Kinds and Locations of Mutations Induced in the Hypoxanthine-Guanine Phosphoribosyltransferase Gene of Human T-Lymphocytes by 1-Nitrosopyrene, Including Those Caused by V(D)J Recombinase

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ABSTRACT

The detection of an increase in the frequency of mutants in the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene of circulating T-cells has been proposed as a method to evaluate the biological effects of human exposure to environmental mutagens. We exposed adult human T-cells in vitro to 1-nitrosopyrene (1-NOP), a partially reduced metabolite of 1-nitropyrene, a ubiquitous environmental carcinogen. In populations of T-cells from two unrelated donors, a dose of 1-NOP that reduced survival to 40% of the untreated cells increased the HPRT mutant frequency 6 to 7 times over the background frequency of 5 $\times$ 10$^{-4}$. The coding region of 35 independent mutants was amplified by polymerase chain reaction and sequenced. Single base substitutions were found in 63% of the mutants (22 of 35). These were distributed randomly throughout the gene. Most of the substitutions (82%) involved G-C base pairs, mainly G-C-A-T transitions and G-C-T-A transversions. Fifteen mutants were lacking one or more exons; 9 of the 15 were lacking exons 2 and 3. Examination showed that at least four of the latter had resulted from V(D)J recombinase acting illegitimately to recombine sites located in introns 1 and 3 of the HPRT gene. T-cells from a second unrelated donor were exposed to 1-NOP and 38 additional independent mutants were analyzed. The results indicated that such mutations occurred at a frequency of 2.4 $\times$ 10$^{-6}$ compared to a background frequency of less than 0.3 $\times$ 10$^{-4}$. This recombinase, which plays an important role in leukemogenesis, is normally present in developing, but not mature, B- and T-cells as such as target cells for 1-NOP. The present study is the first report showing that exposure to an environmental carcinogen can cause mutations induced by the action of this enzyme.

INTRODUCTION

Nitro derivatives of polyaromatic hydrocarbons are produced primarily as a result of incomplete combustion processes (1) and are, therefore, ubiquitous in urban environments. Among this group of compounds, the nitropyrenes are of particular importance because they have been found to be the primary mutagenic component of diesel emission particulate in microbial assays (2). The prototype compound in this group, 1-NP, has been shown to induce mammary (3) and liver (4) tumors in rodents. 1-NP is metabolized into a reactive intermediate via cellular nitroreductases (5).

The first reduction step yields 1-NOP, which must then undergo further enzymatic reduction to form N-hydroxy-1-aminopyrene. This compound yields an unstable intermediate which reacts principally with the C-8 position of guanine to form N-(deoxyguanosin-8-yl)-1-aminopyrene (6, 7) and to a much lower extent with that of adenine (8), yielding N-(deoxyadenosin-8-yl)-1-aminopyrene. These adducts have been shown to be potentially mutagenic lesions in a shuttle vector replicating in human cells (9) and in the endogenous HPRT gene of the CHO cell line (10). They are rapidly repaired by cellular nucleotide excision repair processes (9, 11).

Although excellent chemical and physical methods exist to quantitate the levels of mutagens such as 1-NP in the environment, less is known about the mutagenic effects of human exposure to these compounds. Albertini et al. (12, 13) have proposed that somatic mutations occurring in vivo can be assessed by measuring the mutant frequency at the HPRT locus of circulating T-lymphocytes. For instance, breast cancer patients treated with genotoxic chemotherapeutic agents showed an elevated HPRT mutant frequency in circulating T-cells as compared with patients treated with surgery or surgery plus radiotherapy (14). For risk assessment, it would be useful to be able to determine if an individual who has been exposed to a genotoxic agent has suffered genetic mutations as a result of such exposure. This is particularly important since one problem associated with the use of the T-cell assay to evaluate mutant frequencies is that there is wide variability in the baseline frequency of mutants among normal healthy individuals (15). One approach is to determine if the environmental mutagen of interest induces a characteristic mutational spectrum. Studies from this laboratory show that when a shuttle vector carrying adducts formed by 1-NOP replicates in human cells, the locations of mutations induced are characteristic of that compound (9) and differ significantly from mutations induced in that assay by the structurally related adducts formed by 1-nitro-6-nitrosopyrene (16), N-acetoxy-2-acetylaminoacrylfluorene (17), N-acetoxy-N-trifluoracetyl-2-aminofluorene (18), and (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(α)pyrene (19). Those studies support the feasibility of developing genetic biomarkers of exposure.

