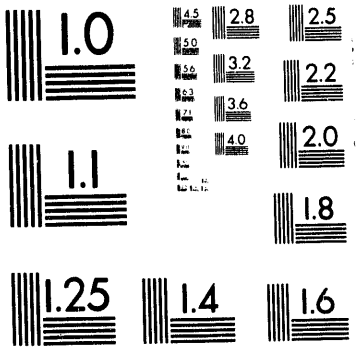
1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
5.6
6.3
7.1
8.0
9.0

1 of 1

The Genotoxicity Studies of Mild Gasification Product, IST#10, in Mammalian Cells

INTRODUCTION

The major hypothesis of carcinogenesis is that malignancy is due to an alteration (mutation) of the genetic material in a somatic cell. Reactive electrophilic metabolites are generated from many chemicals by the action of endogenous mixed function oxidases. These reactive metabolites may bind to cellular macromolecules, such as DNA, and can, therefore, initiate a mutagenic or carcinogenic event. Prokaryotes and non-mammalian eukaryotes are used in mutation assays, while cultured mammalian cells are generally used for mutagenic as well as clastogenic tests examining alterations and damage to the DNA and/or chromosomes of somatic cells. One of the first mammalian cell lines used in genotoxicity studies is V79, which was derived from Chinese hamster lung cells.

According to the test plan on toxicity studies of mild gasification products, mammalian cell *in vitro* assays are to be performed on selected samples displaying mutagenic activity in the Ames assay. The results of the Ames testing of the mild gasification sample IST#10 indicate significant mutagenic activity. Hence, assays for the induction of gene mutation, sister chromatid exchange and micronucleus formation in V79 cells have been carried out for the sample. This paper reports the results of these assays.

METHODS AND MATERIALS

Sample description

IST#10, a product of the mild gasification of coal, is a composite of materials from a wide range of boiling points with a tar odor and thick consistency. The sample was obtained from SGI Fuels in La Jolla, California.

Sample preparation

IST#10 was melted by warming to 80°C in a water bath. 0.236 g of the sample was put into a sterile vial, and 11.6 ml dimethyl sulfoxide (DMSO) was added. The combination was warmed to 50°C and sonicated in a Branson Model 3200 water bath/sonicator for 30 minutes and vortexed until a homogenous solution with a concentration of 20 mg/ml was achieved. The sample was further diluted with DMSO by one half dose in turn (12.5, 6.25, 3.12, 1.56 mg/ml etc.).

DMSO was purchased from Fisher Scientific Company, Fair Lawn, New Jersey. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), used as a positive control, was purchased from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin.

DISCLAIMER

Cell line and culture conditions

The Chinese hamster lung fibroblast (V79) cell line was kindly supplied by C.C. Chang (Michigan State University). Cells were certified to be free of mycoplasma contamination by the standard mycotect assay (Gibco). Stock cultures are maintained in a monolayer in a 75 cm^2 flask with growth medium - minimum essential medium (MEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 100u penicillin and 100 μg streptomycin (Gibco)/ml - at 37°C and 100% humidity in 5% CO_2/95% air. The cells are passaged every 3-4 days by treatment with a trypsin-EDTA solution (Gibco) in phosphate buffered saline (PBS) to prevent the cultures from becoming confluent. Working cultures of this cell line were maintained under the same conditions for the described assays.

Assay procedures

HGPRT gene mutation assay

Hypoxanthine-guanine-phosphoribosyl transferase (HGPRT) is an enzyme, consisting of 2-4 protein subunits, that catalyzes the salvage of hypoxanthine and guanine by the conversion of these purines to the corresponding nucleoside-5-monophosphates (necessary components of DNA, required for cell division and culture growth) upon reaction with phosphoribosyl pyrophosphate. The enzyme also catalyzes the conversion of purine analogs (poisons) 6-mercaptopurine (MP), 6-thioguanine (TG), and 8-azaguanine (AG) into their lethal nucleoside-5-monophosphate forms. V79 cells with mutations that inactivate the product of the HGPRT gene locus, on the X chromosome in humans and rodents, will grow in medium containing the poisonous purine analogs that kill cells with normal HGPRT levels, because they can't metabolize exogenous purines or their deadly analogs. In order to survive, however, these mutants must rely on their ability to synthesis the required purines by a denovo pathway. Aminopterin is a substance that inhibits the denovo synthesis of nucleotides, making a cell dependent on exogenous hypoxanthine, glycine and thymidine. When cultured in medium containing hypoxanthine, aminopterin, thymidine and glycine (HAT medium), mutated V79 cells die because they cannot utilize the exogenous hypoxanthine or synthesize their own purines via the denovo pathway. V79 clones with a mutation inactivating the HGPRT gene product are selected by both a 6-thioguanine resistance (TG^r) and an inability to survive in HAT medium. Induction of the TG^r mutation will be carried out according to the protocol recommended by E.R. Nestman (1991), based on a survey of current practice in genotoxicity testing laboratories.

