CORNING Hazleton

MUTAGENICITY TEST ON

T-6564

MEASURING CHROMOSOMAL ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS: WITH A CONFIRMATORY ASSAY WITH MULTIPLE HARVESTS

FINAL REPORT

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LABORATORY PROJECT IDENTIFICATION

CHV Study No.: 17750-0-437CO

SUBMITTED TO

3M Corporation
Building 220-2E-02
3M Center
St. Paul, Minnesota 55144-1000

STUDY COMPLETION DATE

September 16, 1996

QUALITY ASSURANCE STATEMENT

Project Title: Chromosomal Aberrations in Chinese Hamster Ovary (CHO) Cells: With a Confirmatory Assay With Multiple Harvests

Project No.: 20990 Assay No.: 17750

Protocol No.: 437CO Edition No.: 4, Modified for 3M Corporation

Quality Assurance inspections of the study and review of the final report of the above referenced project were conducted according to the Standard Operating Procedures of the Quality Assurance Unit and according to the general requirements of the appropriate Good Laboratory Practice regulations. Findings from the inspections and final report review were reported to management and to the study director on the following dates:

Inspection/Date	Findings Reported	Auditor
Dosing/06/20/1996	06/21/1996	C. Orantes
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STUDY COMPLIANCE AND CERTIFICATION

The described study was conducted in compliance with the Good Laboratory Practice regulations as set forth in the Food and Drug Administration (FDA) Title 21 of the U.S. Code of Federal Regulations Part 58, issued December 22, 1978, (effective June 20, 1979) with any applicable amendments. There were no significant deviations from the aforementioned regulations or the signed protocol that would affect the integrity of the study or the interpretation of the test results. The raw data have been reviewed by the Study Director, who certifies that the evaluation of the test article as presented herein represents an appropriate conclusion within the context of the study design and evaluation criteria.

All test and control results in this report are supported by an experimental data record and this record has been reviewed by the Study Director. All raw data, documentation, records, protocol and a copy of the final report generated as a result of this study will be archived in the storage facilities of Corning Hazleton Inc. for at least one year following submission of the final report to the Sponsor. After the one year period, the Sponsor may elect to have the aforementioned materials retained in the storage facilities of Corning Hazleton Inc. for an additional period of time, or sent to a storage facility designated by the Sponsor.

Submitted By:

Study Director:

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7/16/96

Study Completion Date

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ABSTRACT

The objective of this in vitro assay was to evaluate the ability of T-6564 to induce chromosomal aberrations in Chinese hamster ovary (CHO) cells with and without metabolic activation.

The specific gravity of the neat test article was evaluated as 1.22 g/ml in the testing laboratory and this was used to calculate the dosing stock preparation. Dilutions of the test article were prepared in sterile deionized water. For the dose rangefinding assays with and without metabolic activation, concentrations of 0.169, 0.508, 1.69, 5.08, 16.9, 50.8, 169, 508, 1690, and 5080 µg/ml were tested. All dosing was achieved using a dosing volume of 1.0% (10 µl/ml). Complete cytotoxicity was observed in the cultures dosed with 5080 µg/ml with and without metabolic activation. A reduction of 92% was observed in the mitotic index of the culture dosed with 1690 µg/ml without metabolic activation as compared with the solvent control culture. Reductions of 44%, 15%, and 38% were observed in the mitotic indices of the cultures dosed with 169, 508, and 1690 µg/ml with metabolic activation, respectively, as compared with the solvent control culture.

Based on the data from the dose rangefinding assay, replicate cultures of CHO cells were incubated with 62.5, 125, 250, 500, 1000, 1500, and 2000 µg/ml without metabolic activation and with 250, 500, 1000, 2000, 3000, and 4000 µg/ml with metabolic activation in 20.0 hour assays. Cultures treated with 125, 250, 500, and 1000 µg/ml from the assay without metabolic activation assay and with 250, 500, 1000, and 2000 µg/ml with metabolic activation were analyzed for chromosomal aberrations. No significant increase in cells with chromosomal aberrations was observed at the concentrations analyzed.

In the confirmatory trial, replicate cultures of CHO cells were incubated with 100, 200, 400, 600, 800, 1000, and 1200 µg/ml and with 50.0, 100, 200, 400, 600, 800, 1000, and 1200 µg/ml without metabolic activation with 20.1 and 44.2 hour harvests, respectively. In the assay with metabolic activation, cultures were incubated with 500, 1000, 1500, 2000, 2250, 2500, 2750, and 3000 µg/ml with 20.1 and 44.2 hour harvests. Cultures treated with 200, 400, 600, and 800 µg/ml from the 20.1 hour nonactivation assay, with 100, 200, 400, and 600 µg/ml from the 44.2 hour nonactivation assay, with 1500, 2000, 2250, and 2500 µg/ml from the 20.1 hour activation assay, and with 2000, 2250, 2500, and 2750 µg/ml from the 44.2 hour activation assay were analyzed for chromosomal aberrations. No significant increase in cells with chromosomal aberrations was observed at the concentrations analyzed except for the highest dose analyzed (2500 µg/ml from the 20.1 hour activation assay and 2750 µg/ml from the 44.2 hour activation assay) from the activation.

T-6564 was considered negative for inducing chromosomal aberrations in CHO cells with and without metabolic activation, except at a single dose level with metabolic activation that induced significant toxicity.

Chromosomal Aberrations in Chinese Hamster Ovary (CHO) Cells:
With a Confirmatory Assay With Multiple Harvests
With T-6564

1.0 SPONSOR: 3M Corporation

2.0 MATERIAL (TEST ARTICLE):

2.1 Client's Identification: T-6564 L-13167 FC-1015-X

2.2 Date Received: May 30, 1996

2.3 Physical Description: Clear, colorless liquid

2.4 Genetics Assay No.: 17750

3.0 TYPE OF ASSAY: Chromosomal Aberrations in Chinese Hamster Ovary (CHO) Cells: With a Confirmatory Assay With Multiple Harvests

4.0 PROTOCOL NO.: 437CO, Edition 4, Modified for 3M Corporation

5.0 STUDY DATES:

5.1 Initiation Date: June 4, 1996

5.2 Experimental Start Date: June 13, 1996

5.3 Experimental Termination Date: July 26, 1996

6.0 SUPERVISORY PERSONNEL:

6.1 Study Director: Hemalatha Murli, Ph.D.

6.2 Laboratory Supervisor: Carol S. Spicer, B.S.

7.0 OBJECTIVE:

The objective of this in vitro assay was to evaluate the ability of T-6564 to induce chromosomal aberrations in Chinese hamster ovary (CHO) cells, with and without metabolic activation.

