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Mutagenesis associated with nitric oxide production in macrophages

(*hprt*/mutational spectrum/spontaneous mutations/free radicals)

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ABSTRACT **To better understand the mechanisms through which persistent infections/inflammation increase cancer risks, we assessed the potential genotoxic properties of NO produced by macrophages. We recently showed that mouse macrophage RAW264.7 cells were capable of resuming exponential growth after stimulation for NO production by interferon-γ (IFN-γ) and/or lipopolysaccharide. Here, we report that increases in mutant fraction (MF) in the endogenous, X-linked, *hprt* gene of the cells are associated with NO exposure. Cells stimulated with 100 units/ml IFN-γ continuously for 14 and 23 days produced a total of 9.8 and 14 μmol of NO per 10^7 cells, respectively. MFs in the *hprt* gene of NO-producing cells were 16.6 and 31.3 × 10^{-5}, respectively, compared with 2.2 and 2.5 × 10^{-5} in untreated cells. Addition of an NO synthase inhibitor, *N*-monomethyl-L-arginine, to the culture medium decreased NO production and MF by 90% and 85%, respectively. Reverse transcription-PCR and DNA sequencing revealed that NO-associated *hprt* mutations did not differ significantly from those arising spontaneously, with the exception that certain small deletions/insertions and multiple exon deletions were observed only in the former. MF also increased significantly in cells stimulated for only 4 days with lipopolysaccharide plus IFN-γ for higher rates of NO production. The types and proportion of *hprt* mutations induced under these conditions were strikingly similar to those associated with long-term NO exposure. These results indicate that NO exposure results in gene mutations in RAW264.7 cells through mechanisms yet to be identified and may also contribute to spontaneous mutagenesis.**

A large fraction of cancer cases globally may be associated with chronic microbial infection or parasitic infestation (1). Although precise mechanisms through which persistent infections and the accompanying inflammation increase cancer risks remain unidentified, it is well established that macrophages and neutrophils infiltrate inflamed tissues, where they produce large quantities of reactive oxygen species and NO. Reactive oxygen species produced by inflammatory cells have been shown to induce gene mutations and cell transformation in target cells cocultured with them (reviewed in ref. 2). Recent studies of several *in vitro* experimental systems have demonstrated that NO is capable of inducing DNA damage and mutations (reviewed in refs. 3 and 4). Recent work by our group showed colocalization of genotoxicity with increased NO production in transgenic SJL mice (5), providing evidence for the involvement of NO in mutagenesis *in vivo*.

We also reported recently that growth and viability of stimulated mouse macrophage-like RAW264.7 cells are strongly influenced by NO (6). Under certain stimulation conditions, cells continued to divide and to produce NO over many generations, a capability making them a useful experimental system for characterization of toxicity resulting from long-term exposure to NO. Furthermore, because RAW264.7 cells were initially isolated from a male mouse (7), the endogenous hypoxanthine-guanine phosphoribosyltransferase (*hprt*) gene provides a convenient selectable target for mutagenesis. In work reported here, we evaluated genotoxicity associated with NO production and found significant elevations of mutant fraction (MF) in cells exposed continuously over many cell generations. Molecular characterization of *hprt* mutations revealed that they did not differ significantly from those arising spontaneously, with the exception that small numbers of small deletions and insertions were observed only in NO-associated mutants. These results indicate that gene mutations arise in NO-producing cells through mechanisms as yet uncharacterized and also suggest that NO may contribute to spontaneous mutagenesis.

MATERIALS AND METHODS

Spontaneous Mutation Rate and Mutagenesis by 4-Nitroquinoline-*N*-Oxide (4-NQO) in the Endogenous *hprt* Gene of RAW264.7 Cells. Hygromycin B-resistant RAW264.7 macrophages were cultured at 37°C in DMEM supplemented with 10% heat-inactivated calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM L-glutamine (all from BioWhittaker), and 100 μg/ml hygromycin B (Boehringer Mannheim), as described (6). To establish a population with a low background MF, six populations, each consisting of ≈10^8 cells, were produced by expansion of initial cultures of ≈200 cells. Aliquots containing 1.65 × 10^7 cells each were transferred to five 96-well plates and cultured for 2 weeks at 37°C in medium containing 2 μg/ml 6-thioguanine (6-TG) (Sigma). The population with a minimum number of *hprt* mutants per well was identified for use in subsequent studies.

