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Mutant spectra of irradiated CHO A_L cells determined with multiple markers analyzed by flow cytometry

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Abstract

We have previously developed a sensitive and rapid mammalian cell mutation assay which is based on a Chinese hamster ovary cell line that stably incorporates human chromosome 11 (CHO A_L) and uses flow cytometry to measure mutations in *CD59*. We now show that multiparameter flow cytometry may be used to simultaneously analyze irradiated CHO A_L cells for mutations in 5 CD genes along chromosome 11 (*CD59*, *CD44*, *CD90*, *CD98*, *CD151*) and also a GPI anchor gene. Using this approach, 19 different mutant clones derived from individual sorted mutant cells were analyzed to determine the mutant spectrum induced by ionizing radiation. All clones analyzed were negative for *CD59* expression and PCR confirmed that at least *CD59* exon 4 was also absent. As expected, ionizing radiation frequently caused large deletions along chromosome 11. This technology can readily be used to rapidly analyze the mutant yield as well as the spectrum of mutations caused by a variety of genotoxic agents and provide greater insight into the mechanisms of mutagenesis.

Keywords

Mammalian mutation assay; flow cytometry; *CD59*; *CD44*; *CD90*; *CD151*; *CD98*; CHO A_L cells; gamma radiation; aerolysin; FLAER; mutant spectrum; GPI linkage

1. Introduction

DNA damage and mutagenesis are essential aspects of understanding the toxicology of chemical and pharmaceutical products. Mutagenesis assays using mammalian cells are based on mutations in a single gene that can be selected for in specialized media. These assays include the mouse lymphoma assay (mutations in the autosomal thymidine kinase gene) [1;2], the HPRT assay (mutations in the X-linked hypoxanthine-guanine phosphoribosyl transferase gene) [3;4], and a Chinese hamster ovary-human hybrid cell line (CHO A_L) assay (mutations in *CD59* on human chromosome 11) [5;6]. We and others [7-9] developed a flow cytometry mutation assay (FCMA) to rapidly measure mutations in the *CD59* gene on human chromosome 11 by measuring the presence or absence of the *CD59* protein on the surface of

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the cells. The FCMA effectively measures the mutant fraction induced by a wide range of mutagens [10].

While it is important to measure mutations in individual genes, it is clear that large deletions and chromosomal aberrations are involved in diseases, including cancer. The CHO A_L cells are uniquely suited to measuring large deletions because the human chromosome 11 is largely irrelevant for survival of the cells and can thus sustain large deletions involving the majority of chromosome 11 [11]. A mutant spectrum may be defined as a sequence-dependent distribution of the different types of mutations induced by a mutagen along a gene or chromosome [12]. Mutation assays have heavily relied upon PCR or Southern blot of DNA isolated from single mutants to determine the mutant spectrum [12-14]. Even though these methods are effective, they are not very efficient as it takes at least 2 months for analysis, including the time to isolate individual clones. Thus mutant spectrum analysis is not routinely done for mutagenic compounds. In this paper we show that a flow cytometry mutation assay (FCMA) can be used to determine the mutant spectrum of mutagenic agents within a two week period for mutagenized cell populations and one month for individual clones.

The FCMA measures the presence or absence of CD59, a GPI-linked cell-surface protein that is encoded by *CD59* on human chromosome 11. We have shown that the FCMA effectively measures mutations from a variety of mutagens [10] and we now demonstrate the capability of this system to measure mutations in 4 other genes located on chromosome 11 using flow cytometry. The CHO A_L cell line expresses at least four additional human cell surface proteins that are not encoded in normal Chinese hamster cells: CD44, CD90, CD98 and CD151. *CD59* and *CD44* genes are adjacent to each other (1.4 Mbp apart), but differ in that CD44 is a transmembrane protein whereas CD59 is a GPI-linked, lipid raft-associated protein [15]. *CD151*, a gene encoding an integral membrane protein, is on the distal end of the p-arm near a gene required for CHO survival [16]. *CD98* is on the q-arm of chromosome 11 close to the centromere and codes for a transmembrane protein. *CD90* is located on the distal end of the q-arm and codes for a GPI-linked protein. (See Figure 4 for a cartoon of chromosome 11 with the respective gene locations).

