenicity of CP was the cumulative increase in mutant frequency with successive CP doses obtained...
for a patient who received five doses, each 1 month apart. This clear dose–response, shown in Figure 2, suggests that the observed increases were real and induced by treatment. Although unlikely, it is possible that the increases in mutant frequency were a consequence of an over-representation of existing pre-treatment mutants due to selective cell killing of non-mutant cells by cytotoxic drug treatment. Interestingly, the sensitivity to detect in vivo mutagenesis may depend on the target cell type examined. In a mouse model, Gorelick et al. (1999) found significant increases in mutant frequency of the lungs and urinary bladder, but not in bone marrow, kidney or splenic T-lymphocytes.

Only one of the two patients treated with CAB in this study showed a detectable increase in mutant frequency following three monthly treatments. Palmer et al. (1986) failed to find a difference in mutant frequency in three patients who had been treated with multiple doses of CAB. The earlier study differed from this current study in that patients were not studied prospectively, and a different assay for thioguanine resistance, a non-clonal assay, was used. Clearly more patients need to be studied before conclusions can be drawn about the in vivo effects of CAB. It would be useful to include analysis of post-treatment mutational spectra in future studies to aid our understanding of the nature of chlorambucil mutagenesis in vivo. This may provide insight into the mechanism underlying the increased risk of leukemia observed following treatment of non-Hodgkin’s lymphoma with CAB. Travis et al. (1994) studied six CAB-treated patients and 22 controls and found risk of acute non-lymphocytic leukemia increased with increasing cumulative dose of CAB, with an overall relative risk of 2.4 [95% confidence interval (CI) 0.7–8.6].

The results for CP-treated patients in this study are generally consistent with those of a prospective study by Ammenheuser et al. (1988), using an alternative assay for TG^R^ mutants and a different monitoring schedule. Those authors reported that five of six patients treated with 750 mg of CP had greater mutant frequencies 2 weeks after therapy, but that the mutant frequencies had returned to normal by 4 weeks after therapy. The results of the current study and the previous study by Ammenheuser et al. (1988) together suggest that the increased TG^R^ mutant frequency in blood lymphocytes seen after exposure to CP is largely transient and peaks ~2 weeks after exposure. A similar transient pattern has been observed for cells carrying sister chromatid exchanges (SCEs) (Jacobson-Kram et al., 1991). The reason for disappearance of TG^R^ T-lymphocytes from the blood is not known, but there are several possible explanations. TG^R^ lymphocytes in the blood may be selected against, either because of their HPRT deficiency or because of other genetic damage they have sustained. Alternatively, the TG^R^ lymphocytes may be members of a relatively short-lived pool of blood lymphocytes which is replaced over a period of a few weeks by lymphocytes which were less susceptible to CP-induced mutagenesis at the time of CP treatment. Another explanation for a transient increase in mutant frequency is that it represents the outgrowth of a single clone subsequently selected against. Although this is considered unlikely, it could be investigated by determining the mutational spectra of the lymphocyte populations at multiple time points before and after treatment using such techniques as multiplex PCR (Gibbs et al., 1990) and PCR from ~20 cells (Grant et al., 1999). A molecular approach is feasible, since it is expected that a change in the mutational spectra would be observed, as was the case in a transgenic mouse model where the spectrum of hprt mutations in CP-treated mice was significantly different from that in control mice (Walker et al., 1999). Both CP and CAB are classified as carcinogenic to humans (IARC, 1987). CAB principally induces single base-pair mutants with 64–75% being at G-Cs, while CP induces crosslinks and only 22% of monoadducts are at G-Cs (for review see Sanderson and Shield, 1996). It should also be possible to compare the mutational spectra of CP and CAB both in vivo and in vitro to elucidate their mechanisms of action further.

The transient increases in mutant frequency observed may be related to changes in cell cycling and selection of different classes of T-lymphocytes. CP acts by inhibiting DNA synthesis, principally in the S phase of the cell cycle, causing lymphopenia in both B- and T-lymphocytes. The predominant T-lymphocyte phenotype changed from CD8^+^,TCR^γδ^+ to CD4^+^,TCR^αβ^+ in TG^R^ cell lines from a patient with subacute cutaneous lupus after treatment with an intravenous pulse of CP (Wood et al., 1994). It is suggested that the CD8^+^,TCR^γδ^+ subgroup of T-lymphocytes rebounds after CP treatment and are implicated in the pathogenesis of the disease and responsible for reappearance of symptoms. Regardless of the reason for the disappearance of TG^R^ lymphocytes, this phenomenon may limit the use of this assay as a method for monitoring individuals for long-term exposure to mutagens.

