Detection of Chromosomal Breakage in the 1cen–1q12 Region of Interphase Human Lymphocytes Using Multicolor Fluorescence in Situ Hybridization with Tandem DNA Probes

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ABSTRACT

A novel multicolor fluorescence in situ hybridization approach, using an α satellite probe which labels the centromeric region on chromosome 1 and a classical satellite probe which targets an adjacent breakage-prone region (1q12), has been used to detect both hyperdiploidy and chromosomal breakage in interphase human cells. With the use of this technique significant increases in chromosomal breakage were observed in interphase and metaphase lymphocytes irradiated in vitro. Metaphase analysis indicated that a significant proportion of these breakage events represented potentially stable aberrations such as translocations and inversions. A comparison of frequencies using a single classical satellite probe and the adjacent α and classical satellite probes indicated that this tandem label procedure allowed chromosomal breakage to be detected and distinguished from hyperdiploidy in untreated interphase lymphocytes, indicating the potential of this procedure for human biomonitoring. To determine whether this hybridization approach could detect alterations in humans, peripheral blood lymphocytes were obtained from a group of pesticide applicators and mixers and compared with a nonexposed control group. Significant increases in both hyperdiploidy and chromosomal breakage affecting the labeled region on chromosome 1 were observed in the pesticide-exposed group. These results indicate that this hybridization strategy allows hyperdiploidy and chromosomal breakage to be detected rapidly in interphase human cells and may facilitate the detection of chromosomal alterations in human populations exposed to carcinogenic and genotoxic agents using tissues which have not been previously amenable for cytogenetic analysis.

INTRODUCTION

An increasing number of molecular and cytogenetic studies have emphasized the importance of genetic alterations such as deletions, translocations, recombination, and aneuploidy in the carcinogenic process (1–4). The involvement of these chromosomal aberrations in other genetic disorders such as infertility, spontaneous abortion, and mental retardation is also widely recognized (5–7). For many years, cytogenetic analyses of metaphase cells have been relied on to detect structural and numerical aberrations in the peripheral blood lymphocytes of exposed individuals to identify populations with increased risk of developing cancer and other genetic diseases (8, 9). However, the usefulness of conventional cytogenetic analyses for screening populations with occupational or environmental exposure is limited in that such analyses are restricted primarily to actively dividing tissues or cell types which divide readily in culture (10, 11). In addition, such studies are labor intensive, require highly skilled personnel, and are prone to technical artifacts such as chromosomal loss during metaphase preparation.

FISH with chromosome-specific DNA probes is a recently developed molecular cytogenetic technique which allows cytogenetic information to be obtained rapidly from interphase cells. Hybridization with fluorescently labeled DNA probes results in a compact staining of the chromosomal region targeted by the DNA probe, permitting the copy number and location of the chromosome of interest to be determined rapidly in a large number of cells (12–14). With the use of this approach, cells containing abnormal numbers of chromosomes can be readily identified. Additionally, FISH has been applied to detect specific structural chromosomal aberrations in interphase cells (15–17). However, these applications have largely been limited to prenatal or cancer diagnostics in which a large proportion of the cells manifest the structural alteration (18). Recently we have developed a new multicolor FISH approach to more accurately identify hyperdiploid nuclei in interphase cells exposed to chemical agents (19). In this report, we demonstrate that this approach is also highly effective at detecting chromosomal breakage and exchanges in both metaphase and interphase cells. In addition, we have extended these studies to show that this new multicolor approach allows hyperdiploidy and chromosomal breakage to be detected in interphase cells of a chemically exposed human population.

MATERIALS AND METHODS

For the in vitro studies, peripheral blood was collected from healthy male volunteers in heparinized vacutainers. Lymphocytes were isolated in Leuco-prep tubes (Becton Dickinson) and cultures were established in RPMI 1640 (GIBCO-BRL) supplemented with 10% FCS, 5% l-glutamine, 1% gentamicin, and 2.36% phytomethagglutinin (type M). The cells were exposed to 0 and 300 cGy of X-irradiation (27.7 cGy/min) in two separate experiments 24 h after culture initiation and harvested 48 h later using previously described procedures (14). The comparison of the single and the tandem labeled probes was performed on archived slides collected for different experiments from one male donor (14). For the pesticide exposure studies, blood samples were collected from 26 nonsmoking pesticide-exposed males and 19 nonsmoking nonexposed males and cultured for 48 h as described previously (20).