To determine if a characteristic mutation spectrum was induced in the HPRT gene of human T-lymphocytes, cells in exponential growth were exposed to 1-NOP, and the kinds and locations of mutations induced in the coding region of the gene were determined. The mutations were mainly base substitutions, targeted primarily to guanine residues. They were distributed randomly throughout the HPRT gene. A striking observation was that a significant proportion of the mutants induced by 1-NOP contained deletions of the genomic DNA spanning 20 kilobases, i.e., from a region of intron 1 to a region of intron 3. Analysis showed that these deletions are consistent with the illegitimate activity of V(D)J recombinase. Such recombination, as a result of exposure of T-cells to 1-NOP, could have implications in the genesis of leukemias and lymphomas associated with exposure to mutagens.
MATERIALS AND METHODS

Cells and Culture Conditions. T-lymphocytes were isolated from leukocyte-enriched "buffy coats" obtained from normal male donors through the American Red Cross. The blood was negative for antibodies to hepatitis B surface antigen and human immunodeficiency virus. A detailed description of the various protocols used with T-lymphocytes has been published (20). Briefly, the peripheral blood mononuclear cells, including the T-lymphocytes, were separated from the "buffy coat" by Ficoll-Hypaque centrifugation and suspended in flasks at a concentration of 1 × 10^6/ml in priming medium (see media below) and incubated for 48 h at 37°C in an atmosphere of 95% air/5% CO_2 to allow PHA polyclonal stimulation of the T-lymphocytes ("priming"). Forty-eight h after priming, when the cells had undergone blast formation, a portion of the population was harvested, centrifuged, and stored in liquid nitrogen for future use. The rest of the cells were resuspended in fresh growth medium to continue exponential growth for 24 h. The cells were then centrifuged, resuspended in the basic medium, and exposed to the carcinogen as described below.

Media for T-Cells. The basic medium used to stimulate the cells to begin blast formation (priming medium), to propagate the T-cells (growth medium), or to expose the T-cells to carcinogen was RPMI 1640 supplemented with penicillin G (100 units/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM). For priming, this medium was supplemented with 20% HL-1 (Ventrex; Endotronics, Minneapolis, MN), 10% HI-FCS, and 1 μg/ml PHA-17 (Burroughs-Wellcome). For growth, the basic medium was supplemented with 20% HL-1, 10% HI-FCS, 0.25 μg/ml PHA-17, and 10% crude h-I-2 (a gift from R. J. Albertini, University of Vermont), and allogenic stimulator cells (see below). For clonal assays, basic medium was supplemented with 20% HL-1, 20% h-I-2, 10% HI-FCS, 0.25 μg/ml PHA-17, and allogenic stimulator cells (cloning medium). Cloning medium was made by mixing equal volumes of basic medium and final dilution medium which contained L-glutamine (2 mM), penicillin G (100 units/ml), streptomycin (100 μg/ml), 40% HL-1, 40% IL-2, 20% HI-FCS, 1 × 10^7 allogenic stimulator cells, and 0.25 μg/ml PHA-17. For selection, 20 μM 6-TG was included in this medium. Enriched medium for refecting cloning assays contained 2 × 10^5 allogenic stimulator cells, 40% h-I-2, 40% HL-1, 20% HI-FCS, and 0.25 μg/ml PHA-17 (and 10 μM TG, if used for selection).

Allogenic Stimulation of T-Lymphocytes. Male-derived B-lymphoblastoid TK6 cells (21) with a complete deletion of the HPRT gene, designated 392X2 cells, were a gift from H. Lieber, University Venezuela. These cells were lethally irradiated with 90 Gy 48°C and were included at the indicated cell densities to serve as allogenic stimulators, i.e., 4 × 10^4 cells/ml in growth medium (to provide a total 4 × 10^5 stimulator cells in a T-25 flask) or 5 × 10^6/ml in cloning medium (to provide 1 × 10^7 stimulator cells in each of the round-bottomed 96-well microtiter plates).