Since two of the markers (CD59 and CD90) are GPI-linked, it is possible that some putative mutations in these genes are actually mutations in one of the ten different genes for GPI anchor formation. The most likely candidate is *Pig-A*, a gene located on the X-chromosome, that is well known to cause GPI defects in paroxysmal nocturnal haemoglobinuria (PNH) [17;18]. A specific bacterial toxin, aerolysin, has been utilized to detect the presence of GPI anchors [19-21]. Aerolysin binds to the GPI anchor and then allows cells to be lysed by enzymatic activity. A particular mutant variant, proaerolysin, lacks the lytic capability but still binds to the anchor. When proaerolysin is conjugated to Alexa-488 (FLAER) [19], it can be used as a marker for the presence or absence of a GPI linkage. FLAER can thus be used to rule out false negative results due to a mutation in the GPI genes.

Using commercially available directly-conjugated monoclonal antibodies, we can simultaneously analyze CD59, CD44 and CD90 using multicolor fluorochromes and separately measure CD98, CD151 and GPI-linkage. Using these markers together, the FCMA may be used to determine the mutant spectra for various mutagens rapidly without compromising cell survival or requiring laborious, time consuming and sensitive techniques such as PCR. In this paper we show the feasibility of using flow cytometry to measure mutations in 5 genes as well as the GPI anchor and compare the mutant spectrum obtained with flow cytometry to that obtained by PCR.

2. Materials and Methods

2.1 Cell Culture

The CHO A_L cells were originally obtained from C.A. Waldren (Colorado State University). They have a standard set of Chinese hamster ovary K1 chromosomes along with a single copy of human chromosome 11. Cells designated CHO A_L(N), which we used in these experiments, contain a neomycin resistance gene on chromosome 11 which confers resistance to the antibiotic G418 [22]. Spontaneous background mutants were reduced via periodic treatment (alternate passages) of the cells with 800 µg/ml G418 antibiotic (Sigma-Aldrich, St. Louis, MO). Cells were cultured in Ham's F-12 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 maintained in T75 tissue culture flasks at 37° C in a humidified 5% CO₂ incubator. The cells were passed every 3 to 4 days to avoid confluence. After 3 to 4 months of continual use, the flasks of stock cells were discarded and new cells were thawed and maintained as above.

2.2 Monoclonal antibody labeling

Cells were trypsinized, washed once in PBS and then again in staining buffer (PBS with 1% BSA and 0.1% sodium azide). Cells were resuspended at a concentration of 1×10^6 cells/ml and 1 ml was aliquoted into three separate 1.2 ml microcentrifuge tubes to analyze three different marker combinations: CD59/CD44/CD90, CD98/CD151, and GPI anchor control. Other marker combinations were also utilized to determine the interrelatedness among markers. Control samples were required with individual staining of each marker and combinations of untreated control A_L cells. After centrifugation at 1500 rpm (450g) in an IEC table-top centrifuge, buffer was aspirated and the cells were resuspended in the residual buffer.

To stain cells simultaneously with CD59, CD44 and CD90 antibodies, 1.25 µl CD59-PE (Caltag, Burlingame, CA), 10 µl CD44 biotin (Serotec, Raleigh, NC), and 10 µl CD90-Alexa 647 (Serotec, Raleigh, NC) directly-conjugated monoclonal antibodies were added to a total volume of 50 µl in staining buffer. The cells were resuspended in the antibody solution and incubated for 30 min on ice; then 1 ml staining buffer was added. After centrifugation and aspiration, cells were resuspended in 99 µl staining buffer and 1 µl Alexa 488 Streptavidin (Molecular Probes, Invitrogen, Carlsbad, CA, 1:100) and incubated for 30 min on ice. All buffers and staining solutions were kept on ice throughout the experiments.