8.0 RATIONALE:

The assay is designed to establish whether the test article or its metabolites can interact with cells to induce chromosome breaks. Chemically induced lesions may result in breaks in chromatin that are either repaired by the cell in such a way as to be undetectable or result in visible damage. Aberrations are a consequence of failure or mistakes in repair processes such that breaks do not rejoin or rejoin in abnormal configurations (Evans, 1962).

9.0 EXPERIMENTAL DESIGN:

Results from the rangefinding assay were used to determine the dose range to be used in the chromosomal aberrations assay. In the rangefinding assay, the cultures were harvested 20.0 hours after initiation of treatment. Mitotic index and visual observation of the cultures were evaluated for evidence of toxicity. A summary of the treatment schedule for the rangefinding assay is given below.

Summary of Dose Rangefinding Assay Treatment Schedule in Hours

Test	Test Article	Wash	Colcemid®	Fixation
- S9	0	17.8	18.0	20.0
+ S9	0	3.0	18.0	20.0

In the chromosomal aberrations assays, replicate cultures were used at each dose level, and negative and solvent controls. Single cultures were used for each of two doses of the positive control. The aberrations assays were conducted with a 20.0 hour harvest time in the initial trial and with 20.1 and 44.2 hour harvest times in the confirmatory trials. Chromosomal aberrations were analyzed from the cultures treated at four dose levels and from one of the positive control doses. A summary of the treatment schedule for the chromosomal aberrations assays is given on the following page.

Summary of Chromosomal Aberrations Assay Treatment Schedule in Hours

Test	Test Article	Wash	Colcemid®	Fixation
Initial Trial				
- S9	0	17.8	18.0	20.0
+ S9	0	3.0	18.0	20.0
Confirmatory Trial				
- S9	0	17.8	18.1	20.1
+ S9	0	3.0	18.1	20.1
- S9	0	41.8	42.2	44.2
+ S9	0	3.0	42.2	44.2

10.0 MATERIALS AND METHODS:

10.1 Test Cells:

The Chinese hamster ovary cells (CHO-WBL) used in this assay were from a permanent cell line and were originally obtained from the laboratory of Dr. S. Wolff, University of California, San Francisco. The cells have since been recloned to maintain karyotypic stability. This cell line has an average cycle time of 12 to 14 hours with a modal chromosome number of 21.

10.2 Cell Culture Medium:

The CHO cells were grown in McCoy's 5a culture medium which was supplemented with 10 % fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin and streptomycin, at approximately 37°C, in an atmosphere of about 5% CO_2 in air.

10.3 Negative and Solvent Controls:

In the nonactivation assays, negative controls were cultures which contain only cells and culture medium. Solvent controls were cultures containing the solvent

for the test article, sterile deionized water, at the highest concentration used in test cultures, 10.0 µl/ml. In the activation assays, the negative and solvent controls were the same as described in the nonactivation assays but with the S9 activation mix included.

10.4 Positive Control Agents:

The positive control agents which were used in the assays were mitomycin C (MMC) for the nonactivation series and cyclophosphamide (CP) in the metabolic activation series. Mitomycin C (CAS# 50-07-7, Sigma, Lot # 25H0619) is a clastogen that does not require metabolic activation. Cyclophosphamide (CAS # 6055-19-2, Sigma, Lot # 67F0155) does not act directly but must be converted to active intermediates by microsomal enzymes. In the chromosomal aberrations assays, two concentrations of MMC (0.08 and 0.1 µg/ml) and CP (5.0 and 10.0 µg/ml) were used to induce chromosomal aberrations in the CHO cells. One of the dose levels was analyzed in each of the aberration assays. Both MMC and CP were dissolved in water.

10.5 Rangefinding Assays:

In these assays, the cells were cultured for approximately 24 hours prior to treatment by seeding approximately 0.3×10^6 cells per 25 cm^2 flask into 5 ml of complete McCoy's 5a culture medium.

10.5.1 Assay Without Metabolic Activation:

The cultures were incubated with the test article for 17.8 hours at ≈37°C. Then, the test article was washed from the cells with phosphate buffered saline and fresh complete medium containing Colcemid® (final concentration 0.1 µg/ml) was added. The cultures were then trypsinized and harvested 2.0 hours later. (See Sections on Harvest and Slide Preparation and Staining).

10.5.2 Assay With Metabolic Activation:

In this assay, the CHO cells were exposed to the test article for three hours at ≈37°C in the presence of a rat liver S9 reaction mixture (S9 15 µl/ml, NADP 1.5 mg/ml, and isocitric acid 2.7 mg/ml). The S9 fraction (Molecular Toxicology, Inc., Lot #0583 used for dose rangefinding assay and initial trial of chromosomal aberrations assay; Lot # 0667, used for the

confirmatory trial of chromosomal aberrations assay) was derived from the liver of male Sprague-Dawley rats which had been previously treated with Aroclor 1254 to induce the mixed function oxidase enzymes which are capable of metabolizing chemicals to more active forms. The three hour incubation time was used because prolonged exposure to the S9 mixture might be toxic to the cells and the enzyme activity of S9 is lost rapidly at about 37°C. The medium did not have FBS during the exposure period to avoid possible inactivation of short lived and highly reactive intermediates produced by the S9 enzymes by binding to serum proteins.