Treatment with 1-NOP. 1-NOP was dissolved in DMSO and stored under N_2 at −20°C. The compound was a gift from F. Beland (National Center for Toxicological Research, Jefferson, AR). T-cells in exponential growth were centrifuged and resuspended in basic medium at a density of 1 × 10^7/ml. The cells were exposed to 1-NOP (treated) or DMSO (control) for 1 h at 37°C in a 95% air/5% CO_2 atmosphere. After treatment, an aliquot was taken to assay the cytotoxicity of the 1-NOP (see below), and the rest of the cells were centrifuged and resuspended in growth medium at 1 × 10^7/ml (control) or 5 × 10^6/ml (treated). At this point, the treated cells were separated into independent populations consisting of 3.2 × 10^5 RC71091 cells or 13 × 10^5 RC80393 cells, each in a series of T-25 flasks and maintained in exponential growth. Cells were typically subcultured once during the 8-day expression period.

Cytotoxicity Assay. As described in detail previously (20), cytotoxicity was determined from the loss of the ability of the T-cells to form colonies at low density. Immediately following exposure to 1-NOP, an aliquot of the T-cells was diluted appropriately and plated into a series of round-bottomed 96-well microtiter plates at various cell densities such that one plate would contain approximately 2 viable cells/well. The number of cells plated per well depended upon the cloning efficiency of the cells and the expected survival. The number ranged from 2 cells/well in untreated controls to 20 cells/well in treated populations. Each well contained T-cells together with 1 × 10^6 allogenic stimulator cells suspended in 200 μl of growth medium. After 7 days, 100 μl of medium were removed and replaced with 100 μl of enriched medium with the aid of an automatic plate refeeding device (Pro/Pette, Perkin-Elmer Cetus). The mean number of clonable cells per well was calculated from the fraction of wells containing no clones, using the Poisson distribution function; this number, divided by the mean number of cells plated per well, yielded the cloning efficiency (12, 13).

Mutagenicity Assay. After an 8-day expression period, the untreated control cells and the series of independent cultures of treated cells were centrifuged and resuspended in basic medium at 1.5 × 10^6 viable cells/ml. An equal volume of final dilution medium containing 20 μM 6-TG was added and 200 μl of the resulting cell suspension were plated into each well of round-bottomed 96-well microtiter plates. Under these conditions, each well contained 1.5 × 10^5 T-cells and 1 × 10^6 allogenic stimulator cells. At the same time, the cloning efficiency of the cells was determined by plating the cells in medium lacking TKG into 96 microwells at a mean density of two cells/well. After 1 week, the cells were refed with the appropriate enriched medium. After 2 weeks in selective medium, the wells were scanned for TG^+ clones. The mutant frequency was calculated from the fraction of wells containing no TG^+ clones, using the Poisson distribution function. This number was divided by the mean number of cells plated per well and the cloning efficiency (12, 13).

Amplification of HPRT cDNA Directly from Cell Lysates and Direct DNA Sequencing. Cells were suspended in RNA-free phosphate-buffered saline and an aliquot of 100–500 viable TG^+ T-cells was centrifuged at 14,600 × g for 10 min at 4°C. Any remaining cells were frozen. Conditions for first-strand cDNA synthesis directly from mRNA in cell lysates, PCR amplification of HPRT cDNA, and dyeoxy sequencing of the gene have been published (22).

