ABSTRACT

Cyclophosphamide, an extensively used cancer chemotherapeutic agent, requires metabolic activation through a mixed-function oxygenase system. The capacity of this agent to produce oncogenic transformation and chromosomal damage, including increases in sister chromatid exchanges, was investigated in cell culture with or without an exogenous liver metabolic activation system. No oncogenic transformation or chromosomal aberrations were produced by cyclophosphamide in the absence of metabolic activation, whereas significant transformation, chromosomal breaks, and increases in sister chromatid exchanges were observed when the activation system was incorporated into the assays. The oncogenic transformation and chromosomal changes were completely eliminated by removing glucose 6-phosphate and nicotinamide adenine dinucleotide phosphate from the metabolic generating system. These studies emphasize the necessity to incorporate some activation procedure into short-term assays used for evaluating the mutagenic and/or oncogenic potential of various chemicals.

INTRODUCTION

Cyclophosphamide is one of the most widely used chemotherapeutic agents for the treatment of various human cancers as well as an immunosuppressant for certain nonmalignant conditions. However, it also has been implicated recently in producing primary and secondary cancer in humans, including both leukemias and solid tumors (11, 12). In addition since cyclophosphamide requires metabolic activation through a mixed-function oxygenase system primarily in the liver (3, 4, 6–8, 13), it is a rather unique alkylating agent. Cyclophosphamide may thus be an ideal model compound for studying and developing metabolic activation systems for mutagenic and/or oncogenic transformation studies in mammalian cells.

It is essential to develop such mammalian tissue culture assays that incorporate systems allowing metabolism of various classes of chemicals to their active form(s) if short-term assays for evaluating the potential hazards of numerous chemicals or mixtures to man can be utilized without performing long and expensive in vivo carcinogenicity studies. One must also assess these short-term assays for their actual ability to predict human risk. Thus, cancer chemotherapeutic agents may be unique for this purpose since the exact human exposure can be determined, inasmuch as these agents are not part of our normal environmental exposure, and are usually given under strict medical supervision. We illustrate in this paper that, whereas cyclophosphamide produces no oncogenic transformation without the presence of an activation system, significant transformation occurs in the presence of an exogenous activation source. In addition chromosomal aberrations including increases in the production of SCE's are observed only when a similar activation system is used. An increase in SCE's has also been shown by others following cyclophosphamide exposure, although a considerably higher concentration of drug was used (14), and more recently this has been linked to an induction of cytochrome P-450 rather than cytochrome P-448 (5).

MATERIALS AND METHODS

The C3H/10T'1/sCL8 cell line, which is approximately tetraploid, was used for the transformation studies as well as a portion of the chromosomal work. The culture conditions for this cell line and the transformation assay have been described previously (1, 9). The cloned Syrian hamster pseudodiploid cell line A(T)C1-3 developed in our laboratory was also used for some of the chromosomal studies.

The liver activation system was prepared by giving male Sprague-Dawley rats injections of Arochlor 1254, 500 mg/kg (Analabs Inc., New Haven, Conn.), dissolved in corn oil. Five days later the livers were removed, minced, and homogenized under sterile conditions in a cold room. The homogenate was centrifuged at 9000 x g for 10 min, and the resulting supernatant "S-9" fraction was stored frozen in small aliquots at −70°. The specific aryl hydrocarbon hydroxylase activity in these frozen samples was determined to be 210 to 260 pmol/min/mg protein with a standard assay (2).

The complete S-9 system was prepared essentially as previously described by Natarajan et al. (10) and was distributed in 0.5-ml aliquots into sterile plastic tubes. Subsequently, 0.1 ml of cyclophosphamide was added to make a final concentration of 5, 10, 25, 50 or 100 μg/ml/tube and was followed by the addition of 0.1 ml of the appropriate cell suspension. The cyclophosphamide was obtained from Dr. Harry B. Wood, Jr., Drug Development Branch, National Cancer Institute, Bethesda, Md. Approximately 5 × 10⁶ cells...
were present in each tube. The tubes were capped and then incubated at 37° in a water bath with vigorous shaking for 1 hr.

