Mechanisms of the Genotoxicity of Crocidolite Asbestos in Mammalian Cells: Implication from Mutation Patterns Induced by Reactive Oxygen Species

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Asbestos fibers are fibrous mineral silicates that have been associated with the development of pulmonary fibrosis, bronchiogenic carcinoma, and malignant mesotheliomas in both humans and experimental animals (1,2). The fact that asbestos, a well-established carcinogen, has been used extensively in industry and households makes it an important health concern. It has been suggested that the danger of developing asbestos-related diseases extends beyond that of a simple occupational hazard because it has been documented in family members of asbestos workers, in individuals living in the neighborhood of industrial sources of asbestos, and in some schools and public buildings where asbestos is being used as insulation material (3–5). Moreover, resuspension of materials from asbestos-containing ceilings has been shown to be the main source of asbestos pollution in old, poorly maintained buildings (6). The continued discovery of routes through which the general public may be exposed to asbestos suggests a long-term, low-dose exposure of a large number of people.

The mechanisms by which asbestos produces malignancy are not entirely clear. Various in vitro and in vivo studies have suggested that fiber dimension, surface properties, and physical durability are important criteria for the carcinogenicity of the fibers (2,7). Although reactive oxygen species (ROS) have been indicated as one of the key determinants of asbestos-induced mutagenesis and carcinogenesis, the types and origin of these free radicals remain elusive (8,9). It has been shown that ROS such as superoxide anions (O2·−) and hydrogen peroxide originate not only from redox reactions catalyzed on the fiber surface but also from the incomplete phagocytosis of fibers in various cells, such as phagocytic, mesothelial, and rat lung epithelium cells (10–12). The involvement of ROS and the protective effects of antioxidants such as catalase and superoxide dismutase and radical scavengers such as dimethyl sulfoxide (DMSO) and Tempol in asbestos-induced toxicity have been studied in various cell systems as well (8,13,14). These biologically reactive ROS, in particular hydroxyl radicals (OH·), act directly or indirectly to damage neighboring biomolecules, such as DNA and membrane lipid (14,15). There is evidence to suggest that the array of DNA and chromosomal damages induced by ROS such as base substitutions, deletions, rearrangements, insertions, sister chromatid exchanges, and chromosomal aberrations may lead to a broad spectrum of mutations in mammalian cells (16). However, earlier studies on the mutagenicity of asbestos at either the hprt or tua loci in a variety of mammalian cells have resulted in mostly negative findings (17,18). Subsequent studies have suggested that this could be a result of multilocus deletions induced predominantly by asbestos, which are not compatible with the survival of the mutants.

Several mutagenicity assays, which detect either large chromosomal mutations, homologous recombination, or score mutants located on nonessential genes, have demonstrated the mutagenic potential of various fiber types (19–22). Although these findings have indicated a close relationship between chromosomal abnormalities that have frequently been shown in fiber-exposed human and rodent cell lines and carcinogenicity in vivo, there is less direct evidence to illustrate how ROS are involved in these processes.

The human–hamster hybrid (A519) cell, which contains a full set of hamster chromosomes and a single copy of human chromosome 11, is sensitive in detecting mutagens that induce mostly large, multilocus deletions such as ionizing radiation and certain heavy metals (23,24). Because only a small part of region 11p15.5 is required for the viability of A519 cells, mutations in the human chromosome 11 ranging in size up to 140 Mbp of DNA can be detected. Compared with the hamster hprt locus, previous studies have shown that there is a 50-fold increase in mutant yield at the CD59 locus in crocidolite-treated A519 cells (25).

In the present studies we focused on clarifying the role of ROS in mediating crocidolite-induced mutagenicity in mammalian cells. We followed the generation of ROS with the radical probe 5′,6′-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate.
pared fresh each time due to its unstable autoclaving and mixed to ensure a uniform water. The fiber suspension was triturated six times with a 20-gauge syringe needle. We further determined the formation of H$_2$O$_2$ from crocidolite fiber-exposed cells using Amplex Red reagent either in the presence or absence of catalase. Because H$_2$O$_2$ can react with intracellular metals to produce ROS via the Fenton reaction, we speculated that asbestos fibers would induce similar types of mutations as that of chemically generated ROS (27,28). We found that there was a dose-dependent formation of ROS in crocidolite fiber-exposed AL cells, and the protective effects of antioxidants were demonstrated by DMSO and catalase. Furthermore, we found that asbestos fibers were mutagenic and the types of mutants induced were similar to those of chemically generated ROS analyzed at two equitoxic doses. These results provide direct evidence that the genotoxicity of asbestos fibers is mediated by ROS.

