

Quantification of Thioguanine-Resistant Lymphocytes from Mice Irradiated *In Vivo*

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Adult mice were Co-60 gamma irradiated, and 7 months later splenocytes were isolated, cultured in microwells, and the frequency of *hprt*-deficient mutants was determined by measuring the cloning efficiency in media with 6-thioguanine. The mutant frequency at 2, 4, and 6 Gy was 1.6×10^{-5} , 4.4×10^{-5} , and 12.7×10^{-5} , respectively. The frequency of spontaneous mutants was 2.5×10^{-6} . The effect of metabolic cooperation on the cloning efficiency of thioguanine-resistant T-cells in selective medium was evaluated in co-cultures with wild-type T-cells. We found that the growth of *hprt*-deficient T-cells is supported in the presence of thioguanine-inactivated wild-type splenocytes up to a cell density of 5×10^5 cells per well. When cell density was higher, cell growth was inhibited. Possibilities and limitations of cloned lymphocytes for the analysis of somatic mutations that occur *in vivo* are discussed.

Introduction

In vitro assays such as the Ames test or the mouse lymphoma test cannot replace *in vivo* tests as predictors of genotoxicity in mammals. Jones and co-workers (1-3) have demonstrated that cloning mouse lymphocytes permits analysis of mutations in the *hprt* gene that have occurred *in vivo* in T-cell progenitors. Possibilities and limitations of this method were investigated, and preliminary results on the mutant frequency in gamma-irradiated animals will be given.

Methods

Thioguanine-Resistant Mutants and Cultivation of Splenocytes

A resistant clone was isolated from wild-type L5178Y mouse lymphoma cells after treatment with *N*-ethyl-*N*-nitrosourea as described by others (4). Resistant splenocytes were prepared from *hprt*-deficient mice (5).

Splenocytes were cultivated in general as described by Jones et al. (1). In brief, spleen cells are isolated from 3 to 6 adult male C57bl mice and cultured in round-bottomed 96-well microtiter plates in the presence of 60 Gy irradiated feeder cells (10^4 *hprt*⁻ L5178Y mouse lymphoma cells and a variable number of splenocytes). Modified RPMI1640 medium in an atmosphere of 5% CO₂ was used for culture. The medium was supplemented with 10% fetal calf serum, HEPES (25 mM),

mercaptoethanol (5×10^{-5} mM), 0.2 mg/mL sodium pyruvate, recombinant IL-2 (100 U/mL), penicillin (100 U/mL), streptomycin (100 µg/mL), 1 µg/mL indomethacin, and 5 µg/mL Con A. Medium of selective plates contained in addition 2.5 µg/mL 6-thioguanine (TG). The total volume per well was 0.2 mL. In each well, 100 µL medium was replaced twice a week. Fresh medium improves cell growth. Late on day 14 in culture, 0.5 µCi [Me-³H]thymidine (3H-TdR), specific activity 20 Ci/mmole, dissolved in 100 µL fresh medium, was added to each well. Cells were harvested 12 to 16 hr later, and the ³H-uptake was measured by liquid scintillation counting.

Detection of Wells with Cell Growth

Cell growth was detected visually as well as by ³H-TdR uptake. When ³H-TdR uptake is used to evaluate the wells, a cutoff point has to be defined to detect wells with proliferating cells. In nonselective plates the ³H-TdR uptake in wells that contained only feeder cells was used to discriminate between wells without cell growth and wells with growth. Wells were considered positive for proliferation when they exceeded by more than 3 SD the mean value of the ³H-TdR incorporation in wells that contained only feeder cells.

The uptake of ³H-TdR by cells inactivated through irradiation is not the same as in TG-inactivated cells. As a consequence, the cutoff point of nonselective plates cannot be used in selective ones. For these plates the cutoff point was defined as the mean value + 3 SD of at least 100 wells without cell growth in selective medium that were prepared with splenocytes from untreated animals. Since spontaneous TG-resistant mutants are rare, positive wells could be eliminated by inspection of the data.

