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EMS mutant spectra generated by multi-parameter flow cytometry

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Abstract

The CHO A_L cell line contains a single copy of human chromosome 11 that encodes several cell surface proteins including glycosyl phosphatidylinositol (GPI) linked CD59 and CD90, as well as CD98, CD44 and CD151 which are not GPI-linked. The flow cytometry mutation assay (FCMA) measures mutations of the *CD59* gene by the absence of fluorescence when stained with antibodies against the CD59 cell surface protein. We have measured simultaneous mutations in CD59, CD44, CD90, CD98 and CD151 to generate a mutant spectrum for ionizing radiation. After treatment with ethyl methanesulfonate (EMS) many cells have an intermediate level of CD59 staining. Single cells were sorted from CD59[−] regions with varying levels of fluorescence and the resulting clonal populations had a stable phenotype for CD59 expression. Mutant spectra were generated by flow cytometry using the isolated clones and nearly all clones were mutated in *CD59* only. Interestingly, about 60% of the CD59 negative clones were actually GPI mutants determined by staining with the GPI specific fluorescently labeled bacterial toxin aerolysin (FLAER). The GPI negative cells are most likely caused by mutations in the X-linked *pigA* gene important in GPI biosynthesis. Small mutations of *pigA* and *CD59* were expected for the alkylating agent EMS and the resulting spectra are significantly different than the large deletions found when analyzing radiation mutants. After analyzing the CD59[−] clonal populations we have adjusted the FCMA mutant regions from 1% to 10% of the mean of the CD59 positive peak to include the majority of *CD59* mutants.

Keywords

EMS; Mutation; Mutant spectrum; CD59; Flow cytometry; GPI linkage

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Conflict of interest statement

One author (MHF) has a potential conflict of interest as the owner of a small business, Cytomation GTX, Inc., which was the recipient of an NIH STTR grant which supported this work.

1. Introduction

We have previously characterized the flow cytometry mutation assay (FCMA) as a sensitive and rapid method of measuring the genotoxicity of known and unknown chemical and physical agents [1–3]. The FCMA uses the CHO A_L cell line that has an incorporated human chromosome 11 as well as the entire hamster genome. This cell line was originally used in the standard complement based CHO A_L clonogenic mutation assay [4,5]. Chromosome 11 contains genes encoding cell surface proteins including *CD59*, mutations of which are scored by the absence of fluorescence when stained with phycoerythrin (PE)-conjugated monoclonal antibodies against *CD59*. Individual mutants can be sorted and cloned for further analysis. Additional genes on chromosome 11 encode proteins that are expressed on the surface of the cells, including *CD44*, *CD90*, *CD98* and *CD151*. These genes are encoded not by the normal hamster genome, only by human chromosome 11. By measuring the presence or absence of these proteins also, it is possible to generate mutant spectra using flow cytometry [6] more rapidly and efficiently than spectra generated by PCR [7,8] and Southern Blot [9]. The term “mutant spectra” is used here to indicate the distribution of mutations in genes along chromosome 11 indicated by the loss of surface expression of the gene product. We have previously shown that the loss of the surface protein expression is completely correlated with the loss of the respective gene(s) based on PCR results [6].

We previously defined the mutant region of the FCMA as cells with fluorescence less than 1% of the mean fluorescence of the positive peak [2]. This was a conservative measurement and we subsequently showed that many cells mutated in *CD59* by the clastogen gamma radiation [10] were not being scored in that region [11], suggesting that a larger region should be used. In that report we also showed that the mutants that initially have an intermediate level of fluorescence lose *CD59* expression over time to background (unstained) control levels of fluorescence [11]. The complete loss of fluorescence can be explained by large deletions within or encompassing the *CD59* gene so no *CD59* protein is expressed on the plasma membrane.

Since ionizing radiation is a clastogen and is well known to cause large deletions, it is important to identify whether other mutagens that primarily cause small mutations would generate similar mutants. In this report we investigate *CD59*[−] populations and clones generated by exposure to the known alkylating agent ethylmethanesulfonate (EMS) [12,13] that are believed to be mostly point mutations. Point mutations allow for mutated conformations of *CD59* on the cell surface and in turn intermediate staining, suggesting that a larger region should be used for mutant detection. Furthermore, these point mutations should lead to stable phenotypes of *CD59* fluorescence that would not reduce to background levels over time.

