ORIGINAL ARTICLES

In vivo mutagenesis of the reporter plasmid pSP189 induced by exposure of host Ad293 cells to activated polymorphonuclear leukocytes

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We measured the mutation frequency and spectrum induced by exposure of the mutation reporter plasmid pSP189 in vivo to phorbol myristate acetate (PMA)-activated human polymorphonuclear leukocytes (PMNs). The mutation frequency induced in the supF tRNA gene of pSP189 transfected into human Ad293 cells by a 30 min exposure to 4 × 10^8 activated PMNs/ml was 3- to 9-fold higher than the background mutation frequency of 0.1-1.8 × 10^{-5}. The enhanced mutation frequency caused by activated PMNs required replication of the reporter plasmid in host Ad293 cells. Fifty five unique activated PMN-associated mutants characterized by sequencing included base substitutions (55%) and deletions (45%), however, no small (1-3 bp) deletions were observed. Ninety four percent of point mutations occurred at C:G base pairs, with C:G→T:A transitions (47%) and C:G→A:T transversions (37%) predominating. A prominent hot-spot was observed at d(pCAGAC) on the tRNA strand. Although H_2O_2 generation was required for mutagenesis, the mutation spectrum induced in pSP189 by in vivo exposure to activated PMNs differed from that induced by in vivo exposure to H_2O_2. It also differed from the spectrum induced in single-stranded DNA in vitro by activated PMNs, suggesting that the mutational spectrum is a complex function of the kinetics of reactive oxygen generation and factors contributed by the target cell.

Introduction

Activated leukocytes are among the principal physiological sources of fluxes of oxygen-derived species. They are known to produce superoxide anion, nitric oxide and H_2O_2, as well as other strong oxidizing species, such as hypohalous acids (for reviews see 1,2). Activated leukocytes cause DNA strand breaks (3) and a variety of DNA base modifications (4) in target cells.

Weitzman et al. (5,6) first demonstrated that activated polymorphonuclear leukocytes (PMNs*) are mutagenic and carcinogenic in vitro. These observations have a well-documented clinical correlate, in that sites of chronic inflammation and chronic wounding are predisposed to develop cancer. Also, the tumor promoting capability of first stage tumor promoters

*Abbreviations: PMNs, polymorphonuclear leukocytes; ROS, reactive oxygen species; PMA, phorbol 12-myristate, 13 acetate.

for the mouse skin system correlates well with their ability to recruit leukocytes and stimulate H_2O_2 release (7).

The original work of Stossel and co-workers and recent work by Reid and Loeb (8) strongly implicate reactive oxygen species (ROS) released by activated PMNs as the mediators of mutagenesis/carcinogenesis. Reid and Loeb studied the mutational spectrum induced when naked single-stranded M13mp2 DNA was exposed to activated PMNs and transfected into SOS-induced Escherichia coli (8). The spectrum and distribution of PMN-induced mutations was quite similar to that induced by Fe(II) (9), suggesting commonality of mechanism of mutation induction.

Loeb and co-workers have shown that the spectrum of ROS-induced mutations depends upon the DNA polymerase involved in replicating the damaged template (10), among other factors, therefore, it is likely that the mutational spectrum caused by activated PMNs in target eukaryotic cells will be different from that observed by Reid and Loeb. There is no information yet on mutations induced by activated PMNs in DNA replicating in human cells in vivo, although data on in vivo mutagenesis caused by other oxyradical generating systems have been reported (11-13). Therefore, we have used the mutation reporter plasmid pSP189 replicating in human Ad293 embryonic kidney cells to analyze mutations associated with treatment of intact target cells with activated PMNs.

pSP189 has several important features which make it useful for these studies. Among them are: (i) pSP189 carries a ‘signature sequence’ which permits distinguishing siblings from distinct mutations at any locus with a high level of confidence (14); (ii) mutations at almost every base in supF cause an identifiable phenotypic abnormality; (iii) although pSP189 replicates episomally, it attaches to the nuclear matrix and becomes chromatinized. Also, the closely related plasmids pZ189 and pS189 have been used successfully to study hydroxyl radical-induced mutations in vitro (11,15).