PCR Amplification of Genomic DNA from Cells and Direct Sequencing. PCR amplification of exon flanks was performed by lysing ~5000 cells in 10 μl of lysis buffer (15 mM Tris (pH 8.55)-50 mM KC1-2.75 mM MgCl_2-20 mM dithiothreitol-0.5% Nonidet P-40-0.5% Tween 20–0.1 mg/ml proteinase K), heating to 56°C for 1 h and then 94°C for 10 min. Forty μl of genomic DNA PCR cocktail (15 mm Tris (pH 8.55)-50 mm KC1-2.75 mm MgCl_2-0.5 mm concentrations each of dATP, dGTP, dCTP, and dTTP-1 unit Taq polymerase-5 μM concentrations each of 5’ and 3’ primers) were added, and 30 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min were performed. Primers flanking the exon(s) of interest, i.e., the exon(s) missing from the cDNA, were described by Gibbs et al. (23). If the cDNA of a mutant was found to be missing both exons 2 and 3, and if mutant cells were available, multiplex PCR was undertaken to distinguish splice-site mutations from large deletions in genomic DNA encompassing exons 2 and 3 and the intervening intron. The conditions for multiplex PCR and the two primers to be used to determine if the DNA of introns 1 and 3 had been brought together [V(D)J primers A106 and A107] were kindly provided by J. Fuscoe, Integrated Laboratory Systems, Research Triangle Park, NC. Forty μl of PCR cocktail [67 mm Tris-HCl (pH 8.8); 6.7 mm MgCl_2; 16.6 mm (NH_4)_2SO_4; 0.5 mm 2-mercaptoethanol; 6.8 μM EDTA; 1.9 mm concentrations each of dATP, dGTP, dCTP, and dTTP; 5 μl DMSO; 12 pmol exon 2 flanking primers (23); 18 pmol exon 3 flanking primers (23); and 90 pmol each of primers A106 and A107 (24)] were added to 10 μl of cell lysate from ~5000 cells which had been heated to 80°C for 5 min, when 1.0 unit Taq polymerase (Perkin-Elmer) was added. The samples cycled 33 times through 94°C for 1 min, 59°C for 1 min, and 68°C for 2 min. Under these conditions exon 2 is on a 572-base pair fragment and exon 3 is on a 1029-base pair fragment. The ~20-kilobase deletion of genomic DNA allows primers A106 and A107 to form an ~860-base pair fragment.

Asymmetrical PCR was used to synthesize single-stranded DNA of appropriate polarity, which was then sequenced using a [α^32P]-end-labeled sequencing primer with a cycle-sequencing protocol (Promega, Madison, WI). The primers for sequencing the exon flanks were described by Gibbs et al. (23), and the primer for sequencing the V(D)J product was described by Fuscoe et al. (24).

RESULTS

Cytotoxicity and Mutagenicity of 1-NOP in Cultured Human T-Lymphocytes. We screened the T-cells of several anonymous donors to find cells with a low background frequency of TG^+ cells, as well as the ability to form colonies when plated at very low density. Cells from two such donors, designated RC71091 and RC80393, fulfilled these requirements and were used for the present study.
The frequency of TG' cells in these two populations was 5 × 10⁻⁶ (Table 1). The cytotoxic effect of 1-NOP varies from individual to individual. This may reflect differences in the levels of endogenous cellular nitroreductases required to form the reactive form of the carcinogen. To determine the dose that would lower survival to ~37%, a preliminary determination of the cytotoxic effect of various doses of 1-NOP was made. The results indicated that this dose was 0.75 μM for RC71091 and 1.0 μM for RC80393 (data not shown); therefore, these were the respective doses used for the mutagenesis experiments involving these cells.

Frozen cultures of primed T-cells were thawed and upon entering exponential growth they were treated with the designated dose of 1-NOP. The number of target cells used was 24 × 10⁶ for RC71091 and 168 × 10⁶ for RC80393 (see Table 2). Immediately after treatment the cells were divided into several independent populations. The surviving cells were maintained in exponential growth for an 8-day expression period and selected for TG' cells. During expression, the populations were maintained in separate flasks to ensure that mutants from separate treatment groups were independent. As shown in Table 2, treatment of cells with doses of 1-NOP that resulted in ~40% survival raised the mutant frequency significantly.