To estimate spontaneous mutation rate and to determine response of the *hprt* gene to exogenous mutagen treatment, 1.0 × 10^8 cells were placed into four 150-mm plates. A solution of 4-NQO (Sigma) in dimethyl sulfoxide was added to two plates to a final concentration of 140 ng/ml; controls were treated with dimethyl sulfoxide alone. After incubation for 1 hr, treated cells were washed twice with 25 ml aliquots of medium, resuspended in 25 ml, then incubated until they resumed exponential growth. At intervals over a period of 3 weeks, treated and untreated cells were transferred to 10 96-well plates at densities of 5,000–30,000 cells/well in medium containing 2 μg/ml 6-TG. In parallel, to determine plating efficiency, cells were transferred at a density of 2 cells/well into

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Abbreviations: IFN-γ, interferon-γ; LPS, lipopolysaccharide; MF, mutant fraction; NMA, *N*-monomethyl-L-arginine; *hprt*, hypoxanthine-guanine phosphoribosyltransferase; 4-NQO, 4-nitroquinoline-*N*-oxide; 6-TG, 6-thioguanine; RT-PCR, reverse transcription–PCR

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five 96-well plates containing medium without 6-TG. Two weeks later, colonies were counted and MF was calculated according to a previously described method (8). Spontaneous mutation rate was calculated from the time course of increases in MF in untreated cell populations. The survival rate of 4-NQO-treated cells was estimated by extrapolating the growth curve to zero time.

Mutagenicity in RAW264.7 Cells Stimulated with interferon-γ (IFN-γ). To determine mutagenicity associated with continuous, long-term NO production, a total of 1.5×10^8 cells were incubated for 23 days in six 150-mm plates with 30 ml of medium per plate containing 100 units/ml IFN-γ (Genzyme) plus 10 units/ml polymyxin B (PMB) (Sigma), in the presence or absence of 2 mM *N*-monomethyl-L-arginine (NMA) (Chem-Biochem Research, Salt Lake City, UT), as described (6). Untreated cells were cultured in parallel as negative controls. At the end of 23 days of continuous stimulation, cells were transferred to medium containing only 2 mM NMA and allowed to resume exponential growth. Twenty days later, cells were plated for determination of MF, as described above. Cells treated with IFN-γ in the absence of NMA were later used for isolation of individual *hprt* mutants (see below). Cumulative NO production was determined by measurement of nitrite plus nitrate content of the medium throughout the stimulation period (6). A second experiment of similar design was conducted, in which cells were stimulated for only 14 days.

Mutagenicity associated with short-term, high level NO production was assessed by stimulation of RAW264.7 cells with lipopolysaccharide (LPS) plus IFN-γ. A total of 4×10^7 cells were placed into two 150-mm plates containing 25 ml of medium. NO production was stimulated by addition of 20 ng/ml LPS plus 20 units/ml IFN-γ, in the absence or presence of 2 mM NMA. Unstimulated cells were cultured in parallel as negative controls. After 48 hr of stimulation, the medium was replaced with medium containing only NMA; 20 days later, MF was determined as described above.

Isolation of *hprt* Mutants. Individual *hprt* mutants were isolated from duplicate cultures stimulated for 23 days with IFN-γ in the absence of NMA, as described above. Cells were cultured in the presence of 6-TG in 96-well plates at an initial density of 1,000 cells/well; after 2 weeks, individual clones were picked and expanded in 24-well plates for total RNA isolation. To isolate independent spontaneous *hprt* mutants, ≈200 cells were placed into each well of five 24-well plates and cultured until they reached confluence (≈3×10^6 cells/well). They were then transferred to 6-well plates and grown, with appropriate dilution, for 20 additional divisions. A total of 1–3 $\times 10^6$ cells from each well were placed into a single 96-well plate with growth medium containing 6-TG. Two weeks later, one clone was picked from each plate and the cells were expanded in one well of 24-well plates for total RNA isolation.