Materials and Methods

Cell culture. We used the human–hamster hybrid (A$_5$) cell line containing a standard set of CHO-K1 chromosomes, and a single copy of human chromosome 11. Chromosome 11 contains the CD59 gene (also known as M1C1) at 11p13.5, which encodes the CD59 cell-surface antigen marker (formerly known as S1) that renders A$_5$ cells sensitive to killing by the monoclonal antibodies E7 in the presence of rabbit serum complement (HPR, Denver, PA). Antibody specific to the CD59 antigen was produced from hybridoma culture. Cells were cultured in Ham’s F-12 medium supplemented with 8% heat-inactivated fetal bovine serum (Atlanta Biological, Norcross, GA), 2 × 10^{-4} M glycine, and 25 μg/mL gentamycin at 37°C in a humidified 5% CO$_2$/95% air incubator, and passaged as described (19,24,25).

Preparation of crocidolite fibers. We used International Union Against Cancer standard reference crocidolite fibers (average length 3.2 ± 1.0 μm; average diameter 0.22 ± 0.01 μm) in these studies. The fibers were prepared as described previously (8,25). Briefly, samples of fibers were weighed and suspended in distilled water. The fiber suspension was trituated six to eight times with a 20-gauge syringe needle. A stock solution of the fibers was sterilized by autoclaving and mixed to ensure a uniform suspension before being diluted with tissue culture medium for cell treatment.

Treatment with antioxidants. Catalase (Sigma Chemicals, St. Louis, MO) was prepared fresh each time due to its unstable nature in aqueous solution (8). Stock catalase solution was membrane-filtered and diluted with medium to a working concentration of 1,000 U/mL. DMSO (Sigma) was diluted directly from stock solution with medium to a final concentration of 0.5% (v/v). To demonstrate the involvement of ROS in asbestos mutagenesis, exponentially growing A$_5$ cells were exposed to asbestos for 24 hr with or without concurrent treatment with either catalase or DMSO. To ascertain the mutagenicity of H$_2$O$_2$ (30%; Sigma), we exposed exponentially growing A$_5$ cells to H$_2$O$_2$ in serum-free medium for 15 min with or without concurrent treatment with either catalase or DMSO. The doses of catalase and DMSO used here were nontoxic and nonmutagenic. After treatment, cells were trypsinized and stained for survival and mutation assays.

Determination of intracellular ROS. Exponentially growing A$_5$ cells were plated onto 35-mm glass-bottom microwell dishes (Biotech Inc., Butler, PA). After overnight incubation, cells were pretreated with a 1-μM dose of the nonfluorescent, membrane-permeable dye CM-H$_2$DCFDA (Molecular Probes, Eugene, OR) at 37°C for 40 min as described by Long et al. (26). Subsequently, the cells were washed twice with cold ACAS buffer (12.7 mM NaCl, 0.8 mM KCl, 1.2 mM CaCl$_2$, 1.2 mM KH$_2$PO$_4$, 4.4 mM C$_6$H$_{12}$O$_6$, 10 mM HEPES, pH 7.4) to decrease metabolic activity and remove any excess dye. We added 1 mL ACAS buffer or fiber suspension with or without DMSO to the dishes, which was rapidly warmed to 37°C on the Zeiss Axiovert 100 TV microscope (Carl Zeiss, Thornwood, NY). Cells were viewed by using 100 × 1.4 objective lens equipped with a laser scanning confocal attachment (model LSM410, Zeiss). CM-H$_2$DCFDA was excited with the 488-nm line of an argon/krypton mixed gas laser. Emission was collected with a 510-nm long pass filter. A semiquantitative estimation of ROS-associated fluorescent signals was obtained using the composite images generated by Adobe Photoshop (Adobe Systems, Mountain View, CA). We randomly selected 60–80 individual cells per dose per experiment and quantified the fluorescent images as described by Liu et al. (29). On average, we measured up to 200 cells per exposed group.