Inter-individual differences in frequency of mutations induced by CP were observed both in this study and the study by Ammenheuser et al. (1988). In two of six patients in this study and one of six patients in the study by Ammenheuser and co-workers, no detectable increase in mutant frequency was induced by similar doses of CP. The lack of mutation induction in some patients may be due to genetic or other, as yet unknown, differences between individuals. These possibilities for difference in mutation sensitivity need to be explored. The ability to induce TG^R^ mutations by in vitro exposure of freshly isolated lymphocytes to CP will allow us to design experiments to define further the nature of inter-individual differences in mutagen susceptibility by studies of lymphocytes from normal individuals. If reproducible differences in the in vitro susceptibility of lymphocytes from different
individuals to CP-induced mutagenesis can be demonstrated, then such an assay could be tested prospectively to determine its ability to predict both the levels of mutations induced in vivo and the risk of treatment-related leukemia. The reproducibility and reliability of these assays are important if they are to be used for screening or monitoring. We have shown the assay to be consistent for the cell line WIL2-NS over an extended period under standard assay conditions (see Table I). This and earlier reports have demonstrated that administration of CP is mutagenic for lymphocytes in vivo and that an activated form of CP, 4HCP, is mutagenic for freshly isolated human lymphocytes during in vitro exposure. We have previously shown that 4HCP is mutagenic for a human lymphoblastoid cell line (Sanderson et al., 1991). The levels of exposure required to produce mutations during in vitro chemical exposure can be compared with the estimates of exposure levels of blood lymphocytes in vivo during therapy. Based on the pharmacokinetic studies of CP in humans by Struck and Alberts (1984), the exposure [or area under the curve (AUC)] to an active metabolite of CP, phosphoramide mustard, is ~5 μM·h for a 1 g dose of CP. Therefore, the exposure (AUC) for the patients in this study receiving single high-doses of CP is ~22.5–45 μM·h. The data in Figure 4 allow us to calculate the induced frequency of TG mutants for both the cell line WIL2-NS and the freshly isolated lymphocytes. When these frequencies are corrected for exposure we arrive at induced mutant frequencies of 0.85 × 10^{-6} and 2.1 × 10^{-6} per μM·h exposure for the WIL2-NS and freshly isolated lymphocytes, respectively. For the six patients who were treated with high-dose CP, the average increase in mutant frequency was 2.08 × 10^{-5} and the average estimated exposure was 31.7 μM·h for an estimate of 0.65 × 10^{-6} induced mutants per μM·h exposure. Thus it would appear that the exposure of blood lymphocytes to active CP metabolites in CP-treated patients would be at a level sufficient to produce an observable increase in mutant frequency if the same level of exposure was carried out in vitro. Furthermore, the increases in mutant frequency observed in vivo in this study are of the order that would be expected from the in vitro experiments with fresh lymphocytes or lymphoblastoid cell lines. Bochkov et al. (1986) also found that the effective dose for genetic damage in vivo and in vitro coincided when studying CP-induced chromosome aberrations and SCEs.

Continued analysis of mutant T-lymphocytes in MS patients should not only aid our understanding of treatment response, as in the current study, but should also further our understanding of their role in autoimmune disease. Extending studies to the molecular level should reveal the diversity of cells involved in the pathogenesis of disease, such as recognition of myelin basic protein (MBP). Allegretta et al. (1994) found TG8 lymphocytes that recognized MBP in blood samples from MS patients. In contrast, Trotter et al. (1997) failed to find TG8 cells from MS patients that recognize MBP, although some patients had evidence of cells activated to recognize myelin proteolipid protein. A number of groups are analyzing mutant cells at the genomic level to further the understanding of the disease process of MS (Lodge et al., 1994; Grant et al., 1999).

Acknowledgements
We thank Drs D.Bourdette, R.Whitham, R.Herndon and T.DeLoughery for assistance in patient recruitment. This work was supported by a grant to W.D.H. from the National Institutes of Health (CA55232).

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Received on August 1, 2000; accepted on November 10, 2000