After the exposure period the cells were washed twice with buffered saline. Complete McCoy's 5a medium was added to the cultures which were then incubated for 17.8 hours with Colcemid® (final concentration 0.1 µg/ml) added for the last 2.0 hours to collect metaphase cells. The cultures were then trypsinized, harvested, fixed, and slides were prepared and stained as was described for the nonactivation rangefinding assay.

10.5.3 Assay Evaluation:

Mitotic index was analyzed from the highest four (nonactivation assay) and three (activation assay) surviving dose levels by analyzing the number of metaphases present in 1000 consecutive cells.

10.6 Aberrations Assay Without Metabolic Activation:

Cultures were initiated by seeding approximately 1.2 x 10^6 cells (≈20 hour assay) and 0.8 X 10^6 cells (44.2 hour assay) per 75 cm^2 flask into 10 ml of complete McCoy's 5a medium. One day after culture initiation, the cells were incubated at ≈37°C with the test article at predetermined doses for 17.8 or 41.8 hours. The cultures were then washed with buffered saline and complete McCoy's 5a medium containing 0.1 µg/ml Colcemid® was placed back onto the cells. Two hours later, the cells were harvested and air dried slides were made. The slides were then stained in 5 % Giemsa solution for the analysis of chromosomal aberrations.

10.7 Aberrations Assay With Metabolic Activation:

Cultures were initiated by seeding approximately 1.2 x 10^6 cells (≈20 hour assay) and 0.8 X 10^6 cells (44.2 hour assay) per 75 cm^2 flask into 10 ml of complete McCoy's 5a medium. One day after culture initiation, the cultures that were

treated under the conditions of metabolic activation were incubated at ≈37°C for three hours in the presence of the test article and the S9 reaction mixture in McCoy's 5a medium without FBS. After the three hour exposure period the cells were washed twice with buffered saline and the cells were refed with complete McCoy's 5a medium. The cells were incubated for the rest of the culture period up to the time of harvest with 0.1 µg/ml Colcemid® present during the last 2.0 hours of incubation. The metaphase cells were then harvested and prepared for cytogenetic analysis.

10.8 Harvest Procedure:

Prior to the harvest of the cultures, visual observations of toxicity were made. These observations included an assessment of the percent confluence of the cell monolayer within the culture flasks. The cultures were also evaluated for the presence of mitotic (large rounded cells) or dead cells floating in the medium. The cultures from the dose rangefinding assay were trypsinized first to collect mitotic and interphase cells and were treated with 0.075 M KCl hypotonic solution. This treatment helps to swell the cells and thus disperse the chromosomes. The cultures were then fixed with an absolute methanol: glacial acetic acid (3:1, v:v) fixative and were washed several times before air-dried slides were prepared.

10.9 Slide Preparation and Staining:

Slides were prepared by dropping the harvested cultures on clean slides. The slides were stained with 5% Giemsa solution for the analysis of mitotic index and chromosomal aberrations. All slides were then air-dried and cover slipped using Depex® mounting medium.

10.10 Aberrations Analysis and Assay Evaluation:

Cells were selected for good morphology and only cells with the number of centromeres equal to the modal number 21 ± 1 (range 20-22) were analyzed.

One hundred cells, if possible, from each replicate culture at four dose levels of the test article and from the negative and solvent control cultures were analyzed for the different types of chromosomal aberrations (Evans, 1962; See Section 16.0). At least 25 cells were analyzed for chromosomal aberrations from one of the positive control cultures and from those cultures that had greater than 50% of cells with one or more aberrations. For control of bias, all slides except for the positive controls were coded prior to analysis. Cells with aberrations were

recorded on the data sheets by the microscope stage location. Mitotic index was assessed by analyzing the number of mitotic cells in 1000 cells and the ratio was expressed as a percentage of mitotic cells.

The following factors were taken into account in the evaluation of the chromosomal aberrations data:

1. The percentage of cells with any aberrations.
2. The percentage of cells with more than one aberration.
3. Any evidence for increasing amounts of damage with increasing dose, i.e., a positive dose response.

Chromatid and isochromatid gaps, if observed, were noted in the raw data and were tabulated. They were not, however, considered in the evaluation of the ability of the test article to induce chromosomal aberrations since they may not represent true chromosomal breaks and may possibly be induced by toxicity. Percent polyploidy was analyzed from the 44.2 hour assay and results were tabulated. Historical control data are presented in Table 8.

A cell classified as "GT" is considered to contain 10 aberrations for statistical purposes but a ">" is also included in the tables for this classification to indicate that it is a minimum number.

Statistical analysis employed the Fisher's Exact Test (Sokal and Rohlf, 1981) to compare the percentage of cells with aberrations in each treatment group with the results from the solvent controls. A linear trend test of increasing number of cells with aberrations with increasing dose (Armitage, 1971) was also performed. Test article significance was established where $p<0.01$. All factors as stated previously were taken into account and the final evaluation of the test article was based upon scientific judgement.

11.0 RESULTS:

11.1 Solubility and Dose Determination

The test article was soluble in water. A clear, colorless solution was obtained at a concentration of 500 mg/ml in water. This solution was dosed in the absence of cells using a 1.0% (10 µl/ml) dosing volume in McCoy's 5a culture medium. At a dosed concentration of 5000 µg/ml, a clear solution was obtained and a pH of 8.0 (the same pH as that of McCoy's 5a culture medium) was determined. The

specific gravity of the test article was calculated in the testing laboratory as 1.22 g/ml. This specific gravity was used to prepare dosing stocks for the assay. Sterile deionized water (Batch # 19, prepared at CHV) was used as the vehicle in this assay. All dosing was achieved with a 1.0% (10.0 µl/ml) dosing of the dosing stocks and the solvent control culture was dosed with 10.0 µl/ml of sterile deionized water. Concentrations of 0.169, 0.508, 1.69, 5.08, 16.9, 50.8, 169, 508, 1690, and 5080 µg/ml were tested in the dose rangefinding assays. The stability of the test article under the preparation and dosing conditions used in this assay is the responsibility of the sponsor.

