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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

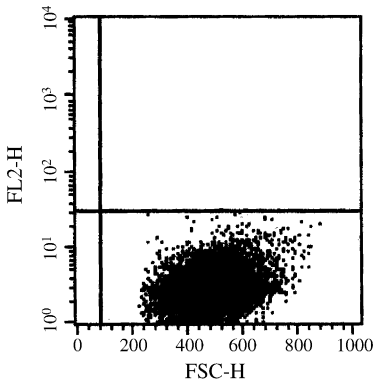
Exponentially growing A_L cells were plated on T75 flasks 2 days before treatment. For MNU (Midwest Research Institute, Kansas City, MO) treatment, cells were treated with 0.125, 0.25 or 0.5 μM for 30 min; for gamma ray irradiation, cells were irradiated with 1, 3 or 5Gy at an absorbed dose rate of 0.96Gy/min 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.

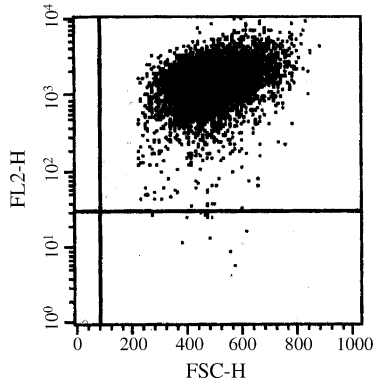
2.4. Preparations of cells for flow cytometry assay

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



Gate: G1
Quad Location: 84,29

Quad	% Gated	X Mean	Y Mean
UL	0.00	***	***
UR	0.04	633.25	29.97
LL	0.00	***	***
LR	99.96	481.35	3.04



Gate: G1
Quad Location: 84,29

Quad	% Gated	X Mean	Y Mean
UL	0.00	***	***
UR	99.88	494.98	1594.94
LL	0.00	***	***
LR	0.12	469.75	19.39

Fig. 1. A typical experiment to detect *CD*59$^-$ mutation using flow cytometry assay. The gate set for this experiment indicated that unstained A_L cells were 99.96% *CD59*-negative (A). In contract, only 0.12% of the stained, wild-type A_L cells were found outside the gate setting indicating that the background *CD59*$^-$ mutation in this experiment is 0.12% or 120 per 10^5 survivors (B).

resuspended in 0.5 ml FACS buffer for flow cytometry assay. Controls included non-stained wild-type A_L cells that served as an auto-fluorescence control; stained wild-type A_L cells (containing CD59 antigen) as a positive control and mutant A_L cells that lack CD59 antigens as a negative control.

2.5. Quantification of mutations at the CD59 locus with flow cytometry

Flow cytometry assay was performed using a flow cytometry (FACSCalibur™, Becton Dickinson). Samples were gated, based on 90° versus forward light scatter characteristics, to eliminate cell clumps and debries from the analysis. Voltage across the photomultiplier tubes was adjusted to obtain a good separation of fluorescent and non-fluorescent cells. The 10^5 cells per sample were collected for analysis. The percentage of cells/events in the non-fluorescent region of untreated, stained wild-type cells was taken as background and was subtracted from the stained, treated samples.

2.6. Statistical analysis

All numerical data were calculated as mean and standard deviation (S.D.). Comparisons of mutation frequency between treatment groups and controls were performed by Student's *t*-test. A *p*-value of 0.05 or less between groups was considered significant.

3. Results

3.1. Separation of cells with or without CD59 antigen using flow cytometry

To test the efficiency in separating *CD59*-negative cells from the positive ones by flow cytometry, stained and non-stained wild-type and known mutant *CD59*$^-$ A_L cells were analyzed. When samples were gated to eliminate debries, we found that unstained cultures of both the wild-type and *CD59*$^-$ mutant A_L cells demonstrated similar background auto-fluorescence as the stained *CD59*$^-$ mutant A_L cells. All of these samples were, therefore, considered to be negative. After repeated flow cytometry analyses until a similar background auto-fluorescent reading was consistently obtained between non-stained wild-type A_L cells and the stained *CD59*$^-$

Table 1
Comparison of gamma ray-induced *CD59*$^-$ mutation in A_L cells using either flow cytometry or traditional complement/antibody assay