Fluorescent microassay of hydrogen peroxide. We measured the release of H$_2$O$_2$ by horseradish peroxidase (HRP)-dependent oxidation of N-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent; Molecular Probes) in 96-well tissue culture plates as described by Mohanty et al. (30). Briefly, 100 μL Amplex Red solution (144.4 mM NaCl, 5.7 mM Na$_2$PO$_4$, 4.86 mM KCl, 0.54 mM CaCl$_2$, 1.22 mM MgSO$_4$, 75 μM Amplex Red reagent, pH 7.35) containing 0.5 U/mL HRP (Molecular Probes) and graded doses of crocidolite fibers in the presence or absence of 1,000 U/mL catalase were added to 96-well microplates and incubated at 37°C for 15 min. We replated 20 μL of exponentially growing A$_5$ cells at a density of 2 × 10$^5$/mL into the microplates and incubated them at 37°C for 4 hr. We measured the fluorescence intensity of each well using a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT) with an excitation wavelength in the range of 530–560nm. The concentration of H$_2$O$_2$ was determined based on a standard curve.

Cytotoxicity of crocidolite fibers and hydrogen peroxide. Exponentially growing A$_5$ cells were treated with graded doses of fibers for 24 hr or with H$_2$O$_2$ in serum-free medium for 15 min. After treatment, cultures were washed with balance salt solution, trypsinized, and replated into 100-mm diameter Petri dishes for colony formation. The dishes were incubated for 7 days, at which time they were fixed with formaldehyde and stained with Giemsa. We counted the number of colonies to determine the surviving fraction as described (19,25).

Quantification of mutations at the CD59 locus. After completion of the various treatments, the cultures were replated into T75 flasks and cultured for 1 week before mutagenesis testing began as described (23,25). This expression period is necessary for the surviving cells to recover from the temporary growth lag induced by either crocidolite fibers or H$_2$O$_2$ and multiply sufficiently, so that the progeny of the mutated cells no longer express lethal amounts of the CD59 surface antigen. To determine mutant fraction, 5 × 10$^5$ cells were plated into each of six 60-mm dishes in a total of 2 mL of growth medium. After incubation for 2 hr, we treated the cultures with 0.2% CD59 antisem and 1.5% (v/v) freshly thawed complement. Controls were composed of identical sites of dishes including antisem alone, complement alone, or neither agent. The cultures were incubated for 7–10 days, at which time they were fixed, stained, and the surviving colonies were scored. We tested the colonies for mutant yield each week for 2 consecutive weeks to ensure the full expression of mutations. We calculated mutant fractions as the number of surviving colonies divided by the total number of cells plated after correction for any nonspecific killing due to complement alone.

Analyses of mutant spectrum by multiplex polymerase chain reaction. CD59$^+$ mutants were isolated by cloning and expanded in culture as described (24). To ensure that all mutants analyzed were independently generated, we isolated only one and, occasionally, no more than two well-separated mutants per dish for analysis. Five marker genes located on either the short arm (WT, PTH, CAT, RAS) or the long arm (APO-A1) of human chromosome 11 were chosen for multiplex polymerase chain reaction (PCR) because of their mapping
positions relative to the CD59 gene and the availability of PCR primers for the coding regions of these genes. PCR amplifications were performed for 30 cycles using a DNA thermal cycle model 480 (Perkin-Elmer/Cetus) in 20-µl reaction mixtures containing 0.2 µg of the EcoRI-digested DNA sample in 1× Stoffel fragment buffer, all four dNTPs (each at 0.2 mM), 3 mM MgCl2, 0.2 mM each primer, and 2 U Stoffel fragment enzyme. Each PCR cycle consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. After the last cycle, samples were incubated at 72°C for an additional 20 min, electrophoresed on 3% agarose gels, and stained with ethidium bromide.

Statistics. We analyzed data using Student’s t-tests. Differences between means were regarded as significant if p < 0.05.

Results

Intracellular ROS production induced by crocidolite fibers. If generation of ROS is one of the major mechanisms for asbestos-induced mutagenesis in mammalian cells, then fiber treatment should be expected to induce ROS production in the A549 cells. To quantify intercellular ROS induced by crocidolite fibers, A549 cells were pretreated with CM-H2DCFDA, which passively diffused into cells and was oxidized by ROS to a fluorescent form (29). Figure 1A and C illustrate CM-H2DCFDA fluorescent imaging in control A549 cells and cells treated with a 6 µg/cm² dose of crocidolite fibers, respectively. These images show a typical field of the various treatment groups, generated from composite confocal images of 11 sagittal sections. Cells exposed to fibers exhibited a higher fluorescence level when compared to the control, indicative of higher intercellular oxidant levels. However, some fluorescence was detected in the control culture, which might be due to the normal oxidative metabolism of cells such as mitochondrial respiration.