For the multicolor FISH experiments, two different chromosome 1-specific DNA probes were used: the pUC1.77 probe, which labels the classical satellite II region within the heterochromatin of chromosome 1 (21, 22), and an α satellite probe targeting a small centromeric region adjacent to the heterochromatin (23). The classical satellite probe was labeled with digoxigenin-11-dUTP by nick translation in our laboratory, and the biotinylated α satellite probe was purchased from Oncor, Inc. (Gaithersburg, MD). Hybridizations were performed using standard procedures (24, 25). Briefly, slides were immersed in a 70% formamide denaturation solution at 70°C for 3 min, put through an ethanol dehydration series (70, 85, and 100%), and then placed on a slide warmer at 37°C. The hybridization cocktail consisted of 1 μl of digoxigenin-labeled classical satellite probe, 1 μl of biotin-labeled α satellite probe, 1 μl of sonicated herring sperm DNA, and 7 μl of MM 2.1 hybridization mix (55% formamide-1× SSC-10% dextran sulfate). This probe mix was heated to 70°C for 3 min to denature the probe DNA. Slides were hybridized overnight at 37°C in a humidified chamber. Posthybridization washes were in

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2 To whom requests for reprints should be addressed, at Environmental Toxicology Graduate Program, Department of Entomology, 5419 Boyce Hall, University of California, Riverside, CA 92521.

3 The abbreviations used are: FISH, fluorescence in situ hybridization; 1× SSC, 0.15 M NaCl plus 0.015 M sodium citrate (pH 7.0); PN buffer, 0.1 M phosphate buffer (pH 8.0) containing 0.5% NP-40; PNM buffer, 0.1 M phosphate buffer containing 5% powdered nonfat milk supernatant and 0.5% NP-40.
60% formamide-2x SSC three times for 5 min, and once in 2x SSC at 45°C. The slides were then rinsed twice in PN buffer (0.1 M phosphate buffer, pH 8.0, containing 0.5% NP40) at room temperature. The digoxigenin-labeled classical satellite probe was detected using the use of a mouse antidigoxigenin antibody (3.2 μg/ml in PNM; PN buffer containing 5% milk supernatant) followed by a Texas Red conjugated goat anti-mouse antibody (10 μg/ml in PNM). The biotinylated α satellite probe was detected using fluorescein-conjugated avidin (5 μg/ml in PNM) amplified once using a biotinylated anti-avidin antibody and then the fluorescein-conjugated avidin. To counterstain the DNA, 4',6-diamidino-2-phenylindole (2.5 μg/ml in phenylenediamine antifade) was used.

All scoring was performed from coded slides using a Nikon Optiphot II microscope with a fluorescence attachment equipped with a triple-band-pass filter (Omega, Brattleboro, VT) for the multicolor FISH studies and a blue filter (Nikon, B-2A filter) for the single label studies.

RESULTS

Hybridization Strategy. Utilizing multicolor FISH techniques, we have developed a new strategy which allows chromosome breakage and other structural aberrations as well as hyperdiploidy to be detected in interphase human cells. The principles underlying this tandem label approach are illustrated in Fig. 1. Multicolor FISH is used to label two adjacent regions on chromosome 1. The pericentric heterochromatic region, which is large and prone to breakage by a variety of chemical and physical agents (26-28), is labeled by a Texas red classical satellite probe. An adjacent centromeric region is labeled by a green fluorescein-labeled α satellite probe, which is somewhat smaller and much less prone to breakage by genotoxic agents. The presence of an interphase nucleus containing three α satellite probes adjacent to three classical satellite probes indicates a nucleus which has 3 copies of chromosome 1. However, if a similar interphase nucleus contains only two α satellite probes adjacent to two of the three classical satellite probes, this indicates that a breakage event has occurred within the chromosomal region targeted by one of the classical satellite probes. Alternatively, a wide separation between the regions labeled by the α and classical satellite probes can also indicate that chromosomal breakage has occurred. Illustrated in Fig. 2, a and b, are a metaphase and an interphase human lymphocyte following multicolor FISH with the tandem probes for chromosome 1. When the fluorescein-labeled probe is adjacent to the Texas red-labeled probe, a shift in the emission spectra occurs so that the fluorescein signal appears yellow rather than green (29). Illustrated in Fig. 2c is a lymphocyte which is hyperdiploid for chromosome 1. Fig. 2d is a photomicrograph of a human lymphocyte in which the yellow fluorescein- and the Texas red-labeled probes are widely separated, indicating that a break has occurred within the region targeted by the Texas red-labeled classical satellite probe.