Group	No. of experiments	S.F.*	Traditional complement/antibody assay					Flow cytometry assay		
			No. of cells	P.E.*	No. of mutants	M.F.* (10^{-5})	Means ± S.D.	Total no. of cells	M.F.* (10^{-5})	Means ± S.D.
Control	5	100	3×10^5	0.92	99	36	56 ± 18	1×10^5	60	120 ± 50
		100	3×10^5	0.90	157	58		1×10^5	130	
		100	3×10^5	0.94	118	42		1×10^5	100	
		100	3×10^5	0.81	199	82		1×10^5	190	
		100	3×10^5	0.88	164	62		1×10^5	120	
1Gy	5	86	3×10^5	0.85	260	102	125 ± 33	1×10^5	160	240 ± 60
		78	3×10^5	0.86	390	151		1×10^5	250	
		64	3×10^5	0.93	237	85		1×10^5	240	
		68	3×10^5	0.81	396	163		1×10^5	320	
		70	3×10^5	0.86	320	124		1×10^5	230	
3Gy	5	71	3×10^5	0.65	332	170	211 ± 47	1×10^5	360	430 ± 120
		60	3×10^5	0.68	530	260		1×10^5	600	
		50	3×10^5	0.61	287	157		1×10^5	300	
		61	3×10^5	0.58	444	255		1×10^5	480	
		42	3×10^5	0.60	383	213		1×10^5	410	
5Gy	5	30	3×10^5	0.45	346	256	334 ± 76	1×10^5	580	790 ± 170
		27	3×10^5	0.38	443	389		1×10^5	1000	
		12	3×10^5	0.40	328	273		1×10^5	730	
		18	3×10^5	0.42	538	427		1×10^5	920	
		15	3×10^5	0.36	362	335		1×10^5	720	

* S.F.: surviving fraction; P.E.: plating efficiency with complement in the medium; M.F.: mutation fraction.

mutant A_L cells, the former was used as a negative control in the present study. In general, stained wild-type A_L cells showed a very strong fluorescent staining pattern with less than 0.15% negatively staining cells in the population (background *CD*59$^-$ A_L cells). Fig. 1 shows a typic A_L cytometry experiment. With the setting gate, 99.96% of the unstained A_L cells were identified to be *CD*59-negative (fluorescence below the line). In contrast, only 0.12% of the stained wild-type A_L cells were found outside the gate setting, indicating that the background *CD*59$^-$ mutation in this experiment was 0.12% or 120 per 10^5 survivors. Furthermore, the mean fluorescent intensity of stained wild-type A_L cells was more than 500 times higher than that of unstained A_L cells (Fig. 1). These data clearly illustrate the potential of using flow cytometry in detecting *CD59*$^-$ mutant fraction in the A_L cell system.

3.2. Calibration of the flow cytometry analysis using pre-mixed samples

To determine the sensitivity and accuracy of CD59 detection by flow cytometry, mutant A_L cells were mixed with wild-type cells in known proportions (0.1–10% mutant cells) and these samples were stained and analyzed. Fig. 2 shows the correlation between the percentage of pre-mixed A_L cultures and the ones identified by FACS analysis. A 2% pre-mixed culture of *CD59*$^-$ mutant cells among wild-type A_L cells was screened as 2.1%, whereas a 0.1% pre-mixed cultures was detected as 0.12%. The results showed an excellent correlation between the pre-mixed ratio and the measured values obtained by flow cytometry. This validated the later approach in mutant quantification.

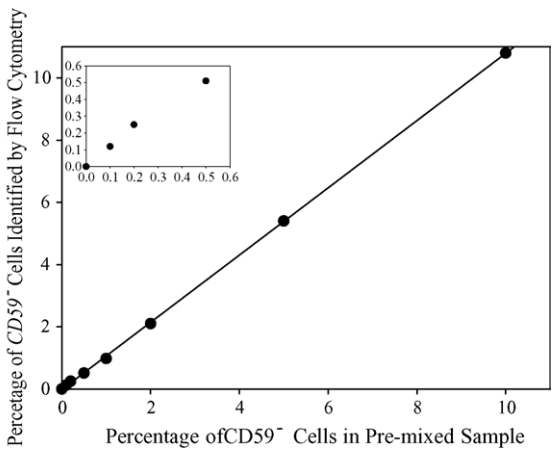


Fig. 2. Identification of CD59-negative mutant cells pre-mixed with wild-type A_L cells in various proportions before antibody labeling. Data were pooled from two independent experiments.