Our results demonstrate that activated PMNs are mutagens, albeit weak ones, for target human cell DNA. Base substitution mutations at C:G base pairs and deletions predominate in the mutational spectrum; base substitutions are distributed non-randomly.

Materials and methods

Mutation reporting plasmid

Plasmid pSP189 was received as a generous gift from Dr Michael Seidman. The construction and properties of pSP189, including its ‘signature sequence’, have been reported (14).

Target cells

Human Ad293 cells were grown as a monolayer in Dulbecco’s modified Eagle’s medium (Gibco, Grand Island, NY) containing 5% heat-inactivated fetal bovine serum in a humidified 5% CO_2 atmosphere at 37°C.

PMN preparation

Fresh human PMNs were isolated from 10 ml anti-coagulated peripheral blood obtained from a single male donor by the method of Boyum (16) as described...
procedure of Hirt (18), purified as previously described (15), then treated with Low molecular weight DNA was recovered from the target cells by the

Mutation analyses

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>PMN concentration</th>
<th>Percent viable target cells</th>
<th>Total no. of transformants</th>
<th>Mutation frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>100</td>
<td>314 000</td>
<td>1.8x10^-5</td>
</tr>
<tr>
<td>2</td>
<td>0 (PMA added)</td>
<td>100</td>
<td>167 000</td>
<td>1.7x10^-5</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>100</td>
<td>98 000</td>
<td>5.8x10^-5</td>
</tr>
<tr>
<td>4</td>
<td>0 (PMA added)</td>
<td>100</td>
<td>1 920 000</td>
<td>0.9x10^-5</td>
</tr>
<tr>
<td>0 (PMA added)</td>
<td>100</td>
<td>1 400 000</td>
<td>0.4x10^-5</td>
<td></td>
</tr>
<tr>
<td>0 (PMA added)</td>
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<td>400 000</td>
<td>5.3x10^-5</td>
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</tr>
<tr>
<td>0 (PMA added)</td>
<td>100</td>
<td>860 400</td>
<td>1.2x10^-6</td>
<td></td>
</tr>
<tr>
<td>0 (PMA added)</td>
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<td>856 000</td>
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</tr>
<tr>
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<td>797 000</td>
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<td>0 (PMA added)</td>
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<td>2.6x10^-6</td>
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<tr>
<td>0 (PMA added)</td>
<td>100</td>
<td>569 000</td>
<td>&lt;1.8x10^-6</td>
<td></td>
</tr>
</tbody>
</table>

*Viability was estimated by the percentage of target cells remaining adherent to the dish after exposure to and removal of PMNs, followed by washing in serum-free medium.

Plated on standard LB medium supplemented with ampicillin, IPTG and X-gal.

Estimated as the zero-order Poisson term derived from plating 16-26 minimal medium/araB plates

No mutants were observed on 26 plates, therefore, this value represents the upper limit of the observed mutation frequency.

In this experiment, plasmid was harvested 2 h after exposure to activated PMNs, therefore no plasmid replication took place in the host Ad293 cells.

Table II.

<table>
<thead>
<tr>
<th>Mutation type</th>
<th>Number</th>
<th>Control</th>
<th>PMN-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deletions</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Large deletions</td>
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<td>20</td>
<td></td>
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<tr>
<td>Small deletions</td>
<td>3</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Total deletions</td>
<td>16</td>
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<td></td>
</tr>
<tr>
<td>Transitions</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C:G-»T:A</td>
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<td>14</td>
<td></td>
</tr>
<tr>
<td>T:A-»C:G</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Transversions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C:G-»G:C</td>
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<td>3</td>
<td></td>
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<tr>
<td>C:G-»A:T</td>
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<td>11</td>
<td></td>
</tr>
<tr>
<td>T:A-»G:C</td>
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<td>1</td>
<td></td>
</tr>
<tr>
<td>Total point mutations</td>
<td>28</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Total mutations</td>
<td>44</td>
<td>55</td>
<td></td>
</tr>
</tbody>
</table>