11.2 Rangefinding Assay Without Metabolic Activation

A dead cell monolayer, no visible mitotic cells, floating dead cells and debris, and <5% cell monolayer confluence were observed in the culture dosed with 5080 µg/ml. An unhealthy cell monolayer, a severe reduction in the number of visible mitotic cells, floating dead cells and debris, and ≈45% reduction in the cell monolayer confluence were observed in the culture dosed with 1690 µg/ml. A slightly unhealthy cell monolayer, floating dead cells and debris, and ≈30% reduction in the cell monolayer confluence were observed in the culture dosed with 508 µg/ml. Floating debris and ≈15% reduction in the cell monolayer confluence were observed in the culture dosed with 167 µg/ml. Floating debris was observed in the culture dosed with 50.8 µg/ml. Mitotic indices were analyzed from the cultures dosed with 50.8, 169, 508, and 1690 µg/ml (Table 1). A reduction of 92% was observed in the mitotic index of the culture dosed with 1690 µg/ml as compared with the solvent control culture. Based on these results, the initial aberrations assay without metabolic activation was conducted with a 20.0 hour harvest testing concentrations of 62.5, 125, 250, 500, 1000, 1500, and 2000 µg/ml.

11.3 Rangefinding Assay With Metabolic Activation

No cell monolayer and no visible mitotic cells were observed in the culture dosed with 5080 µg/ml. No other signs of visual toxicity were observed in any of the other cultures. Mitotic indices were analyzed from the cultures dosed with 169, 508, and 1690 µg/ml (Table 1). Reductions of 44%, 15%, and 38% were observed in the mitotic indices of the cultures dosed with 169, 508, and 1690 µg/ml, respectively, as compared with the solvent control culture. Based on these results, the initial aberrations assay with metabolic activation was conducted with a 20.0 hour harvest testing concentrations of 250, 500, 1000, 2000, 3000, and 4000 µg/ml.

11.4 Chromosomal Aberrations Assay Without Metabolic Activation

Initial Trial

Dead cell monolayers, floating dead cells and debris, no visible mitotic cells, and ≈95% reduction in the cell monolayer confluence were observed in the cultures dosed with 2000 µg/ml. Unhealthy cell monolayers, severe reductions in the number of visible mitotic cells, floating dead cells and debris, and ≈45% reduction in the cell monolayer confluence were observed in the cultures dosed with 1500 µg/ml. Unhealthy cell monolayers, severe reductions in the number of visible mitotic cells, floating dead cells and debris, and ≈30% reduction in the cell monolayer confluence were observed in the cultures dosed with 1000 µg/ml. Slightly unhealthy cell monolayers, floating debris, and ≈15% reduction in the cell monolayer confluence were observed in the cultures dosed with 500 µg/ml. Chromosomal aberrations were analyzed from the cultures dosed with 125, 250, 500, and 1000 µg/ml (Table 2). Reductions of 25%, 30%, 87%, and 96% were observed in the mitotic indices of the cultures dosed with 125, 250, 1000, and 1500 µg/ml, respectively, as compared with the solvent control cultures. No significant increases in cells with chromosomal aberrations were observed at the concentrations analyzed.

Based on the results from this trial, the confirmatory trial of the nonactivation aberrations assay was conducted testing concentrations of 100, 200, 400, 600, 800, 1000, and 1200 µg/ml and 50.0, 100, 200, 400, 600, 800, 1000, and 1200 µg/ml with 20.1 and 44.2 hour harvests, respectively.

Confirmatory Trial

In the 20.1 hour assay, very unhealthy cell monolayers, severe reductions in the number of visible mitotic cells, floating dead cells and debris, and ≈15% reduction in the cell monolayer confluence were observed in the cultures dosed with 1200 µg/ml. Unhealthy cell monolayers, floating dead cells and debris, and severe reductions in the number of visible mitotic cells were observed in the cultures dosed with 1000 µg/ml. Unhealthy cell monolayers, floating debris, and reductions in the number of visible mitotic cells were observed in the cultures dosed with 800 µg/ml. Slightly unhealthy cell monolayers, floating debris, and slight reductions in the number of visible mitotic cells were observed in the cultures dosed with 600 µg/ml. Chromosomal aberrations were analyzed from the cultures dosed with 200, 400, 600, and 800 µg/ml (Table 3). Reductions of 73%, 84%, and 95% were observed the mitotic indices of the cultures dosed with 800,

1000, and 1200 µg/ml, respectively, as compared with the solvent control cultures. No significant increases in cells with chromosomal aberrations were observed at the concentrations analyzed.

In the 44.2 hour assay, dead cell monolayers, no visible mitotic cells, floating dead cells and debris, and ≈45% reduction in the cell monolayer confluence were observed in the cultures dosed with 1000 and 1200 µg/ml. Very unhealthy cell monolayers, floating dead cells and debris, severe reductions in the number of visible mitotic cells, and ≈30% reduction in the cell monolayer confluence were observed in the cultures dosed with 800 µg/ml. Unhealthy cell monolayers, floating dead cells and debris, reductions in the number of visible mitotic cells, and ≈15% reduction in the cell monolayer confluence were observed in the cultures dosed with 600 µg/ml. Slightly unhealthy cell monolayers, floating debris, and slight reductions in the number of visible mitotic cells were observed in the cultures dosed with 400 µg/ml. Chromosomal aberrations were analyzed from the cultures dosed with 100, 200, 400, and 600 µg/ml (Table 4). Reductions of 12%, 61%, 63%, and 92% were observed the mitotic indices of the cultures dosed with 50.0, 400, 600, and 800 µg/ml, respectively, as compared with the solvent control cultures. No significant increases in cells with chromosomal aberrations or in polyploidy were observed at the concentrations analyzed.

The sensitivity of the cell culture for induction of chromosomal aberrations is shown by the increased frequency of aberrations in the cells exposed to mitomycin C, the positive control agent. The test article is considered negative for inducing chromosomal aberrations and polyploidy under nonactivation conditions.