A dose-dependent induction of ROS in A549 cells treated with asbestos fibers is shown in Figure 2. Quantification of relative fluorescence in fiber-treated and control cells indicated that treatment of cells with a 6 µg/cm² dose of crocidolite fibers induced a 5-fold increase in the generation of ROS compared to the control (p < 0.05). However, there was no further increase in fluorescence induction with fiber concentration > 6 µg/cm². The oxyradical nature behind the increase in fluorescence intensity was further supported by including the radical scavenger DMSO in the reaction mixture. Although DMSO alone had little effect on the formation of ROS among control cells (Figure 3), the relative fluorescence level induced by a 6 µg/cm² dose of fibers in A549 cells decreased from 59.01 ± 6.74 to 19.30 ± 0.85 in the presence of DMSO (p < 0.05), which was consistent with our previous studies of a suppressive effect of DMSO on the formation of 8-hydroxy-deoxyguanosine in crocidolite-treated A549 cells (3)1.

Hydrogen peroxide production induced by crocidolite fibers. Figure 4 shows the release of H2O2 from asbestos-treated A549 cells based on HRP-catalyzed oxidation of fluorescent Amplex Red reagent in the presence or absence of catalase. There was a dose-dependent induction of H2O2, reaching a peak of 0.32 ± 0.055 µM at a 6 µg/cm² dose of fibers. Catalase, which directly metabolizes H2O2 to water and oxygen, has been shown to be an effective free-radical scavenger in various cell systems (32). Concurrent treatment of A549 cells with crocidolite fibers at a dose of 6 µg/cm² and catalase (1,000 U/mL) suppressed H2O2 induction by 7- to 8-fold (p < 0.05). The dose of catalase used here had little effect on the level of H2O2 in control cells. Likewise, heat-inactivated catalase (by boiling for 10 min) had little effect on the fluorescence intensity of fiber-treated A549 cells.

Cytotoxicity and mutagenicity of crocidolite fibers and hydrogen peroxide. To show that ROS induced by asbestos fibers actually mediate the mutagenic events, it is necessary...
to demonstrate that chemically generated ROS is mutagenic and induces a similar spectrum of mutants as that of asbestos fibers. Exposure of A549 cells to either graded doses of crocidolite fibers for 24 hr or H2O2 in serum-free medium for 15 min resulted in a dose-dependent increase in toxicity of A549 cells (Figure 5). The normal plating efficiency of A549 cells was 80 ± 5% in the present studies. The surviving fraction of A549 cells treated with a 2 µg/cm² dose of crocidolite fibers was 62 ± 4%, and the value decreased to 26 ± 5% after treatment with a 4 µg/cm² dose of crocidolite fibers. By comparison, survival of A549 cells after exposing to 4.4 mM or 13.2 mM H2O2 was 56 ± 10% and 24 ± 5%, respectively. The background mutant fraction of A549 cells used in these experiments averaged 52 ± 15 mutants per 10⁵ survivors. In contrast, the negative control, titanium dioxide (TiO₂), at doses up to 12 µg/cm² was neither cytotoxic nor mutagenic to A549 cells when tested under similar conditions (data not shown). Both crocidolite fibers and H2O2 led to a dose-dependent induction of CD59 mutants in A549 cells. The mutant fraction increased with the doses of fibers and reached a level that was approximately 4-fold higher than background at a 4 µg/cm² dose of fibers in A549 cells. As shown in Figure 5, the mutant fraction was slightly higher among cells treated with H2O2 than those exposed to crocidolite fibers at equally toxic doses, though the difference was not statistically significant. Furthermore, the mutation yields induced by either crocidolite fibers at a dose of 4 µg/cm² or 13.2 mM H2O2 were dramatically suppressed by either 0.5% DMSO or 1,000 U/mL catalase (p < 0.05; Figure 6). These results confirm that ROS plays a casual role in the mutagenicity of crocidolite fibers and H2O2.