Cells were similarly stained with CD151 and CD98 antibodies using 10 µl CD151-PE (BD Pharmingen, San Jose, CA 1:2 dilution) and CD98-FITC (BD Pharmingen, San Jose, CA 1:2 dilution) in a total volume of 50 µl staining solution.

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 225 µl staining buffer and then 25 µl of 0.1 µM FLAER (Protox Biotech, Victoria, Canada) was added and gently mixed. Cells were then incubated for 30 min on ice.

After incubation, all samples were washed in cold staining buffer and resuspended in 0.5 ml cold buffer, filtered through a 40 µm nylon mesh and kept on ice prior to analysis by flow cytometry.

2.3 Flow Cytometry

A CyAn™ flow cytometer (Dako, Ft. Collins, CO) was used with 488 and 635 nm solid-state lasers for excitation. FITC was detected using a 530/40 nm bandpass filter with a 545 nm long pass dichroic, PE was detected using a 575/25 nm bandpass filter with a 595 nm long pass

dichroic, and Alexa 647 was excited with the 635 nm laser and detected using a 665/20 nm bandpass filter with a 730 nm long pass dichroic. A total of 1×10^5 cells were analyzed for each sample. Gating on forward scatter vs. side scatter eliminated cellular debris, thus reducing false negatives. The photomultiplier voltages were set so that the unstained populations with each marker had the same mean value on the histogram with the mutant region gated on 99% of the negative parental cells which lacked chromosome 11. Compensation was required between the PE and FITC/Alexa 488 stains and was done with the Summit Software v4.2 (Dako, Ft. Collins, CO). Within the PE histogram, the mutant region was defined as those cells with fluorescence intensity less than 1% of the mean fluorescence intensity of the CD59⁺ population of control cells. This enabled quantification of CD59⁻ (mutant) cells for subsequent calculation of the mutant fraction.

2.4 Radiation Treatment and Mutant Analysis

Cells were treated with G418 (800 µg/ml) for 5 days prior to treatment. The day before treatment, 4×10^5 cells were plated in a T75 flask, giving 8×10^5 cells at time of treatment. The cells were then irradiated with doses of 0–4 Gy ^{137}Cs γ radiation, a well-known clastogen [23] (J.L. Shepherd and Associates, Glendale, CA), at a dose rate of 0.93 Gy/min at 22° C. Treatment and control flasks were passed with a minimum of 1.5×10^5 cells per T75 flask and labeled with multiple markers on day 6, the maximum day for CD59 mutation expression. The spontaneous background was subtracted to determine the corrected mutant fraction for each marker.

2.5 Mutant Region Sorting

Cells from the mutant region were isolated by sorting cells labeled with CD59, CD44 and CD90 markers using a Dako MoFlo™ High-Performance Cell Sorter (Dako, Fort Collins, CO) and filter set-up similar to the CyAn™ mentioned previously. Populations were selected by their phenotypes (e.g. CD59⁻CD44⁺CD90⁺) and 1000 cells were sorted into 15 ml sterile conical tubes and later transferred into T75 tissue culture flasks. Compensation for spectral overlap of fluorochromes was done using control samples before sorting began. Individual cells that were primarily CD59⁻ were sorted using the MoFlo CyCLONE™ into 96-well tissue culture plates for clonal analysis. Phenotypes included in the single cell sort were: CD59⁻CD44⁺CD90⁺, CD59⁻CD44⁺CD90⁻, CD59⁻CD44⁻CD90⁺ and CD59⁻CD44⁻CD90⁻.

Cells cultures were expanded 14 days or until enough cells were available for flow cytometry analysis. At that time, clones were screened for CD59 phenotypes and subsequent study of the other markers.