11.5 Chromosomal Aberrations Assay With Metabolic Activation

Initial Trial

No cell monolayers and no visible mitotic cells were observed in the cultures dosed with 3000 and 4000 µg/ml. Slightly unhealthy cell monolayers and ≈30% reduction in the cell monolayer confluence were observed in the cultures dosed with 2000 µg/ml. A reduction of ≈15% in the cell monolayer confluence was observed in the cultures dosed with 1000 µg/ml. Chromosomal aberrations were analyzed from the cultures dosed with 250, 500, 1000, and 2000 µg/ml (Table 5). Reductions of 2% were observed in the mitotic indices of the cultures dosed with 250 µg/ml as compared with the solvent control cultures. No significant increases in cells with chromosomal aberrations were observed at the concentrations analyzed.

Based on the results from the initial trial, the confirmatory trial of the aberrations assay with metabolic activation was conducted testing concentrations of 500, 1000, 1500, 2000, 2250, 2500, 2750, and 3000 µg/ml with 20.1 and 44.2 hour harvests.

Confirmatory Trial

In the 20.1 hour assay, dead cell monolayers, floating dead cells and debris, no visible mitotic cells, and <5% cell monolayer confluence were observed in the cultures dosed with 2750 and 3000 µg/ml. Slightly unhealthy cell monolayers, reductions in the number of visible mitotic cells, floating dead cells and debris, and ≈45% reduction in the cell monolayer confluence were observed in the cultures dosed with 2500 µg/ml. Floating debris, slight reductions in the number of visible mitotic cells, and ≈30% reduction in the cell monolayer confluence were observed in the cultures dosed with 2250 µg/ml. Reductions of ≈15% in the cell monolayer confluence were observed in the cultures dosed with 2000 µg/ml. Chromosomal aberrations were analyzed from the cultures dosed with 1500, 2000, 2250, and 2500 µg/ml (Table 6). Reductions of 13%, 38%, 15%, 11%, and 30% were observed in the mitotic indices of the cultures dosed with 1000, 1500, 2000, 2250, and 2500 µg/ml, respectively, as compared with the solvent control cultures. No significant increases in cells with chromosomal aberrations were observed at the concentrations analyzed, except in the cultures dosed with 2500 µg/ml. In addition, an increase in endoreduplicated cells were observed in the cultures dosed with 2250 and 2500 µg/ml.

In the 44.2 hour assay, dead cell monolayers, floating dead cells and debris, no visible mitotic cells, and ≈95% reduction in the cell monolayer confluence were observed in the cultures dosed with 3000 µg/ml. Unhealthy cell monolayers, severe reductions in the number of visible mitotic cells, floating dead cells and debris, and ≈55% reduction in the cell monolayer confluence were observed in the cultures dosed with 2750 µg/ml. Slightly unhealthy cell monolayers, floating dead cells and debris, and ≈45% reduction in the cell monolayer confluence were observed in the cultures dosed with 2500 µg/ml. Reductions of ≈15% in the cell monolayer confluence were observed in the cultures dosed with 2250 µg/ml. Chromosomal aberrations were analyzed from the cultures dosed with 2000, 2250, 2500 and 2750 µg/ml (Table 7). Reductions of 14%, 17%, 56%, and 53% were observed in the mitotic indices of the cultures dosed with 1000, 2250, 2500, and 2750 µg/ml, respectively, as compared with the solvent control cultures. No significant increases in cells with chromosomal aberrations were observed at the concentrations analyzed, except in the cultures dosed with 2750 µg/ml. In addition, a significant increase in polyploidy was observed in the cultures dosed

with 2250, 2500, and 2750 µg/ml. The dose levels with increased endoreduplication was in the cultures dosed with 2250 and 2500 µg/ml in the 20.1 hour assay.

The successful activation by the metabolic system is illustrated by the increased incidence of cells with chromosomal aberrations in the cultures induced with cyclophosphamide, the positive control agent. The test article is considered negative for inducing chromosomal aberrations except at a single high dose that induced significant toxicity. In addition the test article is considered positive for inducing endoreduplication and polyploidy.

12.0 CONCLUSION:

T-6564 was considered negative for inducing chromosomal aberrations in CHO cells with and without metabolic activation except at a single dose level with metabolic activation that induced significant toxicity.

13.0 REFERENCES:

Armitage, P. Statistical Methods in Medical Research, John Wiley & Sons, Inc., New York, NY, 1971.

Evans, H.J.: Chromosomal aberrations produced by ionizing radiation. International Review of Cytology, 13:221-321, 1962.

Sokal, R.R., and Rohlf, F.J.: Biometry, Ed. 2, W.H. Freeman and Company, New York, 1981.

14.0 DEVIATION FROM THE SIGNED PROTOCOL:

Endoreduplication and polyploidy were analyzed separately since there were some dose levels with increased endoreduplication and some with polyploidy. This added greater scientific information to the study.

15.0 EXPERIMENTAL DATA TABLES

TABLE 1

RANGEFINDING ASSAY FOR ASSESSING TOXICITY

Assay No.: 17750 Trial No.: I Date: 06/13/96
Lab No.: CY6156

Compound: T-6564

Metabolic Activation: -S9

Treatment			% Mitotic Index	Confluence * % Solvent Control
NEGATIVE CONTROL	McCoy's 5a		2.5	100
SOLVENT CONTROL	Water	10.0 µl/ml	2.4	100
TEST ARTICLE		50.8 µg/ml	4.7	100
		169 µg/ml	4.9	85
		508 µg/ml	5.7	71
		1690 µg/ml	0.2	57

Metabolic Activation: +S9

Treatment			% Mitotic Index	Confluence * % Solvent Control
NEGATIVE CONTROL	McCoy's 5a		10.2	100
SOLVENT CONTROL	Water	10.0 µl/ml	10.9	100
TEST ARTICLE		169 µg/ml	6.1	100
		508 µg/ml	9.3	100
		1690 µg/ml	6.8	100