To isolate independent *hprt* mutants from cultures costimulated with LPS plus IFN-γ, 1.2×10^6 cells were placed into each well of 20 6-well plates. Stimulation took place in 2 ml of growth medium containing 20 ng/ml LPS plus 20 units/ml IFN-γ, in the presence or absence of 2 mM NMA. Stimulation continued for 4 days with daily changes of medium, and aliquots of medium from four wells of each treatment group were used for nitrite/nitrate analysis. After 4 days of stimulation, numbers of viable cells in four wells of each treatment group were determined, medium was replaced with growth medium containing NMA, and cells were cultured until they resumed exponential growth. When cell density reached ≈50% confluence, they were transferred to a 10-cm plate and allowed to grow for an additional 18 generations. Approximately 10^6 cells from each population were plated into a single 96-well plate in medium containing 6-TG. Two weeks later, one clone was picked from each 96-well plate and expanded in 24-well plates for total RNA isolation. Numbers of colonies in all 96-well plates from each treatment were combined for calculation of average MF.

RNA Isolation, Reverse Transcription–PCR (RT-PCR) and Sequencing. After cell density reached confluence in 24-well plates (≈3×10^6 cells/well), total RNA from each clone was isolated by using a Promega kit, following manufacturer's recommendations. RNA concentration and purity were determined by using a UV spectrophotometer. RNA solutions were adjusted to a concentration of 500 ng/μl and stored at −20°C. One microgram of RNA was used as template for RT-PCR by using the GeneAmp RNA PCR kit from Perkin–Elmer, with the following modifications: oligo $d(T)_{16}$ primer was used for first strand cDNA synthesis in a volume of 20 μl; one-half of the solution was used for PCR in a final volume of 50 μl containing 400 nM each of primers UP1 and DN2 and was overlayed with 50 μl of mineral oil; PCR profile was 5 min at 95°C, 35 cycles of 1 min each at 95°C, 50°C, and 72°C, and 5 min at 72°C. An aliquot of PCR solution was separated by electrophoresis on 5% polyacrylamide or 2% agarose gel and stained with ethidium bromide.

When PCR product was detected, 5 μl of the solution was treated with 10 units of exonuclease I and 2 units of shrimp alkaline phosphatase (Amersham) at 37°C for 15 min to digest single strand DNA and deoxynucleotides, respectively. The solution was then heated at 80°C for 15 min to inactivate the enzymes. The treated PCR product was mixed with 5 pmol of sequencing primer UP5 or DN4 and submitted for automated cycle sequencing (Whitehead Institute Sequencing Facility, Cambridge, MA). Mutations were identified with the aid of Sequencher 3.0 DNA sequence analysis software (Gene Codes, Ann Arbor, MI). Primers used were based on the published mouse *hprt* cDNA sequence (9) with the adenine residue of the methionine-initiation codon numbered as 1. They were designated as follows: UP1, $_{-87}$TTACCTCACTG-CTTTCCGGA$_{-68}$; DN2, $_{711}$TACTGGCAACATCAACAG-GA$_{692}$; UP5, $_{-47}$GGCTTCCTCCTCAGACCGCT$_{-28}$; DN4, $_{691}$CTCCTCGTATTTGCAGATTC$_{672}$. When no PCR product appeared, the remaining portion of the cDNA solution was used for PCR with a pair of mouse β-actin primers (Strategene) under the same reaction conditions. The few samples that showed no β-actin PCR product were excluded.

RESULTS

Spontaneous Mutation Rate and Response to Mutagen Treatment. Cells of the RAW264.7 line originated from a male mouse (7), and thus possess a single copy of the X-linked *hprt* gene. Because they had not been previously used for mutagenesis studies, it was necessary to determine the spontaneous mutation rate at the *hprt* locus and to characterize the response to a known mutagen. After establishing an effective 6-TG concentration (2 μg/ml) for selection of mutants, the spontaneous mutation rate in untreated cells was determined. As shown in Fig. 1, MF increased with increasing culture time, and from these data the spontaneous mutation rate was estimated to be 10^{-6} per cell/day. Treatment with 140 ng/ml 4-NQO for 1 hr reduced cell viability by 50%. In surviving cells, MF increased exponentially after 8 days following treatment and at 12 days reached a plateau value of 51×10^{-5}, compared with 2.3×10^{-6} in untreated controls. These results established that 12 days were required for expression of the mutant *hprt* phenotype; thus, in all subsequent experiments MF was determined 2 weeks after treatment. These findings verified that the endogenous *hprt* gene was a suitable genetic target for studies of mutagenesis associated with NO production in RAW264.7 cells.