Many cell surface proteins including *CD59* are linked to the cell membrane by glycosyl phosphatidylinositol (GPI) anchors [14,15]. Interestingly, many *CD59*[−] clones, including nearly all *CD59*[−]/*CD90*[−] clones, were found to be negative for GPI anchors when stained with the GPI protein specific fluorochrome conjugated toxin aerolysin (FLAER) [16]. The

lack of GPI linkage is most likely due to the mutation of *pigA*, an X-linked GPI biosynthesis protein [17].

In this paper we analyze clones with intermediate CD59 fluorescence and subsequently generate mutant spectra. Our results show that the mutant region of the FCMA should be enlarged to encompass more of the CD59⁺ region, making an even more sensitive assay. Also, by having the ability to generate mutant spectra using flow cytometry we are able to rapidly and with accuracy determine the nature of mutations generated by novel agents.

2. Materials and methods

2.1. Cell culture

The CHO A_L(N) cell line contains the entire Chinese hamster KI cell line genome with an incorporated copy of human chromosome 11 including a gene conferring resistance to the antibiotic G418 (Sigma–Aldrich, St. Louis, MO). By treating cells periodically with 800 µg/ml G418, spontaneous background mutants were reduced. Cells were cultured in Ham's F12 medium supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA), penicillin/ streptomycin (0.14 and 0.2 g/L, respectively) and 7.5% (w/v) sodium bicarbonate, pH 7.3. Cells were trypsinized and passed every 3–4 days to avoid confluence that can increase the background mutant fraction. Cells were maintained in humidified 5% CO₂ incubators at 37°C.

2.2. Treatment with EMS

One day prior to treatment, 4×10^5 cells were seeded in T-75 flasks, giving approximately 1×10^6 cells at treatment time. Cells were treated with 8 mM EMS (CAS number 62-50-0) diluted in F12 medium for 3 h in 37 °C incubators. This dose results in 20% survival of cells [2]. After the treatment, cells were washed twice with warm PBS and F12 medium was replaced.

2.3. Antibody labeling

One million cells were stained for CD59 using phycoerythrin (PE)-conjugated mouse anti-human CD59 monoclonal antibodies (Caltag Laboratories, Burlingame, CA) as previously described [1,2].

Multicolor staining for CD59/CD90/CD44 and CD98/CD151 was carried out separately as previously described [6]. For CD59/CD90/CD44 analysis, cells were suspended in 50 µl staining solution (1.3 µl anti-CD59-PE, 10 µl anti-CD44-biotin (Serotec, Raleigh, NC), 10 µl anti-CD90-Alexa-647 (Serotec, Raleigh, NC) and 28.7 µl staining buffer), incubated on ice for 30 min, then 1 ml staining buffer (1% BSA, 0.1% sodium azide in PBS) was added to each sample and samples were centrifuged. Samples were resuspended in 100 µl Streptavidin-Alexa-488 (Molecular Probes, Eugene, OR) (1:100 dilution in staining buffer) and were incubated on ice for 30 min. The pellet was resuspended in 0.5 ml staining buffer and passed through a 40 µm mesh filter into flow cytometry analysis tubes. Separately, cells were stained with 50 µl staining solution containing 10 µl anti-CD151-PE (BD Biosciences,

San Jose, CA), 10 μ l anti-CD98-FITC (BD Biosciences, San Jose, CA) and 30 μ l staining buffer.

The GPI anchor was detected using the bacterial toxin aerolysin conjugated to Alexa-488 at a concentration of 10 nM per sample. Cells were resuspended in 250 μ l FLAER (Protox Biotech, Victoria, Canada) (1:10 dilution in staining buffer) and were then incubated for 30 min on ice [6].

2.4. Flow cytometry

Samples were analyzed on a CyAn™ flow cytometer (Beckman Coulter, Fort Collins, CO) with 488 and 635 nm solid-state lasers for excitation. Fluorescence was detected by the following filter configuration: 545 nm dichroic longpass (DLP) and 575/25 nm bandpass (BP), 545 nm DLP and 530/40 nm BP for Alexa-488 and 650 nm DLP and 665/20 nm BP for Alexa-647. The same PMT voltages were used for various experiments and the fluorescence intensities of positive and negative control populations were stable over time. A gating region was set on forward scatter versus side scatter to eliminate debris. When analyzing clonal populations for the presence of CD59, 5×10^4 to 1×10^5 events were collected per sample and all populations were sampled twice at least 1-week apart, while multicolor analysis was completed only once.