Analysis of mutant spectra. To compare the type and size of mutations that caused the CD59 phenotype among A549 cells exposed to either crocidolite fibers or H2O2, multiplex PCR and primer sequences for five marker genes (WT, PTH, CAT, RAS, and APO-A1) located on either the short or long arm of human chromosome 11 were used as described (24). These primers and PCR conditions were selected to amplify only the human genes instead of their CHO cognate. Because there is only one chromosome 11 in A549 cells, the presence or absence of the corresponding PCR products indicates that a particular segment of DNA containing these genes is present or lost, respectively. There is evidence to suggest that a small region of the distal end of human chromosome 11 at 11p15.5, which corresponds to the RAS probe in all mutants, is required for viability of the hybrid cells (33,34). As shown in Table 1, the majority of spontaneous CD59 mutants (22/30 or 73%) showed no detectable changes in any of the marker genes examined, which was consistent with previous studies (24,34). In contrast, only 24/27 or 85% of mutants derived from cells exposed to a 2 µg/cm² dose of fibers retained all of the marker genes examined, and 18/27 or 78% of the mutants had lost at least one additional marker, which included 7/27 or 26% that lost the proximal APO-A1 located on the long arm of the chromosome. In contrast, the proportion of mutants suffering loss of additional chromosomal markers increased with increasing concentration of fibers such that none of the 29 mutants induced by a 4 µg/cm² dose of fibers retained all five of the marker genes, and 19/29 or 66% of them lost the long arm marker in addition to the CAT and WT gene on the short arm of human chromosome 11. The types of mutants induced were similar to those induced by an equivalent cytotoxic dose of H2O2 in that 9/23 or 39% of the mutants induced by a 4.4 mM H2O2 retained all five primers compared to none among those induced by the higher dose of 13.2 mM. These results indicated that equitoxic doses of crocidolite fibers and H2O2 induced similar multilocus deletions that were increased in a dose-dependent manner, providing strong circumstantial evidence that similar mutagenic mechanisms are involved in the mutagenicity induced by fibers and ROS.

Discussion

The mechanisms by which ROS are generated in response to asbestos fiber exposure have been widely studied (10). Recent studies have shown that asbestos-induced toxicity to hamster tracheal epithelial cells, rat lung fibroblasts, and rat alveolar macrophages is suppressed by the antioxidant enzymes, superoxide dismutase, and catalase, as well as the free-radical scavengers, such as cysteine, dimethylthiourea, and ascorbic acid (11,12,36,37). The finding that mesothelioma induction in rats and humans can be correlated with fiber-induced hydroxyl radicals provides further support for the possible role of ROS in fiber carcinogenesis (38). Although crocidolite, the most carcinogenic type of asbestos, has been shown to catalyze the formation of hydroxyl radicals by either Fenton or Haber-Weiss reactions and induces lipid peroxidation, DNA strand breaks, sister chromatid exchanges, and clastogenicity (14,39,40), its carcinogenic and mutagenic mechanisms are still poorly understood.

Several methods, including reduction of cytochrome c by superoxide ion, luminal chemoluminescence, reduction of scopoletin emission, and electron spin resonance, have been used to measure the generation of ROS
induced by asbestos, but such assays are typically performed in large samples of cells (\(10^6\)) and are affected by geometry and number of fibers (41–43). An ideal technique would be to measure ROS released as single-cell interaction with defined doses of asbestos. CM-H2DCFDA is a well-established free-cell interaction with defined doses of asbestos. It would be to measure ROS released as single-cell interaction with defined doses of asbestos. CM-H2DCFDA by ROS, as detected using confocal microscopy, provided strong evidence that asbestos induced a dose-dependent increase of ROS in single cells, which could be inhibited by DMSO.

Although the present data clearly demonstrate the induction of ROS in fiber-induced mutagenesis in A549 cells, these experiments did not specifically identify the source of these radical species. It is generally accepted that the mitochondrial membrane damage induced by asbestos could trigger a cascading event in ROS production involving lipid peroxidation (49). Alternatively, peroxynitrite anions generated as a result of mitochondrial damage might more likely be involved. Recent evidence has indicated that stimulation of an NADH or NADPH oxidase and/or conversion of xanthine dehydrogenase to xanthine in the cytoplasm can occur after contact of fibers with the plasma membrane and their subsequent uptake by the cells (32,50). We observed that ROS were localized mainly in the cytoplasm, especially in spherical organelles, both in the control and fiber-treated cells pretreated with CM-H2DCFDA.