Five chemical concentrations and a negative control were required - each with and without S9 metabolic activation. 5 x 10^5 cells were seeded in 25 cm^2 flasks containing 5 ml growth medium and incubated at 37°C in a humid atmosphere of 5% CO_2. Twenty-four hours later, the medium was replaced with treatment medium containing 4.5 ml serum-free medium and .5 ml S9 mixture (for metabolic activation) or 5 ml serum-free medium without S9 (for testing direct-acting mutagens), and 50 μl IST#10 sample. After a 3-hour exposure, the cells were rinsed twice with PBS and trypsinized, then replated in two 100 mm dishes at 2.5 x 10^5 for the mutation studies and in six 60 mm dishes at 100 cells/dish for

measurement of the survival. Following a 7-day incubation period, the cultures in the small dishes were fixed, stained and counted. Cell survival relative to the untreated controls (i.e., the relative colony forming efficiency; CFE) was determined for each dose level tested; relative CFE = (the number of colonies with treatment/ the number of colonies in the control) x 100%. At least four treatment concentrations should be included which give relative CFEs ranging between 10 and 100%.

The cultures in the 100 mm dishes, prepared for the mutation studies, were also incubated at 37°C for the expression period of 7 days; during which time, the cells were passaged two or three times. After a week, the cells were washed with PBS and trypsinized in preparation for plating another CFE and expression assay. 100 cells/dish were seeded in 60 mm dishes containing 5 ml MEM - six dishes per treatment were used. These cultures were fixed and stained with Giemsa after 7 days to measure the relative CFE. Approximately 2.5 x 10^5 cells were seeded in 100 mm dishes (six per treatment) containing 10 ml of selection medium (94% hypoxanthine-free MEM, 5% dialyzed FBS, 1% 1mM 6-thioguanine solution). The selection plates were incubated for 10 days; after which, the colonies were fixed, stained and counted by eye. The criterion for minimum colony size acceptable was a diameter of 0.5 mm or greater or a minimum of 50 cells per colony. Mutation frequency (MF) = number of mutant colonies/(total cells seeded on all replicate plates x absolute CFE). Solvent and MNNG were tested simultaneously as solvent and positive controls, respectively.

Sister Chromatid Exchange (SCE) Assay

The *in vitro* SCE assay is frequently used for evaluating the potential of chemicals to induce genetic damage. Although the molecular basis of SCE formation remains unknown, SCEs represent the breakage of 4 stands of DNA (2 double helices), a switch of those strands between chromatids of the same chromosome, and the rejoining of those strands in their new location. It is important to know whether the breakage and rejoining events occur faithfully (i.e. without producing any modification in the genetic code). Several studies have demonstrated that the frequency of SCE increases when cells are exposed to known mutagens and carcinogens.

The method followed has been detailed by L. Soler-Niedzieda (1989). Approximately 6 X 10^5 V79 cells were seeded into 100 mm dishes, containing 10 ml growth medium and incubated at 37°C. Twenty-four hours later, the medium was removed and replaced with treatment medium containing 9 ml serum-free medium and 1 ml S9 mixture, or 10 ml serum-free medium without S9 treatment. The 4-6 concentrations of IST#10 used were determined by the survival test included in the gene mutation assay. After a 3-hour treatment, the cells were washed with PBS, trypsinized, and reseeded into two dishes per treatment with 10 ml fresh medium. One dish was used for micronucleus induction; the other for the SCE assay. Solvent and positive (MNNG) controls were run concurrently with each experiment.