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Calculations

The calculation of cloning efficiency (CE) in Equation (1) is based on $P(0)$ the proportion of wells in which a colony has not grown (6). Mutant frequency (MF) was calculated using Equation (2). Confidence limits of MF were determined as described by Furth et al. (7).

$$CE = \frac{-\ln P(0)}{\text{number of cells per well}} \quad (1)$$

$$MF = \frac{CE \text{ in selective medium}}{CE \text{ in nonselective medium}} \quad (2)$$

Results and Discussion

Cloning of T-lymphocytes in microwells was used to determine thioguanine (TG)-resistant mutants (Fig. 1). TG is taken up into the cell by the purine scavenger pathway using the enzyme hypoxanthine phosphoribosyl transferase, the product of the *hprt* gene. TG-resistant variants of T-cells can survive in the presence of thioguanine as a consequence of a loss of function of this enzyme.

Cell growth was quantified by measuring the ^3H -TdR uptake. An uptake of 3 cpm was found per growing cell. Since in our experiments the cutoff point varied be-

tween 150 and 600 cpm, 50 to 200 growing cells per well were the lower limit for the detection of cell growth. In Figure 1, between 500 and 8×10^4 cpm was measured per well with cell growth. This indicates a great variability in the T-cell colony size.

Each well was visually screened for cell growth before harvest. In nonselective plates, 70% of all wells in which a colony had grown, as estimated by ^3H -TdR uptake, could be detected by eye. In selective plates this fraction was not more than 27%. This indicates that small colonies are often covered with inactivated cells. In rare cases, cell growth was detected visually even though the ^3H -TdR uptake in the well was lower than the cutoff point.

Metabolic cooperation by transfer of purine metabolites through the medium or via cell contact might inhibit the growth of *hprt*-deficient mutants in medium with thioguanine when wild-type cells are present (8,9). We have studied the role of metabolic cooperation in T-cell culture. Figure 2 demonstrates that wild-type mouse lymphocytes inhibit the growth of thioguanine-resistant L5178Y mouse lymphoma cells, whereas the growth of *hprt*-deficient splenocytes is supported by the presence of thioguanine-inactivated wild-type splenocytes up to a concentration of 5×10^5 cells/well. However, when the cell density is higher than 5×10^5 , wild-type splenocytes may inhibit the growth of TG-resistant