Breakage in Irradiated Metaphase and Interphase Cells. The effectiveness of this approach in detecting structural chromosomal aberrations in interphase cells was determined by comparing the frequency of chromosomal breakage affecting the labeled chromosomal region in interphase and metaphase lymphocytes following in vitro exposure to X-rays. The frequency of structural aberrations affecting the region targeted by the tandemly labeled probes for chromosome 1 for both control and irradiated cells is presented in Table 1. Breakage events in the metaphase cells were classified as breaks which should have been clearly recognizable in an interphase cell, and those which might possibly have been visible in an interphase cell. This latter category resulted almost exclusively from breakage occurring within or distal to the targeted region but where the acentric fragment was not present in the metaphase cell. In untreated cultures, the frequency of breakage was 1/1000 interphase cells, a frequency which did not differ significantly from the 0/1000 metaphase cells observed in Colcemid-arrested cultures. The frequency of breaks observed in the irradiated interphase cells (18/1000) was remarkably similar to that classified as clearly recognizable in the irradiated metaphase cells (17/1000). These results indicate that the interphase analysis accurately identified a majority of the breakage events occurring within or proximal to the targeted region.

The breakage events affecting the hybridized regions in the irradiated cells were classified further into stable and unstable types of aberrations (Table 2). Interestingly, under these treatment conditions, over 50% of the breakage events affecting the labeled heterochromatin (1q12) region were translocations or inversions. An example of radiation-induced aberration in a metaphase cell is shown in Fig. 3; a portion of the classical satellite region on chromosome 1 has been translocated to another chromosome. In ambiguous cases, the location of the fluorescein-labeled α satellite region can be easily and accurately determined by shifting to a blue filter, which permits only the fluorescein signal to be seen (Fig. 3b). Translocations and inversions represent potentially stable aberrations which could be transferred to the progeny of the cell and persist for relatively long periods of time.

Breakage in Untreated Interphase Lymphocytes. To determine whether this tandem label procedure was detecting additional genetic damage in interphase cells or was simply more accurately characterizing damage detected with a single probe, the frequency of nuclei containing chromosomal breakage and hyperdiploidy was compared using a single fluorescein-labeled classical satellite probe for chromosome 1 and the tandem label approach using both the classical and the α satellite probes for chromosome 1. As illustrated in Table 3, using the single probe technique the frequency of nuclei containing 3 and 4 hybridization regions was 3.3 and 0.3/1000, respectively, which would be interpreted as indicating there were 3.3 and 0.3 cells/1000 which were trisomic and tetrasomic for chromosome 1. Using the tandem label approach, 0.2/1000 cells would be considered tetrasomic for chromosome 1, a number which did not differ significantly from that seen with the single probe. In contrast, the frequency of cells considered trisomic for chromosome 1 using the tandem label approach was 1.5/1000 cells, a frequency significantly smaller than that observed with the single label approach (P = 0.006; 1-tailed Fisher exact test). However, an additional 1.5/1000 cells were observed which were characterized as resulting from chromosomal breakage. The difference between these single and the tandem label approaches
was due almost entirely to cells containing chromosomal breakage within the heterochromatin region targeted by the classical satellite probe which were incorrectly identified as trisomic using the single probe approach. These results indicate that this new multicolor FISH approach more accurately identifies hyperdiploidy in untreated cells and can also be used to detect chromosomal breakage occurring in cells from normal individuals.