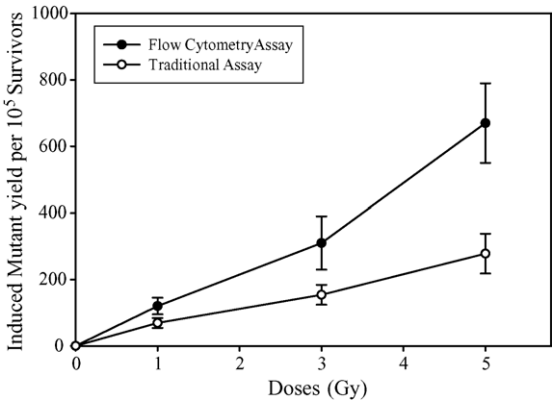


Fig. 3. Dose-dependent mutagenesis in A_L cells induced by graded doses of gamma ray. Data were pooled from five independent experiments. Bar represents ±S.D.

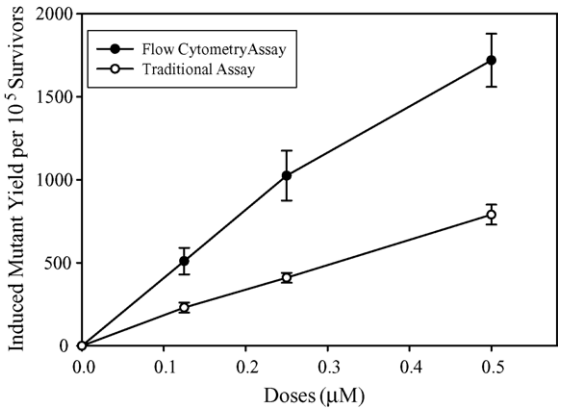


Fig. 4. Effects of MNU on the mutation yield in A_L cells detected by either the traditional complement/antibody mutation assay or flow cytometry method. Data were pooled from three independent experiments. Bar represents ±S.D.

3.3. Determination of radiation-induced mutation using flow cytometry and traditional assay

Using the traditional complement/antibody assay, we showed previously that gamma irradiation induces a dose-dependent mutagenesis in A_L cells after a 7- to 10-day expression period [8,19,20]. In the present study, the dose response survival levels of A_L cells to gamma rays were found to be comparable to previously published reports [8,19,20]. After a 7-day expression period, progeny of irradiated cells were prepared for mutation assay using both the traditional complement/antibody and flow cytometry assays. The background mutant fraction of A_L cells used in these experiments, based on the complement/antibody assay and flow cytometry assay, was 56 ± 18 and 120 ± 50 mutants per 10^5 survivors, respectively (Table 1). As shown in Fig. 3, both methods demonstrated clear a dose-dependent mutagenesis with increase in radiation dose. However, flow cytometry assay showed a higher induced mutation yield than that of traditional complement/antibody assay ($p < 0.01$). These data suggested that flow cytometry approach might be more sensitive than the traditional method for scoring *CD*59$^-$ mutations.

3.4. Comparison of MNU-induced mutation using flow cytometry and traditional assay

To further characterize the accuracy of flow cytometry method in detecting *CD*59$^-$ mutations, A_L cells were treated with a well-known mutagen, MNU. After a 7-day expression period, cells were prepared as described before. Fig. 4 shows the mutant yield of A_L cells treated with graded dose of MNU and analyzed either by flow cytometry or the traditional complement/antibody assay. Similar results were found in MNU-induced mutagenesis as those described above with radiation-induced mutagenesis. In addition to being able to detect a dose response induction, flow cytometry method also showed higher induced mutant yields compared with the traditional assay (Fig. 4). For example, the induced mutation fraction of 0.25 μM MNU treatment was 413 ± 32 and 1020 ± 150 mutants per 10^5 survivors based on the complement/antibody assay and flow cytometry assay, respectively ($p < 0.001$). These results further confirmed that flow cytometry analysis was more sensitive in mutant detection than the traditional complement/antibody assay.