NO-Associated Mutagenicity. Mutagenesis was studied in cells continuously stimulated with IFN-γ to produce NO over many generations (6) and also in those stimulated simultaneously with IFN-γ and LPS under conditions that induced maximal NO production. Increases in MF associated with continuous stimulation are summarized in Fig. 2. Cells stim-

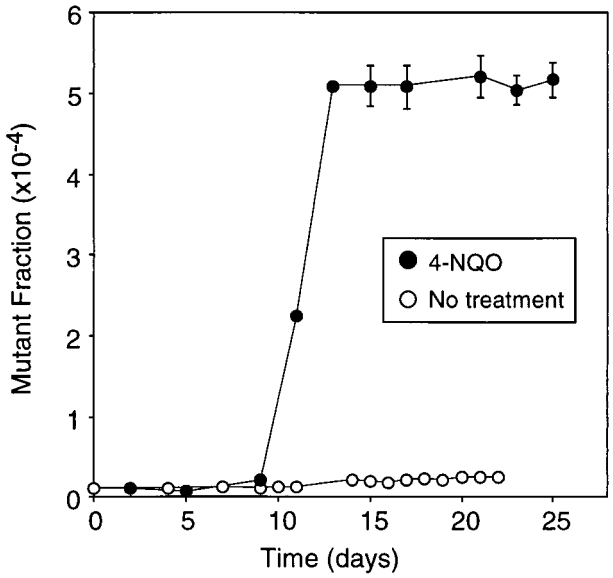

FIG. 1. Time course of MF in the *hprt* gene of RAW264.7 cells with and without 4-NQO treatment. Results are mean ± SD of duplicate experiments.

ulated for 14 days produced a total of 9.8 μmol of NO/10^7 cells (70 nmol/10^6 cells/day). The MF in these cells was 16.6×10^{-5}, compared with 2.2×10^{-5} in untreated controls. Cells stimulated for 23 days produced a total of 14 μmol per 10^7 cells (61 nmol/10^6 cells/day), and the MF was 31.3×10^{-5}, compared with 2.5×10^{-5} in untreated cells. In both cases, exposure of cells to NMA during stimulation decreased NO production by at least 90% (data not shown) and also reduced MFs to 4.6×10^{-5} and 6.8×10^{-5} in cells stimulated for 14 and 23 days, respectively. These data suggest that ≈85% of the mutagenicity observed in stimulated cells was attributable to genotoxic products derived from NO.

A second experiment determined the mutagenic response in cells stimulated with LPS plus IFN-γ for 48 hr. The MF in treated cells was 4.7×10^{-5} compared with 1.4×10^{-5} in untreated controls; in cells stimulated in the presence of NMA,

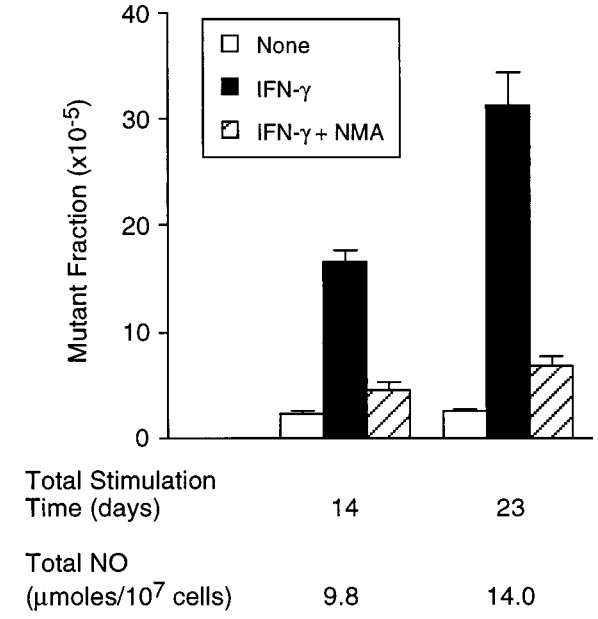

FIG. 2. MF in the *hprt* gene of RAW264.7 cells stimulated with IFN-γ for protracted production of NO. Results are mean ± SD of duplicate experiments.

the MF was 3.1×10^{-5}. NO production thus resulted in significant mutagenicity under these conditions ($P < 0.05$, two-tailed t test, ref. 10).