*This endpoint is based upon visual observations which are made prior to the harvest of the metaphase cells. Actual cell counts are not taken and any hypertrophy of the attached cells cannot be evaluated. At the time of the confluence observation the flasks are also evaluated for the appearance of floating mitotic cells and dead cells.
McCoy's 5a = culture medium

TABLE 2
CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS
Cells Fixed 20.0 Hours After Treatment

Assay No.: 17750 Trial #: 1 Date: 06/20/96 Lab #: CY6196 Metabolic Activation: -S9

Compound: T-6564

				NUMBER AND TYPE OF ABERRATION																	
					NOT COMPUTED			SIMPLE		COMPLEX								OTHER	# OF ABERRATIONS PER CELL	% CELLS WITH ABERRATIONS	% CELLS WITH >1 ABERRATIONS	% MITOTIC INDEX
			CELLS SCORED	TG	SG	UC	TB	SB	ID	TR	QR	CR	D	R	CI	DF	GT				
CONTROLS																					
NEGATIVE:	McCoy's 5a		A 100	2	1													0.00	0.0	0.0	5.3
			B 100	5	1			2										0.02	1.0	1.0	4.2
			A+B 200	7	2			2										0.01	0.5	0.5	4.8
SOLVENT:	Water	10.0 µl/ml	A 100															0.00	0.0	0.0	5.7
			B 100	4									1					0.01	1.0	0.0	4.8
			A+B 200	4									1					0.01	0.5	0.0	5.3
POSITIVE:	MMC	0.080 µg/ml	50	1	1		8	1	2		2				1			0.28	22.0*	4.0*	6.9
TEST ARTICLE		125 µg/ml	A 100					1					1					0.02	2.0	0.0	3.9
			B 100										1					0.01	1.0	0.0	4.1
			A+B 200					1					2					0.02	1.5	0.0	4.0
		250 µg/ml	A 100	2									1					0.01	1.0	0.0	2.7
			B 100	2														0.00	0.0	0.0	4.6
			A+B 200	4									1					0.01	0.5	0.0	3.7
		500 µg/ml	A 100		1													0.00	0.0	0.0	6.2
			B 100	3			1											0.01	1.0	0.0	5.6
			A+B 200	3	1		1											0.01	0.5	0.0	5.9
		1000 µg/ml	A 100	2									1					0.01	1.0	0.0	0.8
			B 100	2														0.00	0.0	0.0	0.5
			A+B 200	4									1					0.01	0.5	0.0	0.7
		1500 µg/ml**	A+B 0																		0.2

* Significantly greater than the solvent controls, $p<0.01$.
McCoy's 5a = culture medium MMC = Mitomycin C

** Chromosome aberrations not analyzed due to excessive toxicity.

TABLE 3
CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS
Cells Fixed 20.1Hours After Treatment

Assay No.: 17750 Trial #: 2 Date: 07/10/96 Lab #: CY7116 Metabolic Activation: -S9

Compound: T-6564

					NUMBER AND TYPE OF ABERRATION																	
					NOT COMPUTED			SIMPLE		COMPLEX								OTHER	# OF ABERRATIONS PER CELL	% CELLS WITH ABERRATIONS	% CELLS WITH >1 ABERRATIONS	% MITOTIC INDEX
				CELLS SCORED	TG	SG	UC	TB	SB	ID	TR	QR	CR	D	R	CI	DF	GT				
CONTROLS NEGATIVE:	McCoy's 5a		A	100	1			1											0.01	1.0	0.0	7.6
			B	100	2	1		1											0.01	1.0	0.0	8.7
			A+B	200	3	1		2											0.01	1.0	0.0	8.2
SOLVENT:	Water	10.0 µl/ml	A	100	2	1		1	1		1			1					0.04	2.0	1.0	4.3
			B	100		1													0.00	0.0	0.0	4.4
			A+B	200	2	2		1	1		1			1					0.02	1.0	0.5	4.4
POSITIVE:	MMC	0.080 µg/ml		50	2			4	4		5	7	1			1			0.44	28.0*	12.0*	3.1
TEST ARTICLE		100 µg/ml**	A+B	0																		6.4
		200 µg/ml	A	100	5									2					0.02	2.0	0.0	7.2
			B	100	6									1					0.01	1.0	0.0	5.7
			A+B	200	11									3					0.02	1.5	0.0	6.5
		400 µg/ml	A	100	4									2					0.02	2.0	0.0	8.7
			B	100	4	1													0.00	0.0	0.0	6.9
			A+B	200	8	1								2					0.01	1.0	0.0	7.8
		600 µg/ml	A	100	3														0.00	0.0	0.0	5.0
			B	100															0.00	0.0	0.0	5.2
			A+B	200	3														0.00	0.0	0.0	5.1
		800 µg/ml	A	100	3										1				0.01	1.0	0.0	1.0
			B	100	4	1													0.00	0.0	0.0	1.3
			A+B	200	7	1									1				0.01	0.5	0.0	1.2
		1000 µg/ml***	A+B	0																		0.7
		1200 µg/ml***	A+B	0																		0.2

* Significantly greater than the solvent controls, $p<0.01$.
** Chromosome aberrations not analyzed due to higher doses available for analysis.
*** Chromosome aberrations not analyzed due to excessive toxicity.
McCoy's 5a = culture medium MMC = Mitomycin C

TABLE 4
CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS
Cells Fixed 44.2 Hours After Treatment

Assay No.: 17750 Trial #: 2 Date: 07/10/96 Lab #: CY7116 Metabolic Activation: -S9