2.5. Cell sorting

Using an Epics V cell sorter, 1000 anti-CD59-PE stained cells were sorted into 35 mm Petri dishes from six CD59 negative regions and one region of the CD59 positive peak 9 days after treatment with 8 mM EMS. Sorted populations were expanded in dishes for 7 days and then transferred to T-75 flasks to keep in culture. When the populations were stable they were stained again with anti-CD59-PE and single cells were sorted into 96 well plates from four regions using a MoFlo™ Cyclone (Beckman Coulter, Fort Collins, CO). Clones were allowed to grow for 18–21 days with one refeeding of 200 μ l F12. Clones were transferred to T-25 flasks for continuous culturing.

In a separate experiment, a MoFlo™ Cyclone was used to sort single cells into 96 well plates from four regions of the CD59 negative population and one region of the positive peak 9 days after treatment with 8 mM EMS. Clones were grown for 18–21 days with one refeeding and then were transferred to T-25 flasks for culturing.

3. Results

3.1. Mutant populations

One thousand cells were sorted from six CD59⁻ regions (Fig. 1) 9 days after treatment with 8 mM EMS to determine if they remain negative and have fluorescence levels similar to their sorted regions. The sorted cells were expanded into populations and were stained several times over the course of several weeks for CD59 and were indeed found to be comprised of stable populations (Fig. 2). Population A is almost completely negative while the opposite is true for populations E and F. The populations sorted from the middle regions of B, C and D contain the entire spectrum of CD59⁻ to CD59⁺ cells. It is important to note

that these populations remained stable and had very similar profiles while remaining in culture for several months.

The populations B, C and D were chosen for sorting because they are heterogeneous in fluorescence when stained for CD59. Single cells were sorted from four CD59 negative regions (Fig. 2) into 96 well plates and the expanded populations were stained for CD59 twice over several weeks to determine if clonal populations are stable in fluorescence intensity. All clonal populations remained fluorescently stable over several weeks with intensities well within the same order of magnitude (Fig. 3).

The average CD59 staining fluorescence was calculated for the four regions of populations B, C and D by averaging the intensities of all clones sorted from the regions (Fig. 4). Negative controls were unstained stock cells while positive controls were the same stock cells stained for CD59. The fluorescence of region four of populations B, C and D were all significantly lower than positive controls with values of $p = 0.001$, $p < 0.001$ and $p = 0.03$ respectively. Also, fluorescence of clones sorted from regions one through four increases in order for all three populations, demonstrating that clones have fluorescence levels relative to the region from which they were sorted. After initial analysis of the clones, they were continuously grown in culture for 1–2 weeks and then re-analyzed to determine that the fluorescence intensity was stable.

3.2. Mutant region sorting

In a separate set of experiments we sorted single cells directly from four CD59 negative regions and the positive peak (Fig. 5A) 9 days after treatment with 8 mM EMS, and expanded them into stable clonal populations. Region 1 represents 1% of the mean positive peak or the region used to score for CD59 mutants with the FCMA, while regions 2, 3 and 4 are between the mutant region and the positive peak. Clones were stained for CD59 and average fluorescence was calculated for each region (Fig. 5B). The average fluorescence for regions 1–3 were all significantly less than that of the positive peak while region 4 was nearly significant ($p = 0.055$).

3.3. EMS mutant spectrum

Cells were sorted directly from the mutant region (1% of the mean of the positive peak) of stock CHO_AL cells and were expanded into 23 stable clonal populations. The background mutant fraction in this region is approximately 0.1%. Clones were then stained for the presence of CD59, CD44, CD90, CD151 and CD98, generating a mutant spectrum of spontaneous background mutants (Fig. 6). Of the 23 clones over half are negative for both CD59 and CD44 which can most easily be explained as a deletion encompassing both genes that are less than 2 Mbp apart. Five of the background mutants were found to be positive only for CD151, which could be the result of a large deletion encompassing nearly the entire chromosome. Interestingly, four mutants were negative for both CD59 and CD90 only and, as will be discussed, are likely GPI mutants.

Clones isolated from regions 1–4 of populations B, C and D (Fig. 2) were analyzed in the same manner as background mutants to generate a mutant spectrum of EMS generated

mutants. Along with the five CD surface proteins, clones were also stained with FLAER, which is a GPI anchor specific toxin conjugated to a fluorochrome that can detect the presence of GPI anchors in the cell membrane.

Of the 16 clones isolated from the four regions of population B only one was positive for CD59 with fluorescence slightly less than half of control stained cells (Fig. 7). Nine clones were CD59 negative only while six clones were negative for both CD59 and CD90 but also FLAER, pointing to mutations in the GPI anchor instead of chromosome 11. Twenty clones were isolated from the four CD59⁻ regions of population C. Only one clone was positive for CD59, 5 of the 20 clones were only CD59 negative but 14 of the 20 clones are negative for CD59, CD90 and the GPI anchor (FLAER). Of the 17 clones isolated from the four regions of population D, two clones were complex, including a large deletion and one clone with mutations in both CD59 and CD90 independently (Fig. 7). Also, nine clones from population D were GPI mutants, three were CD59 negative while three were CD59 positive. It is important to note that nearly all of the CD59⁺ clones came from region 4 which is on the tail of the distribution of positive cells. Thus, these may represent cells that are outliers of the normal distribution and not true mutants.

