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Genotoxic properties of 2,4,7-trinitro-9-fluorenone

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Summary

The genotoxic effects of 2,4,7-trinitro-9-fluorenone (TNF) were studied in assays employing procaryotic (*Salmonella typhimurium* and *Escherichia coli*) and eucaryotic (*Saccharomyces cerevisiae*, mouse lymphoma L5178Y and Chinese hamster ovary) cells. The results show that TNF is a potent mutagen for procaryotes. It causes both frame-shift and base-pair substitution mutations, although frame-shift mutations were predominant. In *Saccharomyces cerevisiae*, this compound appeared to be too toxic to permit detection of genotoxic effects. TNF was also toxic to mouse lymphoma cells and Chinese hamster ovary cells but the toxic effects were reduced by metabolic activation. TNF induced a clear increase in sister-chromatid exchanges in CHO cells and in mutant frequency in mouse lymphoma cells both in the presence and absence of metabolic activation.

A number of investigators have shown 2,4,7-trinitro-9-fluorenone (TNF) to be a potent mutagen in bacteria (Levin et al., 1979; McCoy et al., 1981; Probst and Hill, 1980; and Probst et al., 1981), although it was initially reported to be non-mutagenic in *Escherichia coli* (Szybalski, 1958). TNF has also been shown to induce unscheduled DNA synthesis in rat hepatocytes in vitro (Probst and Hill, 1980; Probst et al.,

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Abbreviation: TNF, 2,4,7-trinitro-9-fluorenone.

1981), and to be a powerful carcinogen in female rats (Huggins and Yang, 1962).

TNF has been used in a number of situations where occupational exposure is possible, including its use as a fungicide (Horiguchi et al., 1974), as a complexing reagent in the analysis of indoles by mass spectrometry (Hutzinger and Jamieson, 1970) and its use in the photoconductor film of certain types of photocopying machines (Bagon and Purnell, 1981). For a better understanding of its potential mutagenic hazard to exposed populations, studies were conducted in our laboratories to further investigate the genotoxicity of TNF by a battery of test systems.

Materials and methods

Chemical

2,4,7-Trinitro-9-fluorenone (TNF) was purchased from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin. Fresh stock solutions prepared in dimethyl sulfoxide (DMSO) were used in all experiments.

Test strains and cell lines

In *Salmonella typhimurium* plate incorporation and fluctuation tests, Ames *his*$^-$ testers (TA98 and TA100) were used for detecting reverse mutations from *his*$^-$ to *his*$^+$ (Ames et al., 1975) and an *ara*^{s} strain (SV-50) was employed for scoring forward mutations from *ara*^{s} to *ara*^{r} (Ruiz-Vasquez et al., 1978; Whong et al., 1981). *Escherichia coli* WP2 *uvr*A$^-$ was used to detect reverse mutation from *trp*$^-$ to *trp*$^+$ (Green et al., 1976). *Saccharomyces cerevisiae* strain D7 was used to measure gene conversion and mitotic crossing-over (Zimmermann et al., 1975). The genetic characteristics of these testers have been described previously (Ames et al., 1975; Pueyo, 1978; Whong et al., 1981; Green et al., 1976; and Zimmermann et al., 1975). Chinese hamster ovary (CHO-WBI) cells used in the sister-chromatid exchange (SCE) assay were obtained from Dr. S. Wolff's laboratory, University of California, San Francisco, and cloned in Dr. A. Bloom's laboratory, Columbia University, New York. The mouse lymphoma cell line, L5178Y TK$^{+/-}$, used in the mammalian forward mutation assay, was derived from the Fischer L5178Y line of Dr. D. Clive.