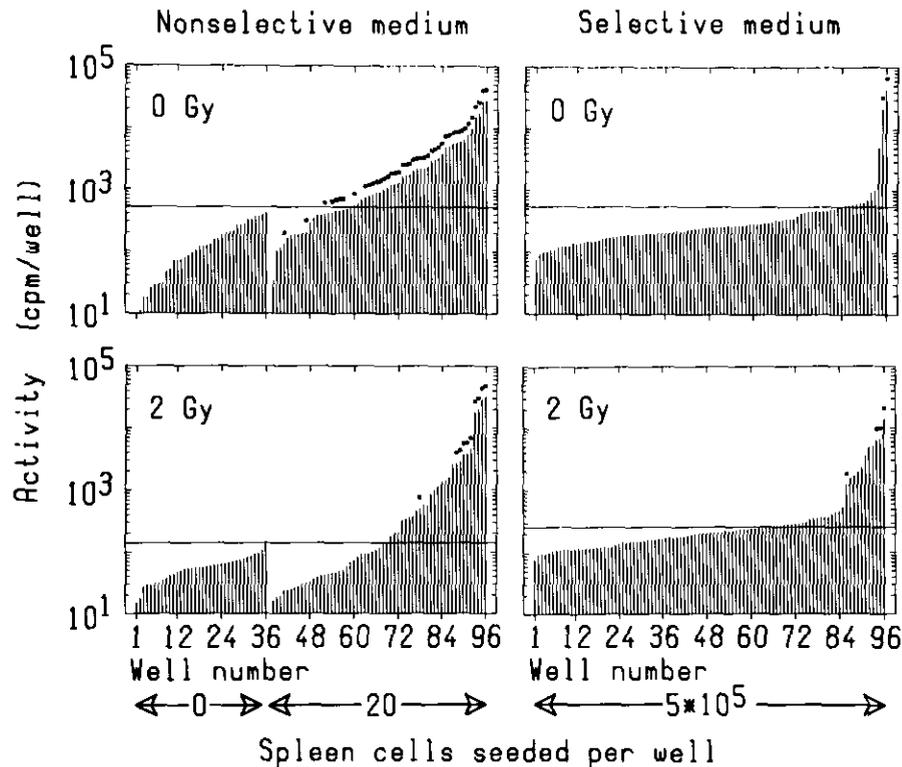


FIGURE 1. Example for the growth of T-cells from 2 Gy irradiated mice and unirradiated controls in nonselective and selective culture medium. Cells were cultured with feeder cells in 96-well microtiter plates for 15 days and labeled with ^3H -TdR 14 to 16 hr before harvesting. Wells with the same average number of seeded spleen cells are arranged according to the ^3H -TdR uptake. Horizontal lines give the cutoff point by which wells with cell growth are discriminated from wells without growth. Details of the method are given in "Methods." (■) Indicates the wells in which cell growth was visually detected.

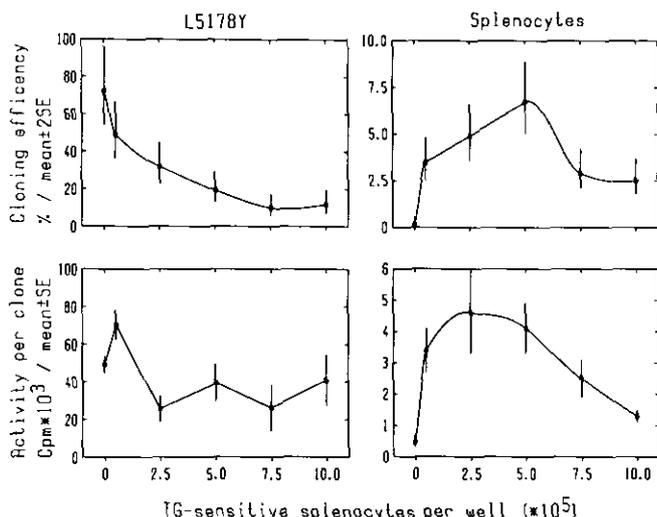


FIGURE 2. Influence of thioguanine-sensitive splenocytes on the cloning efficiency and proliferation of thioguanine resistant cells. (Left) L5178Y mouse lymphoma cells; (right) splenocytes.

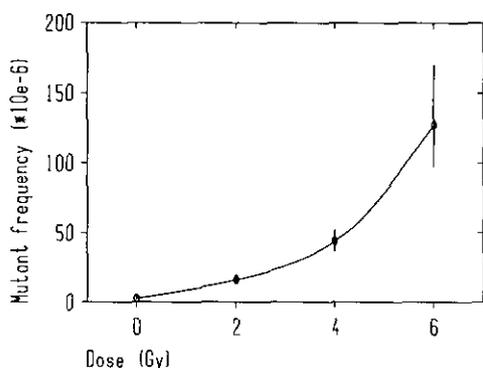


FIGURE 3. Mutations at the *hprt* locus of T-cells 7 months after Co-60 gamma irradiation.

T-cells. Therefore, most experiments were performed with 5×10^5 cells per well.

Figure 3 gives the mutant frequency (MF) of the *hprt* locus in T-cells from mice irradiated 7 months previously. The increase of MF with dose resembles a linear quadratic dose-effect curve. Recently Jones (10) reported about data on the mutant frequency in T-cells from gamma-irradiated animals. In her experiments (10), the dose-effect curve was strikingly curvilinear at weeks 3 to 5 postirradiation. Thereafter the quadratic component was less, and the frequency of mutants present in spleen cells of mice given high doses declined to one-third the maximum observed frequency. At the maximum, the observed frequencies in 4 Gy irradiated animals, measured 4 or 5 weeks after exposure, were 7.2×10^{-5} or 9.5×10^{-5} , respectively. These values are of the same order of magnitude as the results of our investigation. Another relationship between MF in spleen cells and dose was found by Dempsey and Morley (11) 4 weeks after X-ray irradiation. In their experi-

ments, X-rays at a dose of 1.5 Gy produced an approximately 20-fold increase in MF. Thereafter the number of induced mutant cells tended to plateau between 1.5 and 4.5 Gy. An increased MF was also found in T-cells from humans exposed to cytostatica (12) and from atomic bomb survivors more than 40 years after irradiation (13).