NO-Associated Mutational Spectra. We characterized mutational spectra in the *hprt* gene to gain insights into molecular mechanisms underlying the observed genotoxicity of NO. The mutational spectrum was first determined in 84 (of 120) independent cultures that spontaneously gave rise to 6-TG-resistant clones. One clone from each culture was characterized by RT-PCR and DNA sequencing, with results summarized in Table 1. RT-PCR products were produced from 27 mutants (32%). Sequencing revealed that the majority (19/27) of mutations were base substitutions and the remainder (8/27) being single exon deletions. Table 2 summarizes specific types of mutations identified. Among the base substitutions present, all of the possible types were observed at varying frequencies. Localization at A:T base pairs (10/19) was similar to that at G:C sites (9/19), whereas transversions were more abundant (12/19) than transitions (7/19). No small deletions/insertions or multiple exon deletions were found among 84 spontaneous mutants. As shown in Table 3, three independent clones contained mutations at position 1 and two at position 647; the remaining base substitutions were distributed singly from positions 74 to 635.

Continuous stimulation of cells with IFN-γ for 23 days resulted in a 12-fold increase in MF, 85% of which was attributable to NO (Fig. 2). To characterize mutations induced under these conditions, individual *hprt* mutants were isolated from two independent cultures of cells stimulated in the absence of NMA. RT-PCR products were recovered from 29/70 (41%) and 24/58 (41%) mutants from the two cultures, respectively. These results indicated no disparity in growth rates among subsets of clones. The absence of significant clonal selection was verified by the fact that only three base substitutions were observed in four, two, and two clones, respectively, from the same culture (data not shown). Mutation data from the two cultures were therefore combined for analysis after elimination of the putative siblings.

As summarized in Table 1, the types of mutations present in mutants induced by NO exposure for 23 days were similar to those seen in spontaneous mutants, in that base substitutions (23/53) and single exon deletions (17/53) were the major types of change observed. However, substantial differences were also evident, as shown in Table 2. Localization of mutations at A:T sites (13/17) greatly exceeded that at G:C sites (4/17), and transversions represented a greater proportion (13/17) of base substitutions than transitions (4/17). Sequence changes not present in spontaneous mutants were found in those associated with NO production, including small deletions (7/53), insertions and truncations (3/53), and multiple exon deletions (3/53). Three of seven deletions involved absence of only 1 bp, whereas removal of the first 17 bp of exon 9 was found in two independent mutants (Table 4). One NO-associated mutant had double mutations (base substitutions at positions 220 and 269). As in the case of spontaneous mutants, mutations were broadly distributed from positions 1 through 646 of the gene, with no evident hotspots (Table 3).

To determine the nature of mutations associated with high levels of NO exposure over a short time period, independent *hprt* mutants were isolated from cells stimulated with LPS plus IFN-γ for 4 days, under which conditions they produced 300 nmol of NO per 1 million cells. Cell viability decreased by 56% and MF increased fivefold compared with controls (14.0×10^{-5} vs. 2.8×10^{-5}). Mutations found in the 25 mutants of this group showed a striking similarity to those present in mutants derived from more prolonged stimulation; frequencies of all categories of mutations were highly comparable (Tables 1 and 2). One mutational event, deletion of a C at position 503 or 504, was detected in both collections of mutants (Table 4). These results indicate

Table 1. Types of mutations in the coding region of the *hprt* gene in RAW264.7 cells stimulated to produce NO

Type of mutation	Frequency of mutation, %			
	No stimulation	IFN-γ, 23 days	LPS + IFN-γ, 4 days	LPS + IFN-γ + NMA, 4 days
RT-PCR product present	32	41	43	26
Base substitution	22	18	15	11
Small deletion	0	6	5	0
Insertion or truncation	0	2	2	0
Single exon deletion	10	13	12	9
Multiple exon deletion	0	2	9	6
RT-PCR product absent	68	59	57	74
Total mutants analyzed	84	128	58	54

that the induced mutational spectrum was independent of the rate or duration of NO production in this system.