For the SCE assay, 5-bromo-2'-deoxyuridine (BrdU, Sigma) was added to each dish to a final concentration of 20 μM, followed by a 28-30-hour incubation in the dark. Two hours

before harvesting, colcemid was added at a final concentration of 1 μM. The cells were harvested by trypsinization, washed by centrifugation, resuspended in 75 μM KCl hypotonic solution for 20 min at 37°C, and fixed 3 times in a 3:1 mixture of methanol-acetic acid. Two drops of the cell suspension was dropped onto a cold, wet slide which was then waved through a spirit flame. After aging overnight, the slides were stained using the fluorescence-Giemsa method (Soler-Niedzieda, 1989). One hundred metaphases from each treatment were counted for replicative index determination, while 30 cells having 20-23 well-distinguished and well-spread chromosomes were scored for SCEs. The results have been expressed as the mean number of SCEs per metaphase $\pm$ S.D. The student's t test analysis of variance was used to compare the average frequencies of SCEs induced by IST#10.

Micronucleus (MN) assay

Micronuclei are small chromatin-containing bodies arising from acentric chromosomal fragments or whole chromosomes that were not incorporated into the daughter nuclei following mitosis. The presence of MN can be taken as an indication of chromosome breakage and/or spindle dysfunction (Gu, 1992). Treatment of V79 cells with IST#10 and preparation of the cell suspension after exposure is described in the procedures for the SCE assay. After treatment, cells were incubated for 24 hrs, harvested by trypsinization, washed by centrifugation, and resuspended in growth medium to an optimum density. Slides were prepared using cytocentrifugation. An aliquot of cell suspension (50-75 μl) was loaded into a chamber, and the cells were pelleted onto slides at 600 rpm for 7 min, then stained with Diff-Quik stain (Dage Diagnostics Aquada, Puerto Rico). The frequency of micronucleated cells in each treatment is based on 3000 cells scored. The criteria used to scored MN were the following: (1) the diameter of micronucleus must be no larger than one-fifth of the main nucleus; (2) the staining intensity must be equal to that of nucleus; (3) the location in cytoplasm; and (4) there must be no contact with the main nucleus. The results have been expressed as an average number of cells with MN per 1000 cells $\pm$ S.D. Statistical analysis is based on the trend and chi square tests.

RESULTS AND DISCUSSION

Results of the cytotoxicity testing of IST#10 indicate that the test chemical significantly reduced the survival of cultured V79 cells at the concentration range of 15.6 - 62.5 μg/ml without S9 activation (Table 1). There was no killing effect induced by the test sample under the conditions tested in the presence of S9. This may be due to an inactivation of the cytotoxicity by the metabolic enzymes in the S9.

Results of the HGPRT gene mutation assay are shown in Table 2. V79 cells were exposed to IST#10 at doses as high as 62.5 μg/ml, both with and without S9 activation; however, none of the concentrations tested significantly increased the frequency of TG^r mutants. No dose response or minimum of a three-fold increase over the spontaneous level was noted. Therefore, no evidence of induction of gene mutations was noted under the conditions tested. The study was repeated, and the results confirmed.

The frequencies of MN and SCE induced by IST#10 at various concentrations are presented in Tables 3 and 4, respectively. An increase in the frequency of MN formation was seen when cells were treated with IST#10 at concentrations ranging from 7.8 to 62.5 μg/ml, without S9 metabolic activation and only at the highest dose, 62.5 μg/ml, with S9. With out metabolic activation, IST#10 induced a significant increase in the SCE frequency throughout the dose range tested (3.9 - 62.5 μg/ml; $p < 0.01$). In the presence of S9, a significant increase in SCE frequency was observed at the concentrations of 7.8 and 31.2 μg/ml ($p < 0.05$) and at the highest dose, 62.5 μg/ml ($p < 0.01$). The observed increases in both MN and SCE are dose-dependent and statistically significant.

These results indicate that IST#10 can cause DNA damage, chromosome breakage and/or spindle fiber dysfunction in V79 cells without metabolic activation by S9. Therefore, it appears that direct-acting genotoxic agents are present in the sample IST#10. Like it's cytotoxic effect, the genotoxic potential of this agent appears to be detoxified to some extent by metabolic activation with a rat liver preparation.

REFERENCES

Gu, Zu-wei, B.Z. Zhong, et al. (1992) Micronucleus induction and phagocytosis in mammalian cells treated with diesel emission particles, Mutation Research, 279;55-60.