2.6 PCR Analysis

The mutant spectrum of sorted mutant clones was determined by PCR analysis of nine separate genetic loci spanning the length of chromosome 11. After expanding the individual clones, the DNA was extracted and analyzed for the presence or absence of different markers through multiplex PCR. The primer sequences and PCR conditions were adapted from the work of Charles A. Waldren and Diane B. Vannais [11;22;24]. The primers were synthesized by Macro Molecular Resources, Ft. Collins, CO and all the PCR components obtained from Invitrogen (Carlsbad, CA).

The four exons of the *CD59* gene were examined via multiplex PCR for exons 1–3 and an independent PCR reaction for exon 4 because of difficulties running all 4 exons simultaneously. The 20 µl reaction volume contained 200 µM dNTPs, 0.01% gelatin, 1×PCR Buffer, 1.5 mM MgCl₂, 0.5 U Taq DNA Polymerase and the following primer pair concentrations: 4 µM exon 1, 0.5 µM exon 2, 0.2 µM exon 3 and 10 µM exon 4. The PCR program consisted of an initial denaturation at 95°C for 5 min followed by 30 cycles of 94°C, 45 s; 52°C, 45 s; 72°C, 45 s for

denaturation, annealing and extension, respectively and then a final incubation at 72°C for 20 min. Samples were electrophoresed on a 3% agarose gel, stained with SYBR® Gold Nucleic Acid Gel Stain (Invitrogen – Molecular Probes, Eugene, OR) and visualized on a dark reader transilluminator. The PCR product sizes were as follows: 87, 205, 350 and 401 bp for exons 1, 2, 3 and 4, respectively. Exon 4 encodes the majority of the translated sequence, including the GPI anchor binding site [25].

Five additional chromosome 11 markers were analyzed in two separate multiplex PCR reactions. These markers were chosen based on their proximity to relevant *CD* genes and the availability of optimized primer sequences used successfully by other laboratories [14;26]. Lactate dehydrogenase A (LDHA), Wilms tumor 1 (WT) and catalase (CAT) were optimal at 3 mM MgCl₂ while v-Ha-ras Harvey rat sarcoma viral oncogene homolog (RAS) and apolipoprotein A-1 (APO-A1) were optimal at 1.5 mM MgCl₂. The 20 µL reaction volume contained 200 µM dNTPs, 1×PCR Buffer, 1 U Taq DNA Polymerase and the following primer pair concentrations: 0.5 µM for LDHA, WT and CAT, 12.5 µM for RAS and 1 µM for APO-A1. The PCR cycle consisted of an initial denaturation at 95°C for 5 min followed by 30 cycles of 94°C, 1 min; 55°C, 1 min; 72°C, 1 min, and then a final incubation at 72°C for 20 min. As with the CD59 amplicon products, samples were electrophoresed on a 3% agarose gel and stained with SYBR® Gold Nucleic Acid Gel Stain. PCR product sizes were 400 bp for LDHA, 252 for WT, 207 bp for CAT, 63 bp for RAS and 109 bp for APO-A1.

3. Results

3.1 Expression of various genes on chromosome 11

To clearly identify the positive and negative phenotypes of the mutagenized CHO-A_L cells, it was necessary to optimize the staining procedure to give the greatest separation between the unstained and stained control populations. One method of determining the staining efficacy is to measure the mean fluorescence peak intensity of the positive population in comparison to an unstained or negative population. The largest separation was for our standard mutagenesis marker, CD59, with about a 400-fold separation between CD59⁺ and CD59⁻ cell populations (Figure 1). CD90, CD98 and CD151 were also well resolved with 100-, 72- and 60-fold separations between negative and positive populations. CD44 and GPI-linkage (FLAER) were not as well resolved, but still had clear positive and negative populations with 20- and 15- fold separations respectively between the populations. These procedures have been optimized by utilizing the best combinations of antibodies and stains commercially available to allow simultaneous phenotyping of both mutant populations and clones, though not all antibodies can be run at the same time because of similar fluorochromes in some cases.