4. Discussion

Traditional clonogenic mutation assays are based on the ability of mutated cells to survive and multiply when challenged, which in itself can be very time consuming, on the order of several weeks to more than a month [18–20]. Generating a mutant spectrum using PCR with DNA isolated from clones after running a traditional mutation assay [7] or the FCMA [6] is possible but it adds significantly more time and can be difficult to get all reactions working simultaneously. In this paper we propose that detecting mutations caused by genotoxic agents is much less time consuming and more sensitive using the FCMA and the newly defined mutant regions. Also, we suggest as we have previously reported [6] that flow cytometry generated mutant spectra are not only faster, but also simpler and possibly more accurate for DNA damaging agents. Furthermore, we have previously validated the flow cytometry mutant spectra with PCR [6]. Because of the lack of availability of a complete set of labeled antibodies that could be run simultaneously for all five markers plus FLAER, we were not able to actually do a complete mutant spectrum in one analysis. However, we have shown that in principle this could be done, given the availability of the proper antibodies. This would make the flow cytometry based assay even more rapid and flexible.

To determine if varying degrees of DNA damage cause different levels of CD59 expression, CD59⁻ cells were isolated. By sorting populations of cells from the mutant region 9 days after treatment with EMS we show that, unlike previously reported results for ionizing radiation where cells were entirely negative or positive for CD59 [11], EMS generates mutants with intermediate levels of CD59 expression that remain stable. This conspicuous difference in CD59⁻ cells generated by radiation and EMS is likely due to the way each agent acts on DNA. Radiation is known to cause double strand breaks leading to deletions [5,21], leading to the complete loss of CD59 expression on the cell surface while EMS is an alkylating agent often generating base substitutions [12,13], which could lead to changes in protein folding and in turn antibody affinity for CD59 on the protein surface. This in turn

would lead to varying levels of expression of fluorescence in this flow cytometry based assay.

To further understand the intermediate expression of EMS generated *CD59* mutants, and to determine if their fluorescence is stable over time, individual cells were isolated from four *CD59*⁻ regions. The resulting clonal populations were stained twice for *CD59* with cells grown for 1–2 weeks between staining, resulting in very little variation in their fluorescence. This stability further supports our hypothesis that these cells with intermediate *CD59* expression are mutants that consistently express a mutated phenotype of the *CD59* protein and not wild-type *CD59* with protein levels changing on the cell surface over time, which would cause fluorescence to vary. The average fluorescence of these clones increases from regions 1 to 4 and are significantly less than positive controls, meaning all four regions are negative in some degree for *CD59*. Clones isolated from the *CD59*⁻ region of cells 9 days after treatment with EMS support our findings that *CD59* mutants are stable and are not just a result of having been sorted previously into populations.

The background mutant spectrum we obtained from cells sorted from the mutant region of stock CHO A_L cells supports our previous hypothesis [11] that most spontaneous mutants are larger deletions. All of the isolated mutants were found to be mutated in more than just *CD59*: 13 were also *CD44* mutants while 5 were large deletions only positive for *CD151*. It is very unlikely to find many *CD151* mutants since it is relatively close to an essential gene needed for cell survival located on the end of the p-arm of chromosome 11 [8,22]. It is important to note that the four mutants that were found to be negatively stained for both *CD59* and *CD90* are likely not mutated independently in both genes but are instead mutations in an X-linked GPI linkage gene, as discussed below.

The mutant spectra we obtained from individual mutant clones treated with EMS were quite different from either background mutants or those induced by ionizing radiation that we have previously reported [6]. Almost all of the mutants were mutations only in the *CD59* gene or GPI mutants that are also most likely single small mutations. We have previously used the fluorescently labeled inactive variant of the protein aerolysin (FLAER) [6], which is a bacterial toxin that binds GPI anchor proteins [16,23], to distinguish between clonal populations with *CD59* mutations and ones with mutated GPI linkage genes after ionizing radiation. The most probable cause in the loss of GPI linkage would be a mutation in the X-linked hamster gene *pigA*, which encodes an important protein for the biosynthesis of GPI anchors [17]. It can also be assumed that GPI mutants would also be small since *pigA* is X-linked and a large deletion could encompass neighboring genes that are essential for survival. Small mutations in *pigA* would also explain why some cells have an intermediate expression of *CD59*. There could still be partially functional GPI linkage but less efficient than normal, so there is still some expression of *CD59* on the surface of some of the cells that are mutated in *pigA*.