Mutation assays

Bacterial systems .Both the *his* reversion and the *ara*^{r} forward-mutation test assays were carried out using the plate-incorporation test. The procedures for the *his* reversion system were similar to those described by Ames et al. (1975). Metabolic activation was not required. In the *ara*^{r} assay system, SV-50 cells (10^7) from an overnight culture and 0.1 ml of test chemicals or DMSO were added to molten soft agar containing 0.2 ml of 20% L-arabinose and were overlayed onto a M9 bottom plate without metabolic activation (Pueyo, 1978; Ruiz-Vasquez et al., 1978; Whong et al., 1981). All bacterial plates were scored after incubating for 3 days at 37°C. *S. typhimurium his* reversion testers TA98 and TA100 were also used in the fluctuation test along with *E. coli* WP2 *uvr*A$^-$. The media and procedures employed for the fluctuation test are described in detail by Gatehouse (1978). In brief, cell suspensions

containing test bacteria, the chemical or sample under test and growth medium were pipetted into the wells of tissue culture plates. The concentration of the limiting nutrient (histidine or tryptophan, depending on the test strain used) was chosen to permit approximately a 10-fold increase in cell number per well. Brom thymol blue was added to each well after incubation. A positive well is one in which sufficient bacterial growth occurred to produce a color change from blue to yellow. Costar 96-well tissue culture plates (flat-bottomed wells) were used.

Saccharomyces D7 system

Yeast cells (10^5/ml) in 3 ml YEP broth were incubated with 0.1 ml of TNF in DMSO for 24 h at 30°C. The cells underwent several generations of exponential growth during exposure. The cells were then washed and plated on yeast extract-peptone (YEP) and tryptophan-free agar plates as described by Zimmermann et al. (1975), except that 2 series of YEP plates were used per tube. One series received 200 cells/plate and the other received 500 cells/plate. Percentage of survival was determined from the first series and aberrant colonies were scored from both series. All plates were incubated at 30°C. Survivor colonies were counted at 2 days, convertants were counted for 4 consecutive days beginning at the 3rd day and aberrant colonies were scored after 7 days.

Sister-chromatid exchange assay with Chinese hamster ovary (CHO) cells

CHO cells were cultured at 37°C in McCoy's 5a medium supplemented with 10% fetal bovine serum (FBS), L-glutamine and antibiotics. The cells were seeded in 10 ml fresh medium at a concentration of 1×10^6 cells per 75 cm^2 approximately 24 h prior to treatment. Cells were treated in the presence or absence of the S9 reaction mixture. To determine toxicity, cells were incubated 24–26 h after treatment, removed from the flasks by trypsin and counted. The proportion of surviving cells was determined by trypan blue exclusion. The doses selected to score for SCE included the dose that produces approximately a 50% reduction in cell survival, and/or significant delay in cell cycle progression. To assay for SCE in the non-activation assay, cells were treated in growth medium. After a 2-h treatment, 10 μM 5-bromodeoxyuridine (BrdU) was added to the culture tubes and incubation continued in the dark for 26–32 h. In the activation assay with S9, cells were treated in growth medium without fetal bovine serum (FBS). After a 2-h treatment, the cells were washed with buffered saline containing 10% FBS. Fresh medium containing 10 μM BrdU was then added and incubation continued as in the non-activation assay. Colcemid was added for the last 2 h of incubation (0.1 μg/ml), and metaphase cells were collected by mitotic shake-off (Terasima and Tolmach, 1961). The cells were swollen with 0.075 M KCl hypotonic solution, washed 3 times in fixative (methanol : acetic acid, 3 : 1), dropped onto slides and air-dried. Staining for detection of SCE was accomplished by a modified fluorescent plus Giemsa (FPG) technique (Perry and Wolff, 1974; Goto et al., 1978). Following treatment with S9, the cells were washed at least twice with buffered saline containing 10% FBS and then with normal growth medium containing 10% FBS before adding BrdU. Slides were stained for 10 min with Hoechst 33258 (5 μg/ml) in phosphate buffer (pH 6.8),

exposed at 55–60°C to 'black light' and stained with 5% Giemsa for 10–20 min and air-dried.