3.2 Mutant expression in irradiated and sorted cell populations

Cells were irradiated and analyzed 6 days later for mutations in *CD59*. Out of 500,000 cells, about 4000 mutants were detected after a dose of 4 Gy, giving a mutant yield of 0.8%, similar to our previous results [7]. One thousand cells in this mutant region were then sorted and cultured to allow for multiple marker analysis of the mutant population.

Subsequent analysis of the sorted cell populations showed that the mutant region contained 88% CD59⁻ mutants (Figure 2). Since sorting purity is never 100%, a few percent of positive cells were likely included in the sorted region, giving a small positive population. Furthermore, we have evidence that CD59⁺ cells have a higher growth rate than CD59⁻ cells (data not shown), so a small fraction of CD59⁺ cells in the initial sort would become a somewhat larger fraction over time. CD59 mutants were also analyzed for CD44 and CD90 phenotypes. Univariate and bivariate histograms clearly show that the majority of the CD59⁻ mutants were also negative for CD44, and about half of the CD59⁻ mutants were also negative for CD90. By using color

gates from one histogram to another, it is possible to analyze the distribution of mutants in one gene with respect to another gene. For example, the red histogram shown in the CD151 panel is the distribution of CD59⁻/CD98⁻ mutants for the expression of CD151. Thus, about half of the CD59⁻/CD98⁻ mutants are also mutated at CD151 and about half are not. Similar analysis is shown for CD59⁻/CD90⁻ and the GPI-Anchor. Bivariate histograms of all the CD markers compared to CD59 give the frequency of simultaneous mutations in CD59 and other genes and thus the mutant spectrum. A few of these bivariate combinations are shown in Figure 2.

A detailed analysis of these populations is given in Table 1. Cells in the CD59 mutant region were 88% negative for CD59, 56% negative for CD44 and 44% negative for CD90. The mutant yield for the other three markers was much lower (6%, 2% and 2% for GPI-anchor, CD98 and CD151 respectively). There was good agreement between the analysis of univariate and bivariate histograms. CD59⁻/CD44⁻/CD90⁻ triple mutants were about 22% of the entire population. When CD59⁻/CD90⁻ mutants were analyzed for the GPI-anchor control, only 33% of those were negative for the GPI-anchor marker.

3.3 Clones derived from sorting irradiated cells

The bivariate histograms clearly resolve mutant populations into distinct groups. Using these histogram groupings, single cells with varying phenotypic expression were sorted and cloned for phenotypic analysis using all six markers. The clones were then analyzed for the expression of the 6 different markers. A representative sample of one clone with a CD59⁻/CD44⁻/CD90⁺/CD151⁺/CD98⁺/GPI-Anchor⁺ phenotype is shown in Figure 3. The region outlined in each histogram includes 99% of the unstained population. Those cells that fall into that region are negative for the particular marker. Control cells and 19 different sorted mutant clones were analyzed by this methodology.

PCR was also done for genes flanking the CD genes in the same clones analyzed by flow cytometry to validate the flow cytometry analysis of the mutant spectra. The genes were chosen because they have been used previously in CHO A_L cells to determine mutant spectrum by classical mutation methods [11]. The relative locations of the genes along chromosome 11, as well as the PCR and flow cytometry results, are shown in Figure 4.

4. Discussion

We have previously validated the use of the CHO A_L cells to measure mutations induced by a wide variety of mutagens, including point mutagens, using flow cytometry by measuring the loss of CD59 expression as an indicator of mutagenesis [7;10]. Zhou *et al.* [9] also used this assay to measure mutations induced by ionizing radiation and an alkylating agent (N-methyl-N-nitrosurea). These publications demonstrate that this system can be used to rapidly measure mutations caused by various kinds of DNA-damaging agents. Most mutation assays using endogenous genes such as thymidine kinase or HGPRT can only measure point mutations or relatively small deletions because large deletions are usually lethal. However, large chromosomal mutations, including aneuploidy, are very important for carcinogenesis as well [27-29]. The mutant spectrum is a measure of the size of mutations along a chromosome and indicates whether a mutations involves only a single gene locus or several contiguous genes as well [30]. The conventional methods for determining mutant spectra require PCR or Southern blotting. Both of these methods require cloning individual mutant cells, which is very laborious, and interpretation at times may be difficult. In this paper we have shown that CHO A_L cells can be analyzed for mutations in 5 different genes that cover a range of 119 Mbp by flow cytometry to obtain a mutant spectrum in a much faster and less laborious process.