Among those ROS induced by asbestos fibers, \(H_2O_2\) is relatively long lived and directly crosses cell membranes by simple diffusion (42). There is evidence that \(H_2O_2\) induces not only damage to DNA, causing single- and double-strand breaks, base loss, base substitution, and cross-linking, but that it also causes chromosome aberrations, as well as chromatic aberrations (47). Using the spin trap 5,5’-dimethyl-1-pyrroline-N-oxide (DMPO), Weitzman and Graceffa (27) demonstrated that crocidolite fibers catalyze the production of \(OH^\cdot\) from \(H_2O_2\) in a cell-free system. In this study, we examined the role of \(H_2O_2\) generation in A549 cells exposed to crocidolite fibers in the presence or absence of catalase. Our data suggested that \(H_2O_2\) may be an important mediator molecule, which is responsible for fiber cytotoxicity. Because catalase is a relatively large molecule (molecular weight of 250 kD), it is highly unlikely to pass across the cell membrane without being phagocytized. On the other hand, \(H_2O_2\) is freely diffusible both intracellular and extracellular space, and addition of extracellular catalase can reduce the intracellular oxidative stress induced by fibers. In addition to the well-documented iron-catalyzed reactions that generate a variety of ROS such as \(O_2^\cdot\), \(H_2O_2\), \(OH^\cdot\), \(O_2^-\), \(H_2O_2\), and lipid peroxides, another pathway involving reactive nitrogen species may be involved. Several studies have shown that exposure to crocidolite increases the production of nitric oxide in rat macrophages and human lung epithelial (A549) cells, which subsequently reacts with \(O_2^\cdot\) to produce \(OH^\cdot\) and peroxynitrite (ONOO\(^-\)) (51,52). The latter has been shown to cause nitration of proteins, hydroxylation or nitration of DNA, and mutations (53).

Previous studies have suggested that asbestos fibers are strong gene and chromosomal mutagens, inducing predominantly large deletions in A549 cells (8,19,25). To obtain further insight into the role of oxidative DNA and chromosome damage in asbestos-mediated carcinogenesis, it is necessary to compare the mutation pattern between crocidolite fibers and ROS. In the absence of serum, \(H_2O_2\) produced predominantly \(OH^\cdot\) radicals in human fibroblast culture (37). Using this approach, we show that in asbestos-induced CD59\(^-\) mutants, the types of mutation induced are similar to those induced by \(H_2O_2\). From a mechanistic point of view, these data suggest that similar mutagenic mechanisms are involved between asbestos fibers and chemically generated ROS, which provides further evidence that fiber-induced mutagenesis may be mediated through ROS.

Because most ROS are short-lived and can only diffuse short distances in cells, it is still not clear how these radicals reach the nucleus to cause gene and chromosomal mutation (54,55). Compared with the parental Chinese hamster ovary cells, A549 cells contain similar levels of glutathione peroxidase, superoxide dismutase, and total glutathione, but a 50% increase in catalase. This is due, presumably, to the extra copy of CAT gene from the single copy of human chromosome 11 that they contain (56). One possible scenario is that free radicals generated by asbestos fibers lead to a cascading event involving lipid peroxidation. A direct correlation between lipid peroxidation and fiber mutagenesis/carcinogenesis has not been demonstrated, although there is evidence that asbestos fibers induce lipid peroxidation in bacteria and mammalian cells (49,57).

![Figure 6. Effects of catalase (1,000 U/mL) and DMSO (0.5%) on induced CD59\(^-\) mutants per 10^6 survivors in A549 cells exposed to either crocidolite (4 \(\mu g/cm^2\)) or \(H_2O_2\) (13.2 mM)]. Data were pooled from four independent experiments. Error bars indicate SD.

**Table 1.** The number (percent) of CD59\(^-\) mutants either of spontaneous origin or induced by the various treatments that retain the following markers in human chromosome 11 as determined by multiplex PCR analyses.

| Group | Total mutants | AP0-A1 | CAT | WT | PTH | RAS |
|-------|---------------|--------|-----|-----|-----|-----|
| Spontaneous | 30 | 30 (100) | 24 (80) | 22 (73) | 30 (100) | 30 (100) |
| 2 \(\mu g/mL\) Crocidolite | 27 | 20 (74) | 16 (59) | 9 (33) | 27 (100) | 27 (100) |
| 4 \(\mu g/mL\) Crocidolite | 29 | 10 (34) | 11 (38) | 0 (0) | 29 (100) | 29 (100) |
| 4.4 \(mM\) \(H_2O_2\) | 23 | 17 (74) | 10 (43) | 9 (39) | 23 (100) | 23 (100) |
| 13.2 \(mM\) \(H_2O_2\) | 51 | 14 (45) | 8 (26) | 0 (0) | 31 (100) | 31 (100) |

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