To identify the types of mutations associated specifically with NO production, cells were stimulated for 4 days with LPS plus IFN-γ in the presence of NMA. Addition of NMA blocked 85% of NO production, increased cell viability to 62% and reduced the increase of MF to 2.6-fold over untreated controls (7.3×10^{-5} vs. 2.8×10^{-5}). A total of 54 *hprt* mutants, one from each culture, were characterized. As summarized in Table 1, the percentage of samples with a RT-PCR product was comparable to that of spontaneous mutants but somewhat lower than those of NO-associated mutants. Base substitutions and single exon deletions again represented the two major types of mutations, with the remaining being multiple exon

Table 2. Sequence changes in the coding region of the *hprt* gene in RAW264.7 cells stimulated to produce NO

Type of mutation	Number of mutants			
	No stimulation	IFN-γ, 23 days	LPS + IFN-γ, 4 days	LPS + IFN-γ + NMA, 4 days
Base substitution				
GC to AT	3	1	0	1
TA	3	3	2	0
CG	3	0	3	0
AT to GC	4	3	0	1
CG	5	5	3	3
TA	1	5	1	1
Total base substitutions	19	17	9	6
Insertion				
Small (<8 bp)	0	1	1	0
Large (+ exons 4-6)	0	1	0	0
Insertion/deletion	0	1	0	0
Total insertions	0	3	1	0
Deletion				
1 bp	0	3	2	0
Others (<72 bp)	0	4	1	0
Total deletions	0	7	3	0
Single exon deletion				
−Exon 2	2	2	3	2
−Exon 4	2	5	1	1
−Exon 5	2	7	1	1
−Exon 6	1	1	2	0
−Exon 7	1	2	0	0
−Exon 8	0	0	0	1
Total single exon deletions	8	17	7	5
Multiple exon deletion				
−Exon 2–3	0	0	3	1
−Exon 2–4	0	1	0	0
−Exon 3–4	0	1	0	0
−Exon 4–5	0	0	2	0
−Exon 5–6	0	0	0	1
−Exon 4–6	0	0	0	1
−Exon 2–7	0	1	0	0
Total multiple exon deletions	0	3	5	3

Table 3. Positions of base substitutions in *hprt* mutants of RAW264.7 cells stimulated to produce NO

Position	Mutation	Position	Mutation
Spontaneous mutation		IFN-γ, 23 days	
1	AT to GC	1	AT to TA
1	AT to GC	82	AT to CG
1	AT to TA	84	AT to CG
74	GC to CG	133	AT to TA
88	GC to TA	145	GC to AT
104	AT to CG	160	AT to TA
119	GC to TA	220	AT to CG
136	AT to CG	269	AT to GC
229	GC to TA	380	GC to TA
260	GC to CG	397	GC to TA
370	AT to CG	407	AT to CG
407	AT to CG	533	AT to GC
440	AT to GC	542	AT to GC
464	GC to CG	547	AT to TA
569	GC to AT	563	AT to TA
580	GC to AT	594	GC to TA
635	GC to AT	614	AT to CG
647	AT to GC		
647	AT to CG		
LPS + IFN-γ, 4 days		LPS + IFN-γ + NMA, 4 days	
1	AT to TA	1	AT to TA
23	AT to CG	194	AT to CG
65	AT to CG	473	AT to CG
97	GC to TA	473	AT to CG
119	GC to CG	530	AT to GC
299	AT to CG	539	GC to AT
419	GC to TA		
569	GC to CG		
606	GC to CG		

deletions. No small deletions or insertions were observed (Table 2). Thus, no mutations specifically attributable to NO exposure were evident.