To demonstrate that there was adequate separation between the negative and positive populations for each of these markers, we analyzed both stained and unstained cells. All of the

markers were clearly resolved with very little overlap between the negative and positive populations. Our primary mutagenesis marker, CD59, had the largest separation between mutant and normal cells (400-fold). This large separation and corresponding low background of mutants (typically $\sim 70 \times 10^{-5}$) is sufficient to accurately and sensitively measure dose-dependent mutant yield after treating cells with genotoxic agents, as we have previously shown [7;10]. The other CD markers were not resolved well enough to be as useful for primary mutant analysis, but can be analyzed in combination with the loss of CD59 to give additional information. The GPI-anchor marker had the smallest difference (15-fold), but that was sufficient to clearly resolve a positive and negative peak. According to these results, it is possible to determine mutations in these genes by measuring the loss of protein expression by multivariate flow cytometry.

To show the capabilities of multi-gene analysis more clearly, populations were first sorted on CD59 mutants and then analyzed for the expression of mutations in other CD genes. This approach showed that there is a high probability of picking up mutants in CD44 and CD90, as well as CD59, after irradiation. The proportion of double and triple mutants (CD59⁻/CD44⁻ and CD59⁻/CD44⁻/CD90⁻) was much higher than if they were random independent mutational events (Table 1). In the case of CD44 and CD59, 56% of the CD59⁻ cells were also CD44⁻. This high frequency is likely due to the fact that the genes are only 1.4 Mbp apart and ionizing radiation frequently causes deletions of this size or greater. The unexpectedly high frequency of CD90 mutants in this population (44%), which is 85.5 Mbp from the CD59 gene, reflects the common GPI linkage for CD59 and CD90 proteins. Thus, about 33% of the CD59⁻/CD90⁻ mutants were actually GPI mutants. In contrast, only 2% of CD59 mutant cells were also mutated in CD151, which is 33.4 Mbp from CD59.

A further advantage of using flow cytometry to analyze mutations is that individual mutant cells can be sorted and cloned to examine the phenotype of each individual clone and determine the mutant spectrum in different clones. All of the clones that were analyzed as CD59⁻ by flow cytometry were also negative in at least 1 of the four exons for CD59 (exon 4 was negative in all cases). CAT, which is located between CD59 and CD44, was missing from all clones that were negative for CD59 and CD44 by flow cytometry. WT and LDHA are distal to CD59 and their expression varied in different clones. Both clones that lost expression of both CD59 and CD151 were also negative for the WT and LDHA genes that lie between CD59 and CD151. Thus, the loss of surface expression of the CD genes is correlated with the loss of closely associated or intervening genes as measured by PCR, lending support to the idea that the mutant spectrum measured by flow cytometry accurately reflects the underlying loss of the respective genes.

The low frequency of CD151 mutants is most likely because of its proximity to an unknown essential gene. CHO A_L cells have an essential gene at the tip of the p-arm of chromosome 11 (11p15.5) that is required for CHO survival [16]. Since CD151 is located near 11p15.5, those cells lacking the CD151 marker most likely also have mutations in this essential gene and do not survive for mutant analysis. RAS was utilized as a positive control because it is very near the essential gene and was present in all samples.

