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Quantification of *CD59*$^-$ mutants in human–hamster hybrid (A_L) cells by flow cytometry

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Abstract

Mutation assay is an important approach in evaluating the genotoxic risk of potentially harmful environmental chemicals. The human–hamster hybrid (A_L) cell mutagenesis system, based on the complement/antibody-mediated cytotoxicity principle, has been used successfully to evaluate the mutagenic potential of a variety of environmental toxicants. The A_L cells contain a standard set of CHO chromosomes and a single human chromosome 11, which expresses several cell surface proteins including CD59 encoded by the *CD59* gene at 11p13.5. A modified mutation assay by flow cytometry was developed to determine the yield of *CD59*$^-$ mutants after either radiation or chemical treatment. After incubation with phycoerythrin-conjugated mouse monoclonal anti-*CD59* antibody, the *CD59*$^-$ mutant yields were determined by quantifying the fluorescence of the cells using flow cytometry. This method is faster and eliminates the commonly encountered toxicity problems of the complements with the traditional complement/antibody assay. By comparing the mutant fractions of radiation or chemically treated A_L cultures using the two methods, we show here that the flow cytometry assay is an excellent substitute in providing an efficient and highly sensitive method in mutant detection for the traditional complement/antibody assay.

Keywords: A_L cells; *CD59*; Gamma ray; MNU; Mutagenesis; Flow cytometry

1. Introduction

In vitro genotoxic assays to assess the potential DNA damaging and carcinogenic risk of environmental agents are part of an established human health risk evaluation program of the National Toxicology Program because of the close correlation between mutagenesis and carcinogenesis. In contrast to the high cost, societal concern and extensive time frame necessary for animal studies, in vitro genotoxic assay systems represent an attractive alternative.

The human–hamster hybrid (A_L) cell system is a well-established in vitro model for detecting mutagens that induce mutations ranging from large, multilocus deletions to small deletions as well as point mutations. The A_L cells contain a standard set of CHO chromosomes and a single human chromosome 11, which expresses several cell surface proteins including CD59 encoded by the *CD59* gene at 11p13.5 [1–3]. CD59 is a widely distributed, glycosylphosphatidylinositol (GPI)-anchored cell surface protein, which acts as an inhibitor of complement [4,5]. Because only a

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small part of the human chromosome 11 (11p15.5) is required for the viability of the hybrid cells, mutations in the human chromosome ranging in size of up to 140 Mbp of DNA can be readily detected [6–8]. After exposure to mutagens, mutants lacking *CD59* antigen can be scored by using rabbit serum-complement plus anti-*CD59* antibody. While mutants will survive from the treatment and form colonies, wild-type cells are efficiently lysed. There is evidence that some *CD59*$^-$ mutants can maintain small amounts of wild-type CD59 protein for several generations, which results in less efficient mutation detection. Furthermore, the spontaneous mutant fraction increases with the incubation period. On average, it takes 4–6 weeks to complete a mutagenic assay.

A modified mutation assay based on flow cytometry has been developed to determine the yield of *CD59*$^-$ mutations. After incubation with phycoerythrin-conjugated mouse monoclonal anti-*CD*59 antibody, the *CD59*$^-$ mutant fraction was quantified based on fluorescent intensity using flow cytometry. In the present study, A_L cells were treated with graded doses of an alkylating agent, *N*-methyl-*N*-nitrosourea (MNU), a well-known mutagenic and carcinogenic agent [9–11] or irradiated with graded doses of gamma rays. The mutant yields, determined by flow assay, were then compared with those obtained using the traditional complement/antibody mutation assay. We show here that mutant analyses by flow cytometry yields a clear dose response increase in mutation in A_L cells treated with both MNU and gamma rays. The mutant yields detected by flow analyses, however, are consistently higher than the level obtained using the traditional antibody–complement assay.

2. Material and methods

2.1. Cell culture

The human–hamster hybrid A_L cells that contain a standard set of Chinese hamster ovary-K1 chromosomes and a single copy of human chromosome 11 were used in this study. Chromosome 11 encodes cell surface markers that render A_L cells sensitive to killing by a specific monoclonal antibody in the presence of complement. Rabbit serum-complement was from HPR (Denver, PA). Antibody specific to the *CD59* antigen was produced from hybridoma culture as described [2,3]. Cells were maintained in Ham's F-12 medium supplemented with 8% heat-inactivated fetal bovine serum, 25 μg/ml gentamycin and 2×10^{-4} M glycine at 37 °C in a humidified 5% CO_2 incubator and passaged as described [6–8,12–18].

2.2. Cytotoxicity of gamma irradiation or treatment with MNU

Exponentially growing A_L cells were plated on T75 flasks 2 days before treatment. For MNU (Midwest Research Institute, Kansas City, MO) treatment, cells were treated with 0.125, 0.25 or 0.5 μM for 30 min; for gamma ray irradiation, cells were irradiated with 1, 3 or 5Gy at an absorbed dose rate of 0.96Gy/min using a ^{137}Cs irradiator. After treatment, cultures were washed twice with balanced salt solution, trypsinized to remove them from the culture flasks and replated into 100 mm diameter petri dishes for colony formation. Following incubation for 7–8 days, cultures were fixed with formaldehyde and stained with Giemsa. The number of colonies was counted to determine the surviving fraction as described [12–18]. Remaining cells was replated in new flasks for further mutation assay after the expression period.

2.3. Quantification of mutations at the CD59 locus with complement/antibody assay

This 7-day expression period was needed to permit surviving cells to recover from the temporary growth lag caused by MNU or irradiation and to multiply sufficiently so that the progeny of the mutated cells were no longer expressing lethal amounts of *CD*59 surface antigen. To determine the mutant fraction, 5×10^4 cells in 2 ml growth medium were plated into each of the six 60 mm dishes. The cultures were incubated for 2 h to allow the cells to attach, after which 0.3% *CD*59 antiserum and 1.5% (v/v) freshly thawed complement were added to each dish as described [12–18]. The cultures were further incubated for 7–8 days. At this time, the cells were fixed and stained and the number of *CD59*$^-$ mutant colonies was scored. Controls included identical sets of dishes containing antiserum alone, complement alone or neither agent. The mutant fraction at each dose was calculated as the number of surviving colonies divided by the total number of cells plated after correction for any non-specific killing by the complement itself.

2.4. Preparations of cells for flow cytometry assay

While mutations at the *CD*59 locus were analyses by traditional complement/antibody method, the same batch of cells was prepared for flow cytometry assay at the same time. Cells were washed with PBS, counted and 1×10^6 cells were aliquoted into a 15 ml tube on ice. After centrifuged for 5 min at 1000 rpm, cells were suspended in 5 ml cold FACS buffer (1% BSA, 10 mM sodium azide in PBS). After a second round of centrifugation, supernatant was aspirated as thoroughly as possible. The bottom of the tube was then tapped gently to resuspend the cells in the residual buffer. A 1 ml of 1: 200 diluted phycoerythrin-conjugated mouse monoclonal anti-*CD*59 antibody (Catlag Laboratories, Burlingame, CA) was added to the cells and mixed well by pipetting up and down, followed by incubation on ice for 1 h. Cells were subsequently centrifuged and washed twice in 5 ml cold FACS buffer before