DISCUSSION

We recently reported that toxicity in macrophages was strongly influenced by the level and duration of exposure of cells to extracellular NO (6). With increasing rates of exposure, cells sequentially exhibited suppression of growth rate, cytostasis and cell death. Results of this study extend the cascade of damage to include genotoxicity. We characterized mutations in the coding region of the *hprt* gene in spontaneous mutants and in cells stimulated to produce NO, in attempting to elucidate molecular mechanisms through which NO production led to genotoxicity in RAW264.7 macrophages. As summarized in Table 1, mutations occurring in spontaneous mutants comprised only three types: absence of RT-PCR product (68%), base substitutions (22%), and single exon deletions (10%). Specific causes for the absence of RT-PCR product were not identified, but in other cell systems, complete or partial gene deletions affecting either of the PCR primer binding sites have been found to underlie this defect (11). Large deletions also have been detected in spontaneous *hprt* mutants of other cell types. For example, in cultured human B lymphoblastoid (TK6) cells, ≈50% of spontaneous mutants contained large deletions spanning multiple exons (11–13). In mutant peripheral T-lymphocytes isolated from healthy young adults, 15% (125/739) contained gross structural alterations detectable on Southern blots (14). An even larger fraction (85%) was present in mutants isolated from placental cord blood samples of newborns; most of these consisted of deletions of exons 2 and 3 (15). It has been proposed that deletion of exons 2 and 3 occurred as a result of illegitimate action of V(D)J recombinase in an age-dependent fashion (16). It is not known whether a similar mechanism may have been responsible for the high frequency of deletion mutations found in spontaneous mutant RAW264.7 cells, although it is noteworthy that macrophages and T cells are both of hematological origin.

Table 4. Deletions and insertions in *hprt* mutants of RAW264.7 cells stimulated to produce NO

Position	Mutation	Surrounding sequence
Stimulation with IFN-γ, 23 days		
132	−C	GAA(C)AGG
503 or 504	−C	GGA(C)(C)TCT
526 or 527	−C	AGG(C)(C)AGA
643-646	−4 bp	
610-626	−17 bp	
610-626	−17 bp	
160-230	−71 bp	
207-212	+G	GGGGGG
exons 4-6	duplication	
exon 2	replaced by a 116-bp fragment	
Stimulation with LPS + IFN-γ, 4 days		
−22 to 2	24-bp deletion	
503 or 504	−C	GGA(C)(C)TCT
532, 533, or 534	−T	AGAC(T)TTG
606, 607	7-bp insertion	

In spontaneous RAW264.7 mutants in which RT-PCR product was present, the pattern of mutations was comparable with that found in mutant mouse T-lymphoma (GRSL13) cells in two respects (17). First, the proportion of mutants with whole-exon deletions was similar (36% and 30% in GRSL13 and RAW264.7, respectively). Most (7/9 in GRSL13) or all (8/8 in RAW264.7) of these involved loss of a single exon, frequently resulting from point mutations in the splice site(s) for the affected exon (reviewed in ref. 18). Second, base substitutions accounted for most (14/16 in GRSL13) or all (19/19 in RAW264.7) of the remaining mutations. The absence of small deletions/insertions and multiple exon deletions makes the spontaneous mutational pattern of RAW264.7 cells less complex than that of any cell type characterized to date (reviewed in ref. 19). The distribution of base substitutions in RAW264.7 cells was generally similar to that in hamster V79 cells (summarized in ref. 19).

Patterns of *hprt* mutations present in RAW264.7 cells stimulated to produce NO over either prolonged (23 days with IFN-γ) or short (4 days with LPS plus IFN-γ) time periods were remarkably similar, as summarized in Tables 1 and 2. Thus, mutagenesis was evidently unaffected by dose rate and duration of exposure to NO and/or other genotoxic products resulting from stimulation under these conditions. Interestingly, except for small numbers of deletions and insertions, mutations present in cells stimulated with LPS plus IFN-γ were very similar to those found in spontaneous mutants and to those stimulated with LPS plus IFN-γ in the presence of NMA (Tables 1 and 2). χ^2 analysis revealed no significant difference between any two of the four spectra.