In high dose irradiation experiments, most of the T-cells and T-cell progenitors will die because they belong to the most radiosensitive cells of the body (14). Mutants can only be detected in the fraction of surviving T-cells and their progenitors. Therefore, interpretations of dose-effect curves of MF in T-cells have to consider the hierarchical organization of the T-cell renewing system and its radiosensitivity (Fig. 4). The renewing system comprises the virtually quiescent self-renewing pluripotent stem cell population in the bone marrow and two rapidly dividing non-renewing populations of differentiating cells in bone marrow and thymus. A final selective expansion takes place in spleen and lymph nodes subsequent to contact with antigen. Cells of the stem cell compartment are normally released at slow rates. Hence there is relatively more time for repair of any genetic damage in these cells than in any other part of the renewing system. These cells are relatively radio-resistant, whereas all other cells of the renewing system belong to the most radiosensitive cells in mammals. In which compartment mutants are induced is not known. In a preliminary longitudinal analysis of the expression time of *hprt*-deficient mutations, Jones et al. (10) did

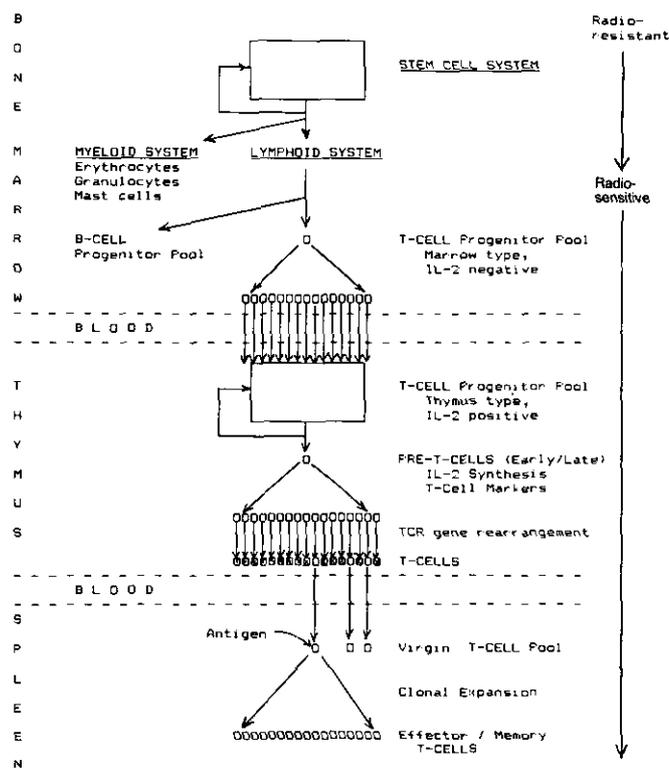


FIGURE 4. The renewing system of T-cells. Details are given in the text.

not find an increase of *hprt*⁻ T-cells in the thymus before 1 week after irradiation and in the spleen not before 3 weeks. This is an argument for the hypothesis that the mutations we measured do not arise in virgin or mature T-cells of primary or secondary lymphoid organs, but in stem cells or pre-T-cells in the bone marrow. The fact that the MF is increased even 40 years after irradiation (13) indicates that mutants are preserved in the stem cell pool.

In most experiments on the mutant frequency in mammalian cells, mutations of the *hprt* locus have been measured. This gene is located on the X chromosome; the *hprt* gene is hemizygous. Genetic damage involving this gene and extending into adjacent essential hemizygous genes results in lethality. Therefore, some *hprt* gene mutations will not give a viable clone, and the mutant frequency will be underestimated. It would thus be more appropriate to use mutational systems employing autosomal markers such as the HLA locus (15).

In summary, the measurement of the mutant frequency in T-cells might be a good system for the bio-monitoring of genotoxic agents under *in vivo* conditions. It would be desirable to screen not only for mutations at the *hprt* locus but also for autosomal genes. The direct DNA sequence analysis of *in vitro* amplified *hprt* cDNA from mutants will lead to a better understanding of the final alterations in DNA by mutagenic events.

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