Mouse lymphoma forward mutation assay: The TK system

The procedure used is based on that reported by Clive and Spector (1975). In brief, cultures ($TK^{+/-}$ cells) exposed for 4 h were washed and placed in growth medium for 2 or 3 days to allow recovery, growth and expression of the induced $TK^{-/-}$ phenotype. At the end of the expression period, 3×10^6 cells for each selected dose were seeded in soft agar plates with selection medium (containing 50 μg BrdU/ml) and resistant (mutant) colonies were counted after 10 days' incubation. Total viable cell number was determined by plating a portion of the cell suspension in non-selective medium. The ratio of resistant colonies to total viable cell number is the mutant frequency. The assay was run with and without S9 activation.

S9 homogenate

The S9 used in the assays for SCE and for gene mutation at the TK locus was purchased from Bionetics Laboratory Products, Litton Bionetics, Inc. It was prepared from the livers of Fischer-344 male rats pretreated with Arochlor 1254.

Statistical analysis

Treated and control cultures in the fluctuation test were compared by the one-tailed χ^2 test as described by Gilbert (1980). The procedure described by Katz (1979) was used in the plate incorporation test. Statistical analysis of sister-chromatid exchange assays was done by a Student *t*-test.

Results

The results of the *his* reversion plate incorporation assay with *S. typhimurium* TA98 are shown in Table 1. Metabolic activation was not required. The results show a dose-dependent response and a dose of 0.003 μg/plate produced a response significant at the 0.01 level. Similarly, the response in the arabinose-resistant forward mutation assay (Table 2) was also dose-dependent but required higher levels of TNF to produce a significant response at the same level of probability (0.042 μg/plate, $p < 0.01$).

TNF produced highly significant mutagenic responses in the fluctuation test ($P < 0.001$, Gilbert, 1980) in *S. typhimurium* tester strains TA98, TA100 and TA100A and in *E. coli* strain WP2 *uvr*A^- (Table 3) but the minimum dose required to produce this response was very different in these strains (0.05 ng/ml in TA98, 15 ng/ml in TA100 and 500 ng/ml in *E. coli*, respectively).

The results with *S. cerevisiae* strain D7 indicate that at the concentrations tested TNF had no significant genotoxic effect (gene conversion and mitotic crossing-over) ((Table 4). Because of its toxicity, however, this compound could only be tested over

TABLE 1

MUTAGENICITY OF 2,4,7-TRINITRO-9-FLUORENONE IN *S. typhimurium* TA98

Concentration (μg/plate)	Revertants/plate [a]
Solvent control (DMSO)	24
Positive control (20 μg TNN) [d]	2466 [c]
0.001	37 [b]
0.003	89 [c]
0.01	216 [c]
0.03	567 [c]
0.1	1279 [c]
0.3	3240 [c]
1.0	8294 [c]

[a] Average of 2 experiments in duplicate.
[b] Significant, $p < 0.05$.
[c] Significant, $p < 0.01$.
[d] 2,3,5-Trinitronaphthalene.

a very narrow concentration range. Consequently, the negative results must be viewed with caution.

The toxicity of TNF to CHO cells is shown in Table 5. Although the data show no consistent toxicity at doses of 3 μg/ml or less, TNF produced complete toxicity at 9 μg/ml or more. The toxic effect was diminished in cultures treated with S9 but this difference may be due in part to the shorter treatment time (2 h) in the activation test compared with about 24 h in the test without S9 mix. The untreated control and solvent control SCE frequencies were normal for this laboratory. There was a highly significant increase in SCEs in cultures exposed to TNF at doses of 0.9 μg/ml or more. The increase was significant even at doses which caused little

TABLE 2

MUTAGENICITY OF 2,4,7-TRINITRO-9-FLUORENONE IN STRAIN SV-50 OF *S. typhimurium* [a]

Test compound	Concn. of test compound (μg/plate)	*ara*^{r} mutants per plate
Solvent control (DMSO)		122
Positive control (MNNG) [c]	2.0	1672
TNF	0.042	247 [b]
	0.084	460 [b]
	0.167	633 [b]
	0.333	584 [b]

[a] Results are the average of 3 independent experiments in duplicate.
[b] Significant, $p < 0.01$.
[c] *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine.