However, when the two NO-associated data sets were combined and compared with combined data from untreated cells plus cells stimulated in the presence of NMA, an increase in NO-associated small deletions/insertions was found to be statistically significant ($P < 0.01$). There was also a significant increase in the number of mutants containing a RT-PCR product ($P < 0.05$), but the difference was no longer significant if those mutants containing small deletions and insertions were excluded from the analysis (data not shown). It is noteworthy that the presence of small numbers of deletions/insertions and multiple exon deletions makes the NO-associated mutational spectrum more comparable to spontaneous *hprt* mutational

spectra reported for other cell systems (Table 5 in ref. 19). Collectively, NO-associated mutations were not different from those arising either spontaneously or as a result of stimulation in the presence of NMA, except for small numbers of deletions and insertions, which may have been NO-specific.

These findings regarding NO mutagenesis are in considerable contrast to previous findings in different experimental systems. NO gas, NO donor drugs, and peroxynitrite have all been shown to induce predominantly base substitutions of various types, all of which were distinct from spontaneous mutations (reviewed in ref. 4). In RAW264.7 cells stimulated with LPS plus IFN-γ, increased deamination and oxidation of DNA was attributed to NO production (20). The absence of any NO-specific base substitution in the present study suggests that RAW264.7 may possess efficient mechanisms for repair of these DNA lesions.

Precise molecular mechanisms through which NO induces mutations are presently unknown. However, it was recently reported that when the *supF* reporter gene was transfected into human cells, then exposed to neutrophils activated to produce superoxide, MF was increased by 10-fold, but the induced mutational spectrum was indistinguishable from the spontaneous spectrum (21). Our findings that NO production similarly increased MF without significantly changing the mutational spectrum in macrophages suggest that the underlying mechanisms in the two systems may be similar. In neutrophils, conversion of superoxide to more reactive species such as hydroxyl radical is largely mediated by high concentrations of myeloperoxidase stored in the azurophil granules of the cells (22). Much of the granule peroxidase is lost in macrophages, but the cells acquire multiple synthetic and secretory activities (23), including the capacity to produce NO. In principle, NO in macrophages can serve a function similar to myeloperoxidase in neutrophils by virtue of its ability to facilitate the transformation of superoxide/hydrogen peroxide into more reactive oxygen species. NO reacts directly with superoxide to form peroxynitrite, leading to the formation of hydroxyl radical-like products (24). NO also can react with hydrogen peroxide to form singlet oxygen (25). Furthermore, NO can cause the release of iron from mitochondrial enzymes (26), thereby augmenting Fenton chemistry in macrophages (27). These reactive oxygen species may directly induce mutations by their ability to induce DNA lesions. Alternatively, they may induce mutations through untargeted mechanisms yet to be identified. Examination of NO mutagenesis in the absence of superoxide production by macrophages, together with investigations of possibilities that NO may lead to other forms of genomic instability, may shed light on the molecular basis of NO mutagenesis in macrophages and also on mechanisms underlying spontaneous mutagenesis.

Among the 14 small deletions and insertions in NO-associated mutants, six were 1-bp frameshifts (Table 4). Small deletions of 3 bp or less have been suggested to be characteristic of H_2O_2 exposure (28), although data from a different experimental system failed to detect a mutation hotspot of this type (29). The 1-bp deletion found in RAW264.7 cells seems unlikely to have been mediated by reactive oxygen species because this type of mutation was not detected in cells activated in the presence of NMA, which blocks NO synthesis but not superoxide production (20) (see Tables 1 and 2). Thus, a mechanistic link between NO production and formation of 1-bp frameshift mutations is not established. However, we note that five of the six frameshifts occurred at positions with two or more identical nucleotides (Table 4), in which small insertion-deletion mismatches can form as a result of strand slippage during DNA replication (ref. 19 and references therein). These small loops are repaired by the mismatch repair machinery in the cell (30), and small numbers of frameshifts are common components of spontaneous mutational spectra (19). Our findings suggest that RAW264.7 cells may be highly proficient in mismatch repair, which can be compromised when cells are exposed to NO. In support of this suggestion, NO has been shown to inhibit enzymes involved in DNA synthesis and repair (reviewed in ref. 3). Similar inhibitory activity of NO also may lead to the formation of other types of NO-associated deletions and insertions.

Our findings also demonstrate the feasibility of isolating individual clones from pools of *hprt* mutants without encountering large numbers of siblings. Contributing factors included the induction of large numbers of mutants (in this study, >40,000 during the 23 days of NO production), use of replicate cultures, and selection of clones of different sizes.

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