Compound: T-6564

					NUMBER AND TYPE OF ABERRATION																	
					NOT COMPUTED			SIMPLE		COMPLEX								OTHER	# OF ABERRATIONS PER CELL	% CELLS WITH ABERRATIONS	% CELLS WITH >1 ABERRATIONS	%[a] POLYPLOID CELLS	% MITOTIC INDEX
			CELLS SCORED		TG	SG	UC	TB	SB	ID	TR	QR	CR	D	R	CI	DF	GT					
CONTROLS NEGATIVE:	McCoy's 5a		A	100															0.00	0.0	0.0	0.0	6.9
			B	100	2														0.00	0.0	0.0	0.0	6.0
			A+B	200	2														0.00	0.0	0.0	0.0	6.5
SOLVENT:	Water	10.0 µl/ml	A	100	5		1							1					0.01	1.0	0.0	2.0	5.8
			B	100	3		1												0.00	0.0	0.0	0.0	4.4
			A+B	200	8		2							1					0.01	0.5	0.0	1.0	5.1
TEST ARTICLE		50.0 µg/ml*	A+B	0																			4.5
		100 µg/ml	A	100	1		1							1					0.01	1.0	0.0	3.0	3.7
			B	100	1														0.00	0.0	0.0	1.0	6.6
			A+B	200	2		1							1					0.01	0.5	0.0	2.0	5.2
		200 µg/ml	A	100	1														0.00	0.0	0.0	4.0	4.1
			B	100					1										0.01	1.0	0.0	3.0	6.1
			A+B	200	1				1										0.01	0.5	0.0	3.5	5.1
		400 µg/ml	A	100	1		1												0.00	0.0	0.0	0.0	2.6
			B	100															0.00	0.0	0.0	1.0	1.3
			A+B	200	1		1												0.00	0.0	0.0	0.5	2.0
		600 µg/ml	A	100	4						1								0.01	0.0	0.0	1.0	2.4
			B	100	2	1	3	1	1										0.02	2.0	0.0	0.0	1.4
			A+B	200	6	1	3	1	1		1								0.02	1.0	0.0	0.5	1.9
		800 µg/ml**	A+B	0																			0.4

* Chromosome aberrations not analyzed due to higher doses available for analysis.
McCoy's 5a = culture medium
[a]Includes polyploidy and endoreduplication

** Chromosome aberrations not analyzed due to excessive toxicity.

TABLE 5
CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS
Cells Fixed 20.0 Hours After Treatment

Assay No.: 17750 Trial #: 1 Date: 06/20/96 Lab #: CY6196 Metabolic Activation: +S9

Compound: T-6564

					NUMBER AND TYPE OF ABERRATION																		
					NOT COMPUTED			SIMPLE		COMPLEX								OTHER	# OF ABERRATIONS PER CELL	% CELLS WITH ABERRATIONS	% CELLS WITH >1 ABERRATIONS	% MITOTIC INDEX
				CELLS SCORED	TG	SG	UC	TB	SB	ID	TR	QR	CR	D	R	CI	DF	GT				
CONTROLS NEGATIVE:	McCoy's 5a		A 100		1	1													0.00	0.0	0.0	9.4
			B 100		5	1													0.00	0.0	0.0	10.1
			A+B 200		6	2													0.00	0.0	0.0	9.8
SOLVENT:	Water	10.0 µl/ml	A 100			2													0.00	0.0	0.0	8.2
			B 100		6									1					0.01	1.0	0.0	9.9
			A+B 200		6	2								1					0.01	0.5	0.0	9.1
POSITIVE:	CP	5.00 µg/ml	25		7			6	1	6	11	6	5			3			1.52	64.0*	40.0*	2.5
TEST ARTICLE		250 µg/ml	A 100		3														0.00	0.0	0.0	9.6
			B 100		3	1					1			1					0.02	2.0	0.0	8.2
			A+B 200		6	1					1			1					0.01	1.0	0.0	8.9
		500 µg/ml	A 100		6			1						1					0.02	2.0	0.0	8.6
			B 100			1					1								0.01	1.0	0.0	10.6
			A+B 200		6	1		1			1			1					0.02	1.5	0.0	9.6
		1000 µg/ml	A 100		3									1					0.01	1.0	0.0	9.8
			B 100																0.00	0.0	0.0	11.5
			A+B 200		3									1					0.01	0.5	0.0	10.7
		2000 µg/ml	A 100		2	1			1		3								0.04	3.0	1.0	8.5
			B 100		2	1													0.00	0.0	0.0	9.8
			A+B 200		4	2			1		3								0.02	1.5	0.5	9.2

* Significantly greater than the solvent controls, $p<0.01$.
McCoy's 5a = culture medium CP = Cyclophosphamide

TABLE 6
CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS
Cells Fixed 20.1 Hours After Treatment

Assay No.: 17750 Trial #: 2 Date: 07/10/96 Lab #: CY7116 Metabolic Activation: +S9

Compound: T-6564

				NUMBER AND TYPE OF ABERRATION																		
					NOT COMPUTED			SIMPLE		COMPLEX								OTHER	# OF ABERRATIONS PER CELL	% CELLS WITH ABERRATIONS	% CELLS WITH >1 ABERRATIONS	% ENDOREDUPLICATED CELLS	% MITOTIC INDEX
			CELLS SCORED	TG	SG	UC	TB	SB	ID	TR	QR	CR	D	R	CI	DF	GT					
CONTROLS NEGATIVE:	McCoy's 5a		A 100	3									1					0.01	1.0	0.0		8.5
			B 100	3			1											0.01	1.0	0.0		8.8
			A+B 200	6			1						1					0.01	1.0	0.0		8.7
SOLVENT:	Water	10.0 µl/ml	A 100	2									1					0.01	1.0	0.0		8.1
			B 100	2	1								1					0.01	1.0	0.0		7.7
			A+B 200	4	1								2					0.01	1.0	0.0		7.9
POSITIVE:	CP	5.00 µg/ml	25	4			4	2	1	1	4				1			0.52	28.0*	12.0*		3.0
TEST ARTICLE		1000 µg/ml**	A+B 0																			6.9
		1500 µg/ml	A 100	1														0.00	0.0	0.0		4.6
			B 100	2	2								1					0.01	1.0	0.0		5.2
			A+B 200	3	2								1					0.01	0.5	0.0		4.9
		2000 µg/ml	A 100	5									1	1				0.02	2.0	0.0		6.9
			B 100	1	1								1					0.01	1.0	0.0		6.5
			A+B 200	6	1								2	1				0.02	1.5	0.0		6.7
		2250 µg/ml	A 100	4					1	2	1		1		2			0.07	4.0	2.0	20.0	7.2
			B 100	2														0.00	0.0	0.0	16.0	6.7
			A+B 200	6					1	2	1		1		2			0.04	2.0	1.0	18.0*	7.0
		2500 µg/ml	A 100	7			4	4	4		1		2		3			0.18	10.0	5.0	32.0	7.3
			B 100	5	4		13	3	2	4	9		1		3			0.35	15.0	10.0	35.0	3.7
			A+B 200	12	4		17	7	6	4	10		3		6			0.27	12.5*	7.5*	33.5*	5.5