After the 1-hr incubation, the C3H/10T/1/2CL8 cells were washed, serially diluted, and plated into 60-mm tissue culture dishes at concentrations of 104 and 200 cells for the transformation and cytotoxicity studies, respectively. Similarly, for transformation studies without S-9 activation, the cells were plated and incubated with various concentrations of cyclophosphamide for 24 hr as previously described (1, 9). Approximately 10 days or 1 month later, the cells were stained and the plating efficiency or the presence of transformed foci were determined, respectively. Only type III morphologically transformed foci were scored, since these transformants usually produce sarcomas when injected into syngeneic hosts (9).

For chromosomal studies 4 ml of complete medium were added to each tube following the 1-hr incubation with S-9 and cyclophosphamide. The cells were centrifuged at 600 x g for 5 min. Cell pellets were resuspended in complete medium and then recentrifuged. All cells from each tube were plated into separate 25-sq cm flasks containing approximately 4 ml of complete medium. The medium was changed 2 hr after the cells were seeded, and 5-bromo-2'-deoxyuridine was added to flasks in which SCE studies were to be done. The cultures were incubated at 37° for 24 or 48 hr to examine chromosomal breakage and SCE's, respectively. Chromosome preparations were then made and scored for chromosomal aberrations (1) or SCE's (14).

A minimum of 50 metaphases were examined for chromosomal breaks at each dose used. The frequency of chromosomal aberrations, excluding gaps, was measured as the number of breaks per metaphase. The number of SCE's per cell was determined on the basis of 4 to 25 intact differentially stained metaphases for each concentration.

**RESULTS**

The ability of cyclophosphamide to produce transformation in C3H/10T/1/2CL8 cells with or without S-9 activation is illustrated in Table 1. No transformation or cytotoxicity was seen without S-9 activation even after a 24-hr exposure to cyclophosphamide, 500 μg/ml. However, with S-9 activation, significant dose-dependent transformation and cytotoxicity were observed after only a 1-hr exposure of cyclophosphamide. In addition the expression of transformation and cytotoxicity produced by cyclophosphamide was inhibited if glucose 6-phosphate and NADP were removed from the activation system.

The effect of a 1-hr exposure to cyclophosphamide with S-9 activation on chromosomal damage and SCE's in both the A(T,Cl-3 and C3H/10T/1/2CL8 cells is shown in Table 2. A dose-response increase in chromosomal breaks and SCE's was found in both cell types. Moreover, elimination of glucose 6-phosphate and NADP from the S-9 activation system completely blocked the expression of cyclophosphamide produced chromosomal breakage and increased SCE's. In addition no increase in chromosomal breaks or SCE's was observed even after a 24-hr exposure to cyclophosphamide, 500 μg/ml, in the absence of S-9 activation (data not shown). Also an increase in SCE's was a more sensitive method for identification of chromosomal changes than was chromosomal breakage, since treatment with cyclophosphamide, 10 μg/ml, produced no significant chromosomal breaks, whereas considerable SCE's were found after similar exposure.
Cyclophosphamide can be activated by liver S-9 to produce oncogenic transformation, chromosomal breakage, and increased SCE’s in mammalian cell cultures. Thus the good correlation found between the ability of cancer chemotherapy agents to produce oncogenic transformation and chromosomal damage in culture and their known carcinogenicity in vivo (1) can now include cyclophosphamide. Unlike the other chemotherapy agents that we have previously tested, cyclophosphamide requires metabolic activation through a mixed-function oxygenase system. Since many other potential carcinogens also produce mutagenic and oncogenic metabolites through a similar mixed-function oxygenase system, these studies should be relevant not only in illustrating the potential usefulness of short-term assays to predict hazards in man but also in the need to incorporate appropriate activation systems into any short-term assay to screen unknown chemicals or mixtures for similar properties.

We do not wish to infer that this particular activation system has been optimized or is optimal for studying potential mutagens and/or carcinogens. Other tissue sources of S-9, the use of intact cells for activation, and the exploration of additional methods must be investigated in depth before conclusions are made regarding the most relevant system(s) for activation of potential mutagens and carcinogens. However, it seems essential that some activation procedure should now be incorporated into any study using in vitro systems to examine potential human hazards of a given chemical.

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Cyclophosphamide-induced Oncogenic Transformation, Chromosomal Breakage, and Sister Chromatid Exchange following Microsomal Activation

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