It is interesting to note that greater than 60% of the clones analyzed when generating the mutant spectra for EMS were found to be GPI mutants and is a much greater fraction than the ~16% found for mutants generated by radiation [6]. This discrepancy may have several explanations; first, radiation causes large deletions which are of no consequence when they

occur along chromosome 11, which for the most part is not essential for survival, while large deletions of *pigA* on the X chromosome would often be lethal. Second, we have reported that CD59⁻ cells that are likely GPI mutants increase from a small fraction to the majority of total CD59⁻ cells measured with the FCMA over several weeks [3]. This increase could be caused by the importance of GPI linkage in the cell membrane for proper cell death signaling [24,25] allowing GPI mutants that would normally die to propagate.

We propose that generating mutant spectra by flow cytometry is not only much faster but can also be more accurate than PCR. Proper antibody binding requires both the presence of protein and correct folding compared to PCR, which can easily miss point mutations since only the presence of the two primer specific regions are necessary for positive results. Although sequencing of the genes of interest to generate a mutant spectrum would be more accurate than PCR or flow cytometry, it would not be time or cost effective for many clones, leaving flow cytometry as the best option, especially when studying novel agents.

When originally developing the FCMA, we defined the mutant region as 97% of unstained control cells which we later changed to 1% of the mean of the positive peak [2]. Here we propose to set the standard mutant region for the FCMA at 10% of the mean of the CD59 positive peak. This change is supported by the results from sorting from the CD59⁻ regions and specifically the mutant spectra where nearly all of the clonal populations from regions 1–3 (Fig. 2) were negative for CD59. We have reported similar results with mutants induced by ionizing radiation [11]. Using this larger region for mutant detection with the FCMA will give a better estimate of the mutant yield and will be less sensitive to day to day fluctuations in antibody staining and detection.

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Fig. 1. CHO A_L cells stained for CD59 9 days after treatment with 8 mM EMS. Six regions (A–F) were set for sorting cells with varying levels of CD59 fluorescence. Region A is the mutant region, 1% of the mean of the positive peak.

Fig. 2. 1000 cells each were sorted from regions A–F (Fig. 1) and expanded into stable populations. Individual cells were then sorted from regions 1–4 from populations B, C and D and were expanded into stable clonal populations for subsequent analysis. The X-axis represents the fluorescence intensity of anti-CD59-stained cells and the Y-axis is the relative number of cells.

Fig. 3.
Examples of individual clonal populations grown from single cells sorted from regions C1–4 shown in Fig. 2. Each column represents four individual clonal populations originally sorted from regions C1 through C4. The *X*-axis in each histogram is the fluorescence intensity of anti-CD59-stained cells and the *Y*-axis is the relative number of cells.

Fig. 4.

The average fluorescence for clones grown from individual cells sorted from the four CD59⁻ regions populations B, C and D in Fig. 2. Each histogram represents from 6–12 separate clones. Positive and negative controls are stained and unstained stock cells respectively. The dark bars are for cells analyzed initially and the light bars are for cells analyzed 1–2 weeks later after continuous culture. (A) Clones from Fig. 2B; (B) clones from Fig. 2C; (C) clones from Fig. 2D. Error bars represent the standard error of the mean (SEM).

Fig. 5.

(A) Individual cells were sorted directly from four CD59⁻ regions and the positive peak of cells 9 days after treatment with 8 mM EMS. Region A1 is the 1% mutant region. (B) Isolated clonal populations were stained for CD59 and the average fluorescence from 8–14 clones was calculated for each region. Error bars represent the SEM.

Fig. 6.
Flow cytometry generated mutant spectrum for clones isolated from the mutant region (1% of the mean of the positive peak) of untreated stock CHO A_L cells. Dark regions stained positive for the CD marker while white regions were negative and are likely mutations. Chromosomal distances are measured from the p-terminus.

Fig. 7.

Flow cytometry generated mutant spectra for clones isolated from four regions of populations B, C and D from Fig. 2. Cells were stained for five surface antigens as well as for the presence of GPI anchors using FLAER. Dark regions stained positive for the CD marker while white regions were negative and are likely mutations. The medium shade represents GPI mutants, which are also negatively stained for CD59 and CD90 since they are GPI-linked proteins.