Nestman, E.R., R.L. Brillinger, J.P.W. Gilman, C.J. Rudd, and S.H.H. Swierenga (1991) Recommended protocols based on a survey of current practice in genotoxicity testing laboratories: Mutation in Chinese hamster ovary, V79 Chinese hamster lung and L5178Y mouse lymphoma cells, Mutation Research, 246;255-284.

Soler-Niedzieda, L., T.M. Ong, et al., (1989) Sister chromatid exchange studies on direct- and indirect-acting clastogens in mouse primary cell cultures, Mutation Research 224;465-470.

Table 1. Cytotoxicity of IST#10 to V79 Cells

Chemical treatment[a]	Concentration (μg/ml)	Relative colony forming efficiency[b] -S9 Mean ± SD	-S9 %	+S9 Mean ± SD	+S9 %
IST#10	0.0[c]	100 ± 12	100	57 ± 11	100
	3.9	160 ± 12	160	85 ± 9	149
	7.8	112 ± 18	112	133 ± 14	233
	15.6	56 ± 5	56	119 ± 16	208
	31.2	48 ± 6	48	88 ± 20	154
	62.5	29 ± 4	29	68 ± 22	119
MNNG[d]	1.0	146 ± 4	146	42 ± 5	72

[a] 3 h exposure

[b] Relative CFE = $\frac{\text{Absolute CFE (treatment)}}{\text{Absolute CFE (control)}} \times 100$

[c] DMSO solvent control

[d] N-methyl-N'-nitro-N-nitrosoguanidine

Table 2. Frequency of 6-thioguanine Resistant Mutants in V79 Cells Treated with IST#10

Chemical treatment[a]	Concentration (μg/ml)	Relative Survival (%) --S9	+S9	Mutants per 10^5 Survivors (Mean + SD) -S9	+S9
IST#10	0.0[b]	100	100	1.9 ± 0.5	0.6 ± 0.8
	3.9	160	149	0.7 ± 0.4	0.7 ± 0.4
	7.8	112	233	0.5 ± 0.8	0.3 ± 0.4
	15.6	56	208	0.2 ± 0.4	0.6 ± 0.4
	31.2	48	154	0.1 ± 0.2	0.1 ± 0.2
	62.5	29	119	0.4 ± 0.6	0.2 ± 0.3
MNNG[c]	1.0	146	74	151.8 ± 45.8	11.7 ± 6.1

[a] 3 h exposure

[b] DMSO solvent control

[c] N-methyl-N'-nitro-N-nitrosoguanidine

Table 3. Frequency of Micronuclei in V79 cells Treated with IST#10

Chemical treatment[a]	Concentration (μg/ml)	Total Number of MN Cells[b]		Frequency of MN Cells ‰ (Mean + SD)	
		-S9	+S9	-S9	+S9
IST#10	0.0[c]	33	39	11.0 ± 1.0	13.0 ± 0.0
	3.9	46	47	15.3 ± 2.1	15.7 ± 3.5
	7.8	69	34	23.0 ± 1.0**	11.3 ± 4.5
	15.6	42	42	21.0 ± 2.8**	14.0 ± 1.0
	31.2	109	54	36.3 ± 8.3**	18.0 ± 1.0
	62.5	104	112	34.7 ± 3.1**	37.3 ± 5.5**
MNNG[d]	2.0	207	288	103.5 ± 12.0**	99.3 ± 26.1**

[a] 3 h exposure
[b] 3000 cells were scored
[c] DMSO solvent control
[d] N-methyl-N'-nitro-N-nitrosoguanidine, 2000 cells were scored.

* $p < 0.05$, trend test
** $p < 0.01$, trend test

Table 4. Frequency of Sister Chromatid Exchange in V79 Cells Treated with IST#10

Chemical treatment[a]	Concentration (μg/ml)	SCE / Cell[b] (Mean + SD)		Replication Index	
		-S9	+S9	-S9	+S9
IST#10	0.0[c]	6.7 ± 1.9	7.7 ± 1.8	2.5	2.8
	3.9	8.7 ± 2.3**	8.2 ± 1.8	2.8	2.7
	7.8	9.3 ± 2.1**	8.9 ± 2.4*	2.5	2.7
	15.6	9.7 ± 2.3**	7.3 ± 2.1	2.6	2.7
	31.2	11.2 ± 3.7**	8.5 ± 1.7*	2.5	2.7
	62.5	11.1 ± 3.4**	11.3 ± 3.7**	2.2	2.5
MNNG[d]	2.0	18.8 ± 5.4**	18.6 ± 4.8**	2.6	2.5