Kinds and Location of Mutations in the HPRT Gene. The TG' cells from 35 unequivocally independent clones derived from 1-NOP-treatment of RC71091 cells were used to prepare and amplify cDNA as described, and the sequence of the coding region of the HPRT gene was determined. In some cases, TG' cells were stored frozen to be used for further analysis if necessary. A total of 22 base substitutions were found. Table 3 summarizes the kinds of base substitutions. Substitutions involving G-C base pairs were found in 82% (18 of 22) of the cases, with G-C→A-T transitions found most commonly (9 of 22, 41%), followed closely by G-C→T-A transversions (7 of 22, 32%). Table 4 presents the results of DNA-sequence analysis of the mutants. Nucleotides are numbered according to the method of Jolly et al. (25). Of the 35 mutants analyzed, 21 had a base substitution that resulted in an alteration in the predicted amino acid sequence of the protein or, in the case of mutant TNP4, in the loss of an entire exon. Most of the cases, with G-C→A-T transitions found most commonly (9 of 22, 41%), followed closely by G-C→T-A transversions (7 of 22, 32%). Table 4 presents the results of DNA-sequence analysis of the mutants. Nucleotides are numbered according to the method of Jolly et al. (25). Of the 35 mutants analyzed, 21 had a base substitution that resulted in an alteration in the predicted amino acid sequence of the protein or, in the case of mutant TNP4, in the loss of an entire exon. The only silent base substitution was found in a mutant that was also two exons. Whenever possible, the region(s) of interest from the genomic DNA of these mutants was amplified and analyzed for mutations as described in "Materials and Methods." In the case of five mutants (TNP5, TNP8, TNP27, TNP28, and TNP30), additional cells were not available for such analysis; therefore the location of the mutations responsible could not be determined. Mutant TNP4 was found to have a G-C→T-A transversion in the splice acceptor site of exon 4, resulting in the loss of exon 4 from the mutant mRNA. The intron sequences flanking the missing exons of the rest of these mutants (TNP3, TNP9, TNP14, TNP16, and TNP33), as well as the sequences of the missing exons, were determined, but no sequence alterations were found in the regions analyzed. The reason for the loss of exons 2 and 3 in the remaining four mutants is discussed below.

Evidence for 1-NOP-induced V(D)J Recombination in the HPRT Gene. Fuscoe et al. (24) showed that large deletions of genomic DNA spanning the ~20-kilobase region from a section of intron 1 to a section of intron 3 are responsible for the majority of HPRT mutations recovered from the circulating T-cells of neonates. Such mutants have the sequence characteristics of recombination events mediated by V(D)J recombinase, an enzyme activity normally present in developing but not mature B- and T-cells, acting illegitimately on the HPRT gene. These sequence hallmarks include incision at consensus sequences present in introns 1 and 3 of HPRT and/or insertion of non-template-encoded nucleotides. Compared with the fetal circulation, in the adult circulation T-cell HPRT mutations mediated by V(D)J recombinase account for a much lower proportion (typically <5%) of TG' cells. This has been interpreted as indicating that V(D)J-mediated deletions occurred during fetal development when V(D)J recombinase is active and that the mutants have persisted in the blood into adult life (26). When we examined the sequences of cDNAs from 1-NOP-induced mutants from donor RC71091, an unexpectedly high proportion were found to be missing exons 2 and 3 (9 of 35, 26%). When we evaluated seven of these mutants using multiplex PCR, as described in "Materials and Methods," we were surprised to observe the ~860-base pair fragment characteristic of V(D)J recombination in four of the mutants. The remaining three produced bands reflecting amplification of exons 2 and 3. Fig. 1 presents the sequence analysis of the putative V(D)J mutants. Nucleotides are numbered according to the method of Edwards et al. (27).
The data confirmed that each mutant has the sequence characteristics of DNA that has been cleaved and repaired by V(D)J recombinase, consistent with loss of one or more exons. The genomic DNA of these T-cells were selected in TO after priming. Of the 38 background mutants identified, 12 (31%) were found to have cDNA of a size preexisting in the blood of the donor, 9.6 X 10^6 unexposed donor the V(D)J primers. Only one mutant was found to have the V(D)J product; the remaining 11 showed exon 2 and exon 3 bands. Thus, the frequency of preexisting background V(D)J mutants in the donor was 0.13 X 10^-6, a value in the range reported by Fuscoe et al. (26) for normal adult volunteers. The fact that the 1-NOP-exposed populations exhibited >18-fold increase in such mutations suggests that they were induced by the carcinogen, but a χ^2 analysis of the data revealed that the difference in the number of V(D)J class mutants between the control and treated populations is not statistically significant (P = 0.301).