Results

The forward mutation reporting assay of Ariza et al. (19) was used in our experiments because of the expectation of a low level of treatment-induced mutagenesis. In this assay, the reporter E. coli strain ML50 (19) was transformed with recovered plasmid by electroporation. For identification of mutated plasmid molecules, transformed ML50 were plated on minimal agar plates containing 0.15% (w/v) glyceral, 0.2% (w/v) l-arabinose, 50 μg/ml ampicillin, 40 μg/ml methicillin, 5 μg/ml thiamine and Vogel-Bonner salts. Mutant colonies were quantified after 48 h growth at 37°C. The presence of mutated supF was confirmed by streaking colonies secondarily on Luria broth plates containing 50 μg/ml ampicillin, 0.1 M isopropyl-p’-D-thiogalactoside and 3% (w/v) 5-bromo-4-chloro-3-indoyl-p-D-galactoside. The total transformant concentrations were determined by plating aliquots of transformed ML50 directly on the Luria broth plates.

Mutation frequencies were determined by counting the percentage of minimal plates with no mutant colonies and applying the Poisson formula:

\[
\frac{\text{total transformants}}{\text{plates}} = \exp\left(\frac{\text{mutant colonies}}{\text{plate}}\right)
\]

The mutation frequency is then

\[
\text{mutation frequency} = \exp\left(-\ln P(O) \times \text{number of plates}\right) \text{ + total transformants}
\]

Mutant colonies confirmed by secondary streaking were amplified, DNA was isolated using Qiagen columns (Chatsworth, CA) according to the manufacturer’s instructions, then sequenced by automated (Applied Biosystems) PCR cycle sequencing.

(4). PMNs obtained by this procedure were routinely 96-98% viable as assayed by exclusion of trypan blue dye. Purified PMNs were used immediately.

Transfection of target cells and PMN exposure

Target Ad293 cells, grown to ~50% confluency in 100 mm dishes, were transfected with 10 μg pSP189 DNA by the diethylaminoethyldextran technique (17). Four hours after transfection, the target Ad293 cells were overlaid with 5 ml serum-free RPMI medium 1640 containing, where appropriate, PMNs ± 2 μg/ml PMA (Sigma Chemical Co., St Louis, MO) dissolved in dimethylsulfoxide and incubated for 1 h at 37°C. After exposure, PMNs, non-viable target cells and medium were poured off and remaining target cells were rinsed with ice-cold serum-free RPMI medium 1640, then overlaid with fresh growth medium and incubated for an additional 44 h.

In some experiments, plasmid DNA was recovered from the target Ad293 cells 2 h after exposure to activated PMNs, rather than 44 h after exposure. This was done because plasmid replication does not begin until ~24 h after transfection; therefore in these experiments, the reporter bacteria were transformed with plasmid molecules which had been damaged by exposure to activated PMNs but had not replicated in the Ad293 cells.

Mutation analyses

Low molecular weight DNA was recovered from the target cells by the procedure of Hart (18), purified as previously described (15), then treated with 0.05 U/μl DpnI to degrade unreplicated plasmid DNA. Escherichia coli strain ML50 (19) was transformed with recovered plasmid by electroporation. For identification of mutated plasmid molecules, transformed ML50 were plated on minimal agar plates containing 0.15% (w/v) glyceral, 0.2% (w/v) l-arabinose, 50 μg/ml ampicillin, 40 μg/ml methicillin, 5 μg/ml thiamine and Vogel-Bonner salts. Mutant colonies were quantified after 48 h growth at 37°C. The presence of mutated supF was confirmed by streaking colonies secondarily on Luria broth plates containing 50 μg/ml ampicillin, 0.1 M isopropyl-p’-D-thiogalactoside and 3% (w/v) 5-bromo-4-chloro-3-indoyl-p-D-galactoside. The total transformant concentrations were determined by plating aliquots of transformed ML50 directly on the Luria broth plates.

Mutation frequencies were determined by counting the percentage of minimal plates with no mutant colonies and applying the Poisson formula:
In vivo mutagenesis of the reporter plasmid pSP189

Fig. 1. The upper panel shows the position of base substitutions induced in the supF gene after exposure to activated PMNs above the wild-type sequence and the position of background base substitutions below the wild-type sequence. The lower panel shows the position of activated PMN-associated deletions above the wild-type sequence and background deletions below. The sequence d(pTAAAT) was reiterated once at position 67. The 17 bp deletion beginning at position 69 was replaced by the sequence d(pTAGCTT). This complex mutation was an 86 bp deletion beginning at position 58 which was replaced by a 103 bp insertion. The 78 bp deletion beginning at position 75 was replaced by the sequence d(pGATGACGGC). This deletion begins at position 120, which is downstream of the 3'-terminus of the tRNA molecule. This mutant contained a 27 bp deletion which was replaced by a 26 bp insertion and a second large deletion beginning at position 99.