[a] 3 h exposure
[b] 30 metaphase cells were scored
[c] DMSO solvent control

* $p < 0.05$, grouped t-test
** $p < 0.01$, grouped t-test
[d] N-methyl-N'-nitro-N-nitrosoguanidine

Mutagenicity of IST#10 Subfractions in the Ames Assay

INTRODUCTION

Mild gasification of coal is a technology being developed by the United States Department of Energy (DOE) and private industry with the hope that a cleaner method of coal use can help meet future energy needs. As the technology develops and its commercial use becomes a more viable possibility, efforts are being made to study the safety and possible toxicity of the mild gasification products. DOE and the National Institute for Occupational Safety and Health (NIOSH) are cooperating through an interagency agreement to examine some of these products for their genotoxic potential.

The Ames Salmonella/microsomal assay (Maron and Ames, 1983) is widely used as a short-term test for the detection of possible genotoxic agents and potential carcinogens. Bacterial tester strains used in the Ames assay contain specific mutations (frameshift or base pair substitution) that cause the bacteria to be dependent on growth medium containing the amino acid histidine. The mutagenic activity of a test substance is measured by the number of reverse mutations eliminating the histidine requirement. DNA mutation is generally accepted to be involved in the initiation stage of carcinogenesis; therefore, the Ames assay has often been used as the first tier of testing in the evaluation of long-term health risks associated with exposure to certain chemicals. NIOSH has used this assay to study the mutagenicity of ten mild gasification products. Several of those samples, including IST#10, have been found to be mutagenic. It is difficult to know which group of chemicals is responsible for the mutagenic activity in complex mixtures. Therefore, such mixtures are often separated by organic solvents into subfractions for testing of mutagenic potentials. This report details the results of testing the subfractions of IST#10.

METHODS AND MATERIALS

Sample description

IST#10 is a mild gasification products with a liquid/tar consistency. It is a composite material, containing materials from a wide range of boiling points. This sample was obtained from Mr. Lan of the DOE, METC.

Chemicals

Positive controls:

2-aminoanthracene (2AA), a chemical mutagen which requires metabolic activation (+S9), and trinitrofluorenone (TNF), a direct-acting mutagen (-S9), were used in the assay system at final concentrations of 2.5 μg/plate and 0.5 μg/plate, respectively. Both were purchased from Aldrich Chemical Company, Milwaukee, Wisconsin.

Solvent control:

Dimethylsulfoxide (DMSO; sterile, spectrophotometric grade) was purchased from EM Science, Cherry Hill, New Jersey.

Sample preparation

Fractionation

The fractionation of IST#10 was performed by Dr. Joseph Ma of the West Virginia University School of Pharmacology. Briefly, the sample was dissolved in ethyl acetate and extracted with sodium hydroxide (NaOH). The NaOH layer was neutralized with HCl and the precipitate (the acidic fraction) was collected. The ethyl acetate layer was extracted again; this time with HCl. The acidic layer was separated and neutralized with sodium bicarbonate. The precipitate, the basic fraction, was collected. The remaining ethyl acetate layer now contained only the neutral fractions of the sample. Crude silica gel was added to the solution. After stirring, the solvent, ethyl acetate, was removed under reduced pressure. An affinity column was prepared and eluted with hexane and cyclohexane to collect the nonpolar neutral fraction. The column was then eluted with cyclohexane/ethyl acetate to collect the polar neutral fraction. The solvents were removed from each fraction before testing was done. The fractions were dissolved in DMSO for the Ames assay. Care was taken to insure that homogeneous mixtures were used while preparing dilutions and during testing.

DMSO Solvation:

Each fraction was dissolved in pure DMSO to the desired concentrations. The mixture was heated to 50°C and sonicated in a Branson model 3200 water bath/sonicator for 30 minutes to facilitate homogenization. The positive controls were prepared in a manner similar to the sample to ensure consistent experimental design.