Application to a Pesticide-exposed Human Population. The ability to detect chromosomal breakage in interphase human cells, particularly persistent and stable rearrangements, indicated that this approach might be suitable for detecting genetic alterations in populations exposed to environmental and occupational chemicals. To determine the applicability of this procedure for human biomonitoring, the tandem labeled method was applied to cultured peripheral blood lymphocytes obtained from a population of pesticide-exposed workers from southern India. The workers chosen for this study were nonsmoking and nondrinking males who work 6–8 h/day, 7–9 months/year, mixing or spraying pesticides (primarily insecticides) to cotton fields in the Guntur region of India. The average worker was 32.2 years of age (range, 18–55 years) and had been employed in this profession for 8.9 years (range, 1–20 years). A group of nonsmoking and nondrinking males with an average age of 30.4 years (range, 18–60 years) from the same village was chosen as controls. A number of studies have been performed previously on this highly exposed population and have shown that the peripheral blood lymphocytes of this group contain significantly higher frequencies of structural and numerical aberrations than does a comparable group without pesticide exposure (20, 30). In situ hybridization with the tandemly labeled probes for chromosome 1 was performed on cultured lymphocytes isolated from these groups, and the frequency of nuclei exhibiting hyperdiploidy or chromosome breakage affecting the 1q12 region was determined. The frequencies of hyperdiploidy and breakage for each of the control and the exposed individuals are presented in Figs. 4 and 5, respectively.

Significantly higher frequencies of hyperdiploidy and breakage were observed in the cells from the pesticide-exposed population when compared to the control group \( P < 0.001; \) Mann-Whitney U test). The median frequency of hyperdiploidy in the exposed workers was 3/1000 (interquartile range, 2–4/1000), whereas the median frequency for the nonexposed controls was 2/1000 (interquartile range, 1–2/1000). A more pronounced difference was observed with chro-
Table 1. Aberrations affecting the centric/pericentric regions of chromosome 1 (Icen-1q12) in irradiated interphase and metaphase human lymphocytes

Cultured human lymphocytes were exposed to X-irradiation at 24 h following initiation of the culture, and the cells were harvested 48 h later. The cells were hybridized with an α satellite probe targeting the Icen region and a classical satellite probe which labeled the chromosome 1 centromeric heterochromatin (1q12). A comparison of the frequencies of aberrations affecting the hybridized regions in both metaphase and interphase cells from irradiated and control cultures is shown.

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>X-irradiated (300 cGy)</th>
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<tbody>
<tr>
<td></td>
<td>Interphase</td>
<td>Metaphase</td>
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<tr>
<td>&quot;Interphase&quot; Aberrations</td>
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<td></td>
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<tr>
<td>Possible</td>
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<tr>
<td>Total cells</td>
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<td>4001</td>
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</table>

* In interphase cells, these represent nuclei with 3 hybridization regions of which only 2 contained adjacent α satellite and the classical satellite probes. In metaphase preparations, these represent aberrant cells exhibiting either a break or an exchange involving the labeled regions which were sufficiently separated that they would be visible in an interphase cell. If a breakage event, the acentric fragment was present.

Table 2. Characterization of aberrations affecting the probe-labeled centric/pericentric regions of chromosome 1 (Icen-1q12) observed in metaphase cells and likely to be detectable in interphase cells

The aberrant metaphase cells observed in untreated and irradiated cultures described in Table 1 were classified by types of aberration.

<table>
<thead>
<tr>
<th>Aberrations</th>
<th>Untreated</th>
<th>X-irradiated (300 cGy)</th>
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</thead>
<tbody>
<tr>
<td>Stable aberrations</td>
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<td></td>
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<tr>
<td>Translocations</td>
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<tr>
<td>Inversions</td>
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<td>3 (4)</td>
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<tr>
<td>Complex rearrangements</td>
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<td>15 (22)</td>
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<tr>
<td>Unstable aberrations</td>
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<td></td>
</tr>
<tr>
<td>Breaks within classical satellite region with fragment present</td>
<td>0</td>
<td>25 (36)</td>
</tr>
<tr>
<td>Breaks between α and classical satellite regions</td>
<td>0</td>
<td>7 (10)</td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
<td>69</td>
</tr>
</tbody>
</table>

* Percentage of total is shown in parentheses.