Because of the unusually long half-life of unreplicated plasmid molecules in Ad293 cells (20), the question arises whether the activated PMN-associated mutations could have arisen as a result of replication of damaged but unreplicated plasmid molecules in the reporter E.coli strain. In order to address this question, we determined the mutation frequency of plasmid molecules exposed to activated PMNs in vivo which were not allowed to replicate in the host Ad293 cells (Table I, experiment 4). Although the amount of damage generated by the activated PMNs in this experiment was similar to previous experiments, as assessed by loss of transformants, the mutation frequency was not enhanced by activated PMN exposure. This indicates that the enhancement of mutation frequency associated with activated PMN exposure occurs during plasmid replication in the host Ad293 cells. These results contradict those of De Togni et al. (21), who noted a low level increase in mutation frequency above background in plasmid DNA replicating in E.coli exposed directly to human PMNs. However, the experiments of De Togni et al. differed from ours in two key details. (i) They exposed bacteria directly to activated PMNs. In bacteria, the target plasmid is "naked", possibly rendering it more susceptible to promutagenic damage than the chromatinized plasmid located in Ad293 nuclei. (ii) De Togni et al. used a 25-fold higher PMN concentration (10^5/ml) than we did, considerably enhancing the exposure of the target cells to ROS.

The mutational spectrum observed after exposure of pSP189 transfected into Ad293 cells to activated PMNs is shown in Table II. One hundred and forty mutant colonies were sequenced to obtain 55 unique mutants. The signature sequence of pSP189 permits distinguishing unique mutants from siblings with confidence; the large number of siblings reflects the low probability of mutational events occurring in these studies. All but one of the mutant colonies contained only a single mutation. Ninety four percent of the base substitutions occurred at C:G base pairs, with C:G->T:A transitions (47%) and C:G->A:T transversions (37%) predominating.

Forty five percent of the observed mutations were deletions, however, there were no 1-3 bp deletions. There were no obvious distinguishing features at the deletion break points. A repeated sequence occurred only once at a break point, that being the sequence d(pTTTTTTTT) at the downstream break point of a 77 bp deletion. Also, one deletion mutant reiterated the sequence d(pTAAAT) at the break point position 67. None of the large deletions contained supF sequences, however, a

mutation frequency in supF 3- to 9-fold above background. Addition of catalase (3000 U/ml) to the serum-free medium completely suppressed the enhancement of mutagenesis caused by activated PMNs (Table I, experiment 3), indicating a significant role for H_2O_2 in this process.
6 bp insertion of sequence d(pTAGCTT) replaced 17 bp of wild-type sequence at position 69 of two distinct mutants.

Forty four unique background mutants were also sequenced (Table II). The background mutational spectrum was quite similar to that observed after activated PMN exposure. None of the background deletions contained repeated sequences at the break points. One 99 bp deletion was flanked by the sequence d(pCGA) on both the 5'- and 3'-ends. None of the background deletions contained supF sequences, however, a 26 bp insertion whose sequence was not derived from supF replaced 27 bp of wild-type sequence at position 54.

The distribution of mutations observed after in vivo exposure of supF to activated PMNs is shown in Figure 1. The distribution is non-random; hot-spots for base substitution occurred at positions 57 of the sequence d(pCAGAC) and 36 of the sequence d(pTTCCC) on the tRNA strand.

Discussion

Activated PMNs are known mutagens to DNA replicating in bacteria (5,8); this report confirms that they are mutagens for DNA replicating in target human cells. There has been general agreement that activated PMN-induced mutations are mediated by H2O2 (5,8). Consistent with this proposed mechanism, Dizdaroglu et al. (4) observed induction of modified bases by activated PMNs in target human cell DNA typical of that induced by the hydroxyl radical.