TABLE 3

MUTAGENICITY OF 2,4,7-TRINITRO-9-FLUORENONE IN THE FLUCTUATION TEST WITH *E. coli* AND *S. typhimurium*

Tester	Concentration (ng/ml)	Positive wells		
		Treated	Solvent Control (DMSO)	p
E. coli WP2 *uvrA*$^-$	500	129 [a]	12 [a]	< 0.001
	150	25	12	< 0.05
	50	6	12	
	MMS [c] 30	43	5	< 0.001
TA98	1.5	509 [b]	18 [b]	< 0.001
	0.5	287	18	< 0.001
	0.15	109	18	< 0.001
	0.05	57	18	< 0.001
	0.015	28	18	
	TNN [d] 150	337	18	< 0.001
TA100	150	191 [a]	12 [a]	< 0.001
	50	147	12	< 0.001
	15	56	12	< 0.001
	5	27	12	< 0.01
	TNN [d] 150	41	12	< 0.001

[a] Total wells in 2 experiments in duplicate.
[b] Total wells in 2 experiments in triplicate.
[c] Positive control; methyl methanesulfonate.
[d] Positive control; 2,3,5-trinitronaphthalene.

toxicity or cell cycle delay. In tests with metabolic activation, there was a significant increase at doses of 0.9 μg TNF/ml or more and a dramatic dose-related increase to a maximum of 46.9 SCE/cell at 30 μg/ml. Metabolic activation, although reducing cytotoxicity, increased the genotoxic activity of TNF.

TABLE 4

MUTAGENICITY OF 2,4,7-TRINITRO-9-FLUORENONE IN *S. cerevisiae* D7 [a]

Test compound	Concentration (μg/ml)	Survival as % control	Aberrants (%)	$trp^+/10^5$ survivors
Solvent control (DMSO)		100.0	0.05	2.19
Positive control (EMS)	1.18	16.3	2.80	80.07
TNF	1.0	82.7	0.04	2.02
	1.5	65.1	0.09	2.60
	2.0	33.8	0.05	2.32
	2.5	7.1	0	2.33

[a] Results are from 3 independent experiments in duplicate.

TABLE 5

SISTER-CHROMATID EXCHANGE INDUCED BY 2,4,7-TRINITRO-9-FLUORENONE IN CHINESE HAMSTER OVARY CELLS

Treatment	Survival as % control		Number of SCEs		SCEs per chromosome		SCEs/cell ± S.E. (21 chromosomes)	
	− S9	+ S9	− S9	+ S9	− S9	+ S9	− S9	+ S9
Untreated control	100	100	325	394	0.31	0.38	6.5 ± 0.4	7.9 ± 0.4
Solvent control (DMSO)	100	100	384	345	0.37	0.33	7.8 ± 0.4	6.9 ± 0.4
Mitomycin C [d] (5.0 ng/ml)			1139		1.09		22.9 ± 0.7	
Cyclophosphamide [d] (1.0 μg/ml)				1372		1.31		27.5 ± 0.7
TNF 0.03 [a]	> 100	100	354		0.34		7.1 ± 0.4	
0.09	39	88	387		0.37		7.8 ± 0.4	
0.3	93	83	435	392	0.42	0.37	8.8 ± 0.4	7.9 ± 0.4
0.9	80	85	466	470	0.45	0.45	9.5 ± 0.4 [c]	9.4 ± 0.4 [c]
3.0	> 100	89	767	579	0.74	0.55	15.6 ± 0.6 [b]	11.6 ± 0.5 [c]
9.0	0	99		788		0.75		15.8 ± 0.6 [c]
30.0	0	32		2326		2.23		46.9 ± 1.0 [b]

[a] μg/ml.
[b] Significantly greater than solvent control, $p < 0.01$.
[c] Significantly greater than solvent control, $p < 0.001$.
[d] Positive control.