4. Discussion

Determining the carcinogenic potential of environmental and occupational chemicals is an important issue related to cancer prevention. Unfortunately, epidemiological and animal studies are expensive to conduct and information is unavailable for most of the chemicals. Mutagenesis tests can help in predicting carcinogenicity, but many short-term assays, such as the *HPRT* and *Oua* are limited in recovering mainly point mutations and small deletions. Among the various mutagenic assays currently in use, the A_L cell assay developed by Puck et al. [1–3] provided a sensitive system for mutation analysis. Although human chromosome 11 in the A_L hybrid resides in a hamster cell, there is no evidence that its function or structure is different than its human cell habitat. Banding patterns for the human chromosome 11 in the A_L cells are indistinguishable from any human chromosome number 11 [21]. Furthermore, there is no evidence that the high frequencies of *CD*59$^-$ muta-

tions induced by a variety of mutagens, such as radiation, which induces predominately multilous deletions, are caused by the intrinsic hypermutability of the A_L cells [4,6,7]. For example, its mutability at the *HPRT* locus is not different from that of other human or rodent cell lines [4,22]. The main reason that more *CD59*$^-$ mutants than ouabain-resistant or *HPRT*$^-$ mutants are obtained are that mutants with large deletions at the *HPRT* locus are poorly recovered.

The A_L cell *CD59*$^-$ mutation complement/antibody assay has been used for more than 20 years in various laboratories to investigate mutagenesis [2,3,11–19]. The main advantage of this system is that mutant cells do not require the human chromosome 11 for survival except a small segment at the tip of the short arm (11p15.5) encoding the *Ras* gene. This made it possible to detect multilocus deletious as well as the small, point mutations. However, the traditional assay of *CD59*$^-$ mutation depends on the antibody–complement complex-mediated cytotoxicity to select *CD59*$^-$ mutants. Since complement is often toxic, it is necessary to pre-test rabbit serum lots and to adjust the mutant fraction based on the increased toxicity [13,19].

The flow cytometry-based mutation assay can easily avoid the complement issue and shortens the assay by about 1 week (colony formation period), thereby, reducing the cost. Although flow cytometry has been used to detect the *CD*59$^-$ mutation in the A_L cell line [23], there were several deficiencies with this study. First of all, the background of the *CD*59$^-$ level in this study was high (∼0.45%), probably, because of higher spontaneous mutation or improper gate set for experiments. Secondly, the gate was manually set at a level such that cells whose fluorescent intensity was weaker than 10% of the maximal peak of CD59-positive cells were considered to be mutants. This resulted in the high background yield as well as the induced mutant fractions reported in the study. Thirdly, without the traditional complement/antibody assay as a parallel control, there was no reference point to compare both the sensitivity and specificity of the flow cytometry assay.

In the present study, results from both radiation and MNU-induced *CD*59$^-$ mutation demonstrated higher induced mutant yields (total mutant yield minus background level) when quantified by flow cytometry in comparison with the traditional complement/antibody assay, which is an indication of increased sensitivity of this new method. However, our data also indicated less precision with flow cytometry compared to the traditional assay based on the large standard deviations in mutant yields in experiments using gamma rays and MNU. This difference may be caused by inherent fluctuations in flow cytometric detection of mutants. In flow cytometry assay, various mutants may reveal various patterns of fluorescence, whereas in traditional assay, mutations that eliminate binding of the CD59 antibody are phenotypically the same, i.e. these cells survive and form colonies after treatment with monoclonal antibody and complement. Point mutations may decrease the binding affinity of the CD59 antibody, resulting in a relative decrease, but not complete elimination of fluorescence as detected by flow cytometry. Large deletions would be expected to completely eliminate antibody binding, and hence,fluorescence. Another possibility is that mutants with variable levels of fluorescence maybe occurred secondary to mutations in the CHO genes encoding the glycosylphoshotidylinositol anchor that secured CD59 to the cell membrane [4,5].

Overall, our present study indicated that flow cytometry assay for *CD*59$^-$ mutation in A_L cells is a reliable method, one that is more sensitive and efficient than the traditional assay. Using other fluorescence that tag to other human chromosome 11 genes, it is possible to determine the *CD*59$^-$ spectrum of the mutants. These studies are currently underway in our laboratory.

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