The mutational spectrum induced by activated PMNs in supF included a substantial number of both deletions and base substitutions. The base substitutions occurred principally at C:G base pairs and included both transitions and transversions. Considering that the control mutational spectrum was quite similar, the question arises as to how much the 'induced' mutagenic spectrum was influenced by admixture of 'spontaneous' mutants, considering the low level of induced mutagenesis. The 3- to 9-fold enhancement of mutation frequency induced by activated PMNs indicates that ~11-33% of 'induced' mutants sampled actually arose from 'spontaneous' processes. Although physiological production of H2O2 has been proposed as one cause of 'spontaneous' mutagenesis (22), several reports have provided evidence to the contrary, at least with respect to base substitutions and small deletions (11-13).

It is instructive to compare the mutation spectrum induced by activated PMNs in supF replicating in human cells with that induced in single-stranded DNA in vitro replicated in bacteria (8). Base substitutions at cytosines or guanines predominated in both spectra, however, G→C transversions were prominent mutations in bacteria, but not in the human situation. Also, d(pCC)→d(pTT) transitions observed by Reid and Loeb, and considered by them to be a possible signature mutation of ROS exposure, were not observed in our experiments. This difference may reflect differences in the polymerases attempting to replicate the damaged DNA, as suggested by Feig and Loeb (10), and/or differences in repair capacity.

Alternatively, damage products themselves may have differed in the in vivo versus in vitro situations. Reid and Loeb implicated Fe-catalyzed Fenton reaction products as the mediators of activated PMN-induced mutagenesis in vitro. The modified bases, particularly modified purines, produced by this reaction are dependent upon the local redox environment. Dizdaroglu and co-workers observed significantly different amounts of activated PMN-induced base modifications in purified DNA exposed in vitro compared with DNA exposed

in vivo (compare 4 and 23). These authors attributed the differences to the fact that DNA in the intact mammalian nucleus is in a much more reducing environment than is DNA treated in vitro under aerobic conditions.

Our data indicate that H2O2 is an important mediator of activated PMN-induced mutagenesis in target human cells. A commonality of mechanism of mutation induction between activated PMNs and the Fenton reaction has been suggested. In this regard, it is useful to compare our data with that of Moraes et al., who studied mutation in supF replicating in CV-1 monkey kidney cells induced by H2O2 (11). The comparison indicates that activated PMN-induced mutagenesis is a more complex process than simply H2O2 generation. The mutation frequency induced by short term exposure to 5–10 mM H2O2 was comparable with that induced by activated PMNs, although the background mutation frequency reported by Moraes et al. was several orders of magnitude higher. However, the mutational spectrum and the mutation distribution induced by activated PMNs differed from that induced by H2O2. Unlike activated PMNs, H2O2 induced a significant proportion (20%) of C:G→G:C transversions.

Also, almost half of the deletions induced by H2O2 were of 1–2 bp, compared with 0/25 PMN-induced deletions. Although H2O2 is a proximal mediator of activated PMN-induced damage in target cell DNA, Shacter et al. (24) demonstrated that there are significant differences in the kinetics of repair of single-strand breaks induced in target mammalian cells by a short term exposure to H2O2 versus activated PMNs. Small deletions in repeated sequences of supF have been thought to occur during repair of single-strand breaks by a slippage-realignment mechanism (11). The kinetic difference in repair of H2O2 versus activated PMN-induced breaks may reflect different repair mechanisms, one of which is more promutagenic than the other. Alternatively, other strong oxidants produced by activated PMNs in the presence of H2O2, e.g. hypohalous acids or secondarily derived chloramines (25), could be contributing to activated PMN-induced mutagenesis.

In summary, we have characterized mutations induced by activated PMNs in DNA replicating in target human cells. Induced and spontaneous mutation spectra were similar, with large deletions and C:G→T:A transitions and C:G→A:T transversions predominating. Although H2O2 generation is required for activated PMN-induced mutagenesis, the resultant mutational spectrum differs from that induced by H2O2 and from the spectrum induced by activated PMNs in single-stranded DNA in vitro. These differences suggest that the mutational spectrum is a complex function of the kinetics of reactive oxygen generation and factors contributed by the target cell.

Acknowledgement

This work was supported by grant CA53115 from the National Cancer Institute.

References


Received on June 3, 1996; revised on July 2, 1996; accepted on July 8, 1996