To evaluate the possibility that the induction of V(D)J class mutants was related to exposure to the carcinogen, the frequency of such mutants in circulating T-cells of donor RC80393 was determined in large populations of T-cells from an unrelated donor (RC80393). As shown in Table 5, 16 TG@mutants were found when 31.7 X 10^6 cells were assayed immediately after priming, resulting in a background frequency of 5 X 10^-6 TG' cells when corrected for cloning efficiency of 11%. No V(D)J mutants were found in this population, so the frequency of such mutants in circulating T-cells of donor RC80393...
was $<0.3 \times 10^{-6}$ (i.e., $<1$ of $16 \times 5 \times 10^{-6}$). However, when we exposed $168 \times 10^6$ cells to $1.0 \mu M$ 1-NOP (40% survival) and selected $17.3 \times 10^6$ cells for TG after 8 days of expression, we found 38 induced mutants ($31 \times 10^6$ clonable cells) and 3 unequivocally independent V(D)J mutants. The number of V(D)J class mutants in the treated population is significantly greater than in the background populations by several tests of statistical significance, including $\chi^2 (0.019)$ and likelihood ratio $\chi^2 (0.012)$. The frequency of V(D)J mutants in the induced population was therefore $2.4 \times 10^{-6}$ (i.e., 3 of 38 of $31 \times 10^{-6}$), representing at least an 8-fold induction over the background of $<0.3 \times 10^{-6}$.

**DISCUSSION**

DNA binding by reactive intermediates of 1-NOP in human cells has been shown to occur principally at the C-8 position of guanine, but in vitro adducts can also be formed with adenine, although at a much lower frequency (8). Studies in a variety of systems indicate that mutations induced by 1-NP or 1-NOP primarily involve G-C base pairs (9, 10, 29). In the present study with human T-lymphocytes, base pair substitutions were the most common form of sequence alteration found among the 35 1-NOP-induced unequivocally independent V(D)J mutants. The number of V(D)J class mutants in the treated population is significantly greater than in the background populations by several tests of statistical significance, including $\chi^2 (0.019)$ and likelihood ratio $\chi^2 (0.012)$. The frequency of V(D)J mutants in the induced population was therefore $2.4 \times 10^{-6}$ (i.e., 3 of 38 of $31 \times 10^{-6}$), representing at least an 8-fold induction over the background of $<0.3 \times 10^{-6}$.

The most unexpected and exciting feature we observed in the spectrum of mutations induced by 1-NOP was that the target for mutations resulting from DNA adducts of 1-NOP, i.e., N-(deoxyguanosyl)-1-aminopyrene, is closely related to the fused adenine adduct of 1-NOP in rodent cells. However, we found that 18 of 25 (72%) of the 1-NOP-induced mutants were $-1$ or $+1$ frame-shifts. This suggests that the molecular mechanism of mutagenesis by this compound differs in mammalian and bacterial systems.

A high proportion (15 of 35, 43%) of the 1-NOP-induced mutants exhibited loss of at least one exon. Nine of these 15 were missing exons 2 and 3. The regions of genomic DNA surrounding the missing exons was analyzed for 10 of the mutants (10). In the latter studies, only 2 of 60 (3%) of the mutants had a $-1$ or $+1$ frameshift. In contrast, Stanton et al. (29) sequenced 30 A-prophage mutants induced by 1-NOP in a nucleotide excision repair-deficient bacterial host and found that 18 of 25 (72%) were $-1$ or $+1$ frame-shifts. This suggests that the molecular mechanism of mutagenesis by this compound differs in mammalian and bacterial systems.

The most unexpected and exciting feature we observed in the spectrum of mutations induced by 1-NOP in cultured adult T-cells was $^{4}$V. M. Maher, manuscript in preparation.
oncogene is brought under the control of an inappropriate promoter on occurs (41). Our finding that 1-NOP, a known mutagen and carcinogen, can induce V(D)J recombinase activity suggests that such a mechanism may play a role in mutagen-induced lymphoid cancer.

ACKNOWLEDGMENTS
We thank Clarissa Stropp for her technical assistance and Connie Williams for typing the manuscript.

REFERENCES

Table 5 Frequency of V(D)J recombinase-mediated HPRT deletions in background and in 1-NOP-treated populations of T-lymphocytes

<table>
<thead>
<tr>
<th>Donor</th>
<th>Population assayed</th>
<th>1-NOP dose (µµM)</th>
<th>Cells selected (X 10^6)</th>
<th>V(D)J mutants observed</th>
<th>Total mutants examined</th>
<th>% of mutants involving V(D)J</th>
<th>TGI mutants/10^6 clonable cells</th>
<th>V(D)J mutants/10^6 clonable cells</th>
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<td>6</td>
<td>5</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
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<td>17.3</td>
<td>3</td>
<td>38</td>
<td>7.9</td>
<td>31</td>
<td>2.4</td>
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*a* Not significant (χ^2 = 0.301).

*b* χ^2 = 0.019.


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