APO-A1 and CD90 had the same expression except in three clones in which CD59 and CD90 were negative but no intervening genes were missing. Both CD59 and CD90 are anchored into the plasma membrane via a GPI tail and are localized to lipid-rafts in the plasma membrane. It is highly probable that one of the ten genes required for GPI-linkage, most likely the X-linked *pigA* gene, was mutated causing a loss of expression of both CD59 and CD90. Mutations in this gene are cited as the reason CD59 expression is lost in the disease PNH [17;18]. We investigated this problem by labeling the CD59⁻/CD90⁻ mutants with fluorescent aerolysin (FLAER) which detects the presence of a GPI-anchor. Thirty-three percent of the CD59⁻ cells

were also negative for CD90 and GPI expression, indicating that mutations in GPI-anchor gene (s) may explain a portion of the elevated mutant yield.

The majority of clones analyzed were also missing some or all of the *WT*, *CAT* and *LDHA* markers and CD44, showing that radiation frequently causes multigene deletions. Ten percent of the clones were missing all markers (Clones R and S), but the entire chromosome was not lost as *RAS* was still present. Overall, these results show that the FCMA mutant spectra directly reflect the genotypic mutant spectra based on PCR.

The mutant spectrum induced by a given agent is independent of the mutation system used but is instead dependent on the mode of action of the specific mutagen [12]. Gamma radiation is known to cause large deletions as shown by analysis not only with the CHO-A_L clonogenic assay [11] but also the mouse lymphoma *tk* gene assay [31]. Other assays are limited in scoring large deletions because they frequently lead to the loss of essential genes and the cells die. Because human chromosome 11 is not essential for CHO A_L cell survival, nearly the entire chromosome may be lost without cell death [5]. Thus much larger deletions can be measured and the mutant spectrum is more representative of the actual chromosomal damage.

The FCMA multiple marker analysis drastically reduces the time and resources required for mutant spectrum development from 1-2 months for conventional methods to a week to 10 days for the FCMA. Clonal populations were used here, but it is possible to do the initial analysis of mutants screening for all markers and determine the yield of mutants for all markers. Because of overlapping fluorochromes, we could not analyze all 5 markers and GPI simultaneously. However, in principle this is quite possible given appropriate tagged antibodies. This new multiparameter flow cytometry mutagenesis assay makes it possible to quickly ascertain the mutagenic potential of novel chemicals and pharmaceuticals as well as clarify the mechanism (s) of mutagenesis.

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Figure 1.

Flow cytometry histogram overlays showing the separation between positively stained and unstained control CHO A_L cells for CD59, CD44, CD90, CD151, CD98 and GPI Anchor (FLAER). The positive populations were labeled with antibodies specific for the various CD proteins, then analyzed by flow cytometry.

Figure 2.

Univariate and bivariate flow cytometry histograms of sorted mutagenized cells. CHO A_L cells were irradiated (4 Gy), grown for 6 days, then labeled with antibodies against CD59 and 1000 cells were sorted on the CD59 mutant region (<1% of positive peak intensity). The sorted cells were then grown for 14 days and analyzed for expression of CD59, CD44, CD90 (upper row), CD98, CD151 and GPI-Anchor (FLAER) (middle row). The red histogram in the CD151 panel shows the subpopulation gated on CD98⁺ cells while the red histogram in the GPI-Anchor histogram shows the subpopulation gated on CD90⁺ cells. The lower panels show the simultaneous analysis of bivariate combinations of CD59, CD44 and CD90.

Figure 3.

An example of a clone isolated by sorting an individual irradiated cell that was negative for expression of both CD59 and CD44. The clone was then analyzed for all of the markers, including CD59, CD44, CD90, CD151, CD98 and GPI-Anchor (FLAER).

Figure 4. Mutant spectra of 19 different CHO A_L clones that had been irradiated and then cloned by cell sorting, as shown in Figure 3. The individual clones were analyzed both by PCR (indicated by white labels) and flow cytometry markers (indicated by the grey labels). Clones were also analyzed for the four exons of CD59 and the presence of a GPI-anchor using FLAER). The presence or absence of markers is indicated by (+) and (-). The cartoon at the left shows the relative locations of the various genes along chromosome 11.