Ames Salmonella/microsomal Assay

The mutagenicity of IST#10 was determined using the pre-incubation variant of the Ames Salmonella/microsomal assay system (Maron and Ames, 1983), which is frequently more sensitive than the standard protocol. The sample was tested on TA98 and TA100 bacterial tester strains. Each concentration on each tester was tested with and without metabolic activation by S9, a preparation made from the livers of Aroclor 1254-treated male Wistar rats. Each of these treatments was tested in triplicate. In a test tube, 0.1 ml of the sample or a control chemical was combined with 0.1 ml overnight bacterial culture and 0.5 ml S9 preparation or 0.5 ml physiological saline. Each test tube was vortexed and incubated on a rotary shaker at 37°C for 30 minutes prior to adding 2.5 ml molten (45°C) top agar and pouring the mixture onto a petri dish. After the top agar solidified, the dishes were inverted and incubated for 48 hours at 37°C. The top agar contained a trace amount of histidine which is required for initial bacterial growth. After several rounds of cell division, the histidine present was depleted. Only those bacteria that had mutated to be histidine-independent continued to grow and form a colony. Those bacteria are called revertants because they have reverted (in that trait only) back to the wild-type Salmonella. The revertant colonies were scored on an automatic colony counter. The criterion for positive mutagenic activity in the Ames assay is a doubling of the solvent control in the number of revertants, accompanied by a dose-related increase in revertant numbers. The positive

controls allow us to be certain that the bacteria grew and the S9 worked properly.

RESULTS AND DISCUSSION

The sample IST#10 displayed mutagenic activity in three of the four subfractions tested, namely, the basic, nonpolar neutral and polar neutral subfractions. The basic and nonpolar neutral fractions were mutagenic on both tester strains, TA98 and TA100, in the presence and absence of S9 metabolic activation. The polar neutral fraction was mutagenic in the presence and absence of S9, but only on TA98 (Table 1). These results were confirmed when the assays were repeated. Only the acidic fraction failed to display mutagenic activity in any of the assays performed.

The histidine dependence in TA98 is due to a frameshift mutation, which shifts the reading frame of the genetic code, while the histidine dependence in TA100 is due to a non-specific base-pair substitution. The reversion of either mutation requires another event of the same type mutation, so the evidence of mutagenic activity in TA98 and TA100 indicates that the basic and nonpolar neutral samples most likely contain frameshift mutagens, but may also contain base-pair substitution mutagens. The polar neutral fraction, displaying activity only on TA98, probably only contains frameshift mutagens. The fact that the activity is present with or without S9 suggests that the samples do not require metabolic activation, in which case they are referred to as direct-acting mutagens.

REFERENCES

Ma, C.Y., C.H. Ho, R.B. Quincy, M.R. Guerin, T.K. Rao, B.E. Allen, and J.L. Epler (1983) Preparation of oils for bacterial testing, Mutat. Res., 118:15-24.

Maron, D.M., and B.N. Ames, 1983. Revised methods for the *Salmonella* mutagenicity test. Mutat Res, 113:173-215.

TABLE I

Mutagenicity Testing of the Subfractions of IST#10

Sample	Concentration µg/pl	Average Number of Revertant Colonies per Plate			
		TA 98		TA 100	
		- S9	+ S9	- S9	+ S9
Basic	0[A]	19	25	92	95
	3.9	18	80*	96	157
	15.6	32	271*	123	171
	62.5	76*	464*	202*	261*
	250	112*	570*	257*	305*
Acidic[B]	0[A]	26	31	118	113
	125	27	40	131	129
	250	33	47	117	141
	500	28	42	127	132
	1000	29	51	128	133
Nonpolar Neutral	0[A]	19	25	92	95
	3.9	30	53*	85	126
	15.6	53*	61*	134	188
	62.5	101*	63*	210*	279*
	250	156*	72*	223*	289*
Polar Neutral	0[A]	19	25	92	95
	31.2	37	63*	124	141
	62.5	41*	89*	110	170
	125	59*	106*	137	165
	250	41[C]	91[C]	103[C]	134[C]
2AA	0[A]	19	25	92	95
	2.5	-[D]	1974	-	1447
TNF	0[A]	19	25	92	95
	0.4	1475	-	343	-

[A] Solvent control [B] Results from a separate assay [C] Cytotoxicity [D] Not tested * Positive response

DATE
FILMED
2/28/94
END