CHROMOSOME BREAKAGE IN INTERPHASE CELLS

DISCUSSION

In these studies we have demonstrated the application of a new multicolor FISH approach to detect chromosomal breakage in metaphase and interphase human cells following exposure to genotoxic agents in vitro and in vivo. The sensitivity of this tandem label method to detect chromosome alterations is enhanced by the use of probes which target regions of the human genome that are highly prone to breakage by genotoxic agents. In these studies, a classical satellite probe targeting the heterochromatin region (1q12) on chromosome 1 was used. A similar strategy could be used with probes for other regions that are highly prone to breakage, such as the heterochromatin region of chromosome 9 or the breakage-prone region on the short arm of chromosome 3. Previous studies have shown that lymphocyte chromosomes exhibit elevated frequencies of breakage in these regions following in vitro exposure to a variety of clastogenic agents such as ionizing radiation, melphalan, busulfan, mitomycin C, triethylenemelamine, nifurtimox, and hydroquinone (19, 26–28, 31–34). In addition, these chromosomal regions have also been shown to be prone to breakage in untreated lymphocytes from normal individuals and patients with Fanconi’s anemia (27, 35, 36) as well as other cell types (37, 38). Although breakage within the probe-labeled region was not observed in the untreated metaphase lymphocytes in this study, we have observed breakage within the 1q12 region in other studies using untreated lymphocytes from this donor as well as other donors.

Following the in vitro exposure of human lymphocytes to X-irradiation, a close correspondence in the frequency of chromosomal breakage and exchanges affecting the probe-labeled regions was observed in metaphase and interphase cells. This correspondence ranged between 100 and 70% depending on whether only metaphase aberrations characterized as definitely visible in an interphase nucleus are used, or whether cells containing both definitely and possibly visible interphase aberrations are combined for the comparison. Most of the aberrations categorized as possibly visible would probably not be detected as aberrations in interphase cells because breakage events adjacent to the targeted region, as well as probe-labeling fragments lost during cell culture, would not be seen in interphase cells. In addition to the close agreement between the interphase and the metaphase results, this technique appears to be fairly sensitive for both hyperdiploidy and breakage. On the basis of the frequencies of alterations observed in the interphase cells of control individuals in these studies (approximately 2/1000 for either breakage or hyperdiploidy), scoring 2000 cells should allow the detection of aberrant cells with an
approximate 7/1000 frequency of either hyperdiploidy for chromosome 1 or breakage affecting the 1q12 region. Another advantage is that scoring can be performed rapidly by a microscopist with relatively little training; scoring 1000 interphase cells requires approximately 90 min. One potential limitation of this approach is that agents inducing breakage specifically at other loci or at low levels throughout the genome may not be detected using probes for only one region such as 1q12. However, ongoing studies in our laboratory indicate that breakage induced by a wide variety of clastogens can be detected using this technique.4

With the use of probes for chromosome 1, this multicolor FISH technique was shown to be capable of detecting both structural and numerical alterations occurring in the peripheral blood lymphocytes of a population occupationally exposed to a variety of pesticides. In recent years a number of pesticides such as endosulfan, acephate, quinalphos, monocrotophos, chlorpyrifos, fenvalerate, and cypermethrin have been used in these cotton fields, suggesting that exposure to one or more of these agents may be genotoxic to humans. In addition, many of these workers have also been exposed to other organochlorine and organophosphate insecticides which were used previously in these fields (20). The question as to whether the observed genotoxic effects are a result of current or previous pesticide exposures cannot be determined from this data because lymphocytes are a relatively long-lived cell type and can exhibit chromosomal alterations for years following exposure to genotoxic agents (8, 39). However, through the application of this tandem label procedure to shorter-lived blood cells such as neutrophils, a temporal relationship to exposures cannot be determined from this data because lymphocytes have a longer life span than neutrophils and are not exposed to pesticides in a manner similar to the chronic, intermittent exposure experienced by the pesticide-exposed workers.

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4 Rupa, Hasegawa, and Eastmond, unpublished results.


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