Results in the mouse lymphoma forward mutation assay were similar to those reported for the SCE assay. TNF was toxic with and without metabolic activation but the toxicity was decreased by treatment with S9 (Table 6). An elevated mutant frequency was observed at a concentration of 1.95 μg/ml in the non-activation test. The trial with metabolic activation confirmed the mutagenic activity and showed an even better dose response effect. The addition of S9 mix resulted in a shift of the toxicity curve toward reduced cytotoxicity and permitted a greater expression of mutagenic activity by TNF.

Discussion

The results of the present study with *S. typhimurium* tester strain TA98 are in agreement with previous studies (Levin et al., 1979; McCoy et al., 1981) in that TNF was seen to be a potent frame-shift mutagen without metabolic activation. Presumptive base-pair substitution mutations were also induced in *E. coli* strain WP2 *uvr*A$^-$

TABLE 6

MUTAGENESIS IN MOUSE LYMPHOMA CELLS INDUCED BY 2,4,7-TRINITRO-9-FLUORENONE

Treatment	Cloning efficiency % control		Total mutant colonies		Total viable colonies		Mutant frequency ($\times 10^{-6}$)	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
Solvent control (DMSO)	90.4	80.5	87.5 [b]	116.0	271.0 [b]	241.5	32.3	47.8
Untreated control	92.7	89.0	95.0	175.0	278.0	267.0	34.2	65.5
EMS, 584 [a,c]	25.3		627.0		76.0		825.0	
DMN, 302 [a,d]		10.7		165.0		32.0		515.6
TNF 0.12 [a]	74.5		87.0		202.0		43.1	
0.24	87.4		72.0		237.0		30.4	
0.49	94.4		59.0		256.0		23.0	
0.98	90.7	100.6	111.0	148.0	246.0	243.0	45.1	60.9
1.95	60.5	67.5	211.0	127.0	164.0	163.0	128.7	77.9
3.91		98.1		170.0		237.0		71.1
7.81		94.0		260.0		227.0		114.5
15.60		41.4		213.0		100.0		213.0

[a] μg/ml.
[b] Average of 2 determinations.
[c] Ethyl methanesulfonate, positive control.
[d] Dimethylnitrosamine, positive control.

(Table 2) but concentrations several orders of magnitude higher were required for an approximately equivalent response ($p < 0.001$). Strain TA100 also responded well to TNF but this cannot be taken to represent base-pair substitution mutation because of the lack of specificity associated with TA100. A concentration approximately 2 orders of magnitude higher was required for TA100 than for TA98 for an equivalent response ($p < 0.001$). TNF also induced forward mutations in the ara^r assay but no attempts were made to characterize these mutations. This system was somewhat less sensitive than TA98 in the plate incorporation test (SV-50 required approximately a 10-fold higher concentration to produce a response at the same level of significance, $p < 0.01$).

TA98 was tested in both the plate incorporation assay and the fluctuation test. This permits a comparison for the relative sensitivity of the 2 systems for TNF. The results suggest a slight advantage in sensitivity for the fluctuation test since mutagenic activity could be detected at 0.05 ng/ml ($p < 0.001$) whereas a concentration of 3 ng/plate was required in the plate incorporation test ($p < 0.001$).

Although TNF is a potent mutagen for bacteria and is highly toxic to strain D7 of *S. cerevisiae*, there appeared to be no significant increase in gene convertants or aberrant frequency as a result of treatment of *S. cerevisiae* with TNF.

The results in CHO cells and in the mouse lymphoma system clearly demonstrate the genotoxicity of TNF to mammalian cells. In each of these systems, the genotoxic effects were observed with and without metabolic activation. Therefore metabolic activation was not a requirement for the expression of mutagenic activity. In both systems the effective concentration was similar regardless of S9 treatment, yet the toxicity of TNF was decreased. This fact suggests that S9 treatment may have reduced the toxic effects of TNF without inhibiting the genotoxic activity resulting in a greater expression of genotoxic effects under S9 activation.

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