* Significantly greater than the solvent controls, $p<0.01$.
McCoy's 5a = culture medium CP = Cyclophosphamide

** Chromosome aberrations not analyzed due to higher doses available for analysis.
* Endoreduplication scored due to noticible increase in incidence.

TABLE 7
CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS
Cells Fixed 44.2 Hours After Treatment

Assay No.: 17750 Trial #: 2 Date: 07/10/96 Lab #: CY7116 Metabolic Activation: +S9

Compound: T-6564

				NUMBER AND TYPE OF ABERRATION																	
					NOT COMPUTED			SIMPLE		COMPLEX								OTHER	# OF ABERRATIONS PER CELL	% CELLS WITH ABERRATIONS	% CELLS WITH >1 ABERRATIONS	%[a] POLYPLOID CELLS	% MITOTIC INDEX
			CELLS SCORED	TG	SG	UC	TB	SB	ID	TR	QR	CR	D	R	CI	DF	GT					
CONTROLS NEGATIVE:	McCoy's 5a		A 100	3	1													0.00	0.0	0.0	0.0	7.0
			B 100	1														0.00	0.0	0.0	1.0	6.6
			A+B 200	4	1													0.00	0.0	0.0	0.5	6.8
SOLVENT:	Water	10.0 µl/ml	A 100		1		1											0.01	1.0	0.0	2.0	6.9
			B 100	1														0.00	0.0	0.0	1.0	5.8
			A+B 200	1	1		1											0.01	0.5	0.0	1.5	6.4
TEST ARTICLE		1000 µg/ml**	A+B 0																			5.5
		1500 µg/ml**	A+B 0																			7.0
		2000 µg/ml	A 100	1														0.00	0.0	0.0	4.0	7.6
			B 100	1	3		1											0.01	1.0	0.0	3.0	8.7
			A+B 200	2	3		1											0.01	0.5	0.0	3.5	8.2
		2250 µg/ml	A 100	3									1					0.01	1.0	0.0	7.0	6.2
			B 100	1	1			1										0.01	1.0	0.0	5.0	4.3
			A+B 200	4	1			1					1					0.01	1.0	0.0	6.0*	5.3
		2500 µg/ml	A 100	3	1								2			1		0.03	3.0	0.0	17.0	1.6
			B 100	1				1					2					0.03	3.0	0.0	14.0	4.0
			A+B 200	4	1			1					4			1		0.03	3.0	0.0	15.5*	2.8
		2750 µg/ml	A 81	6	1	1	2	6			1		4		4	2		0.23	13.6	4.9	46.0	2.8
			B 84	4	1	3		2	2				6	2				0.14	11.9	2.4	39.0	3.1
			A+B 165	10	2	4	2	8		2	1		10	2	4	2		0.19	12.7*	3.6*	42.5*	3.0

* Significantly greater than the solvent controls, $p<0.01$.
McCoy's 5a = culture medium
[a]Includes polyploidy and endoreduplication

** Chromosome aberrations not analyzed due to higher doses available for analysis.

TABLE 8

CONTROL DATA OF CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS
8/95 THROUGH 12/95

	Activation		# Of Aberrations Per Cell	% Of Cells With Aberrations	% Of Cells With >1 Aberrations	% Endore-duplicated Cells	% Poly-ploidy Cells
Negative Control							
	Without	MIN	0.00	0.0	0.0	0.0	0.0
		MAX	0.04	4.0	0.5	5.0	9.5
		AVG	0.012	1.04	0.01	0.19	1.93
		N	36	36	36	39	45
Solvent Control							
	Without	MIN	0.00	0.0	0.0	0.0	0.0
		MAX	0.05	5.0	0.5	2.0	10.0
		AVG	0.012	0.93	0.01	0.14	2.20
		N	36	36	36	39	45
Positive Control Mitomycin C	Without	MIN	0.24	20.0	0.0		
		MAX	2.96	84.0	60.0		
		AVG	0.770	39.70	16.52		
		N	27	27	27		
Negative Control							
	With	MIN	0.00	0.0	0.0	0.0	0.0
		MAX	0.03	2.5	1.0	3.0	12.5
		AVG	0.017	1.40	0.10	0.45	2.33
		N	31	31	31	28	30
Solvent Control							
	With	MIN	0.00	0.0	0.0	0.0	0.0
		MAX	0.12	7.0	4.0	4.0	13.0
		AVG	0.023	1.78	0.26	0.64	2.28
		N	29	29	29	28	30
Positive Control Cyclophosphamide	With	MIN	0.40	32.0	8.0		
		MAX	8.88	100.0	100.0		
		AVG	1.728	57.17	32.83		
		N	24	24	24		

16.0 DEFINITIONS OF CHROMOSOME ABERRATIONS FOR GIEMSA STAINED CELLS

NOT COMPUTED

TG	Chromatid gap:	("tid gap"). An achromatic (unstained) region in one chromatid, the size of which is equal to or smaller than the width of a chromatid. These are noted but not usually included in final totals of aberrations as they may not all be true breaks.
SG	Chromosome gap:	("isochromatid gap, IG"). Same as chromatid gap but at the same locus in both sister chromatids.
UC	Uncoiled chromosome:	Failure of chromatin packing. Probably not a true aberration.
PP	Polyploid cell:	A cell containing multiple copies of the haploid number (n) of chromosomes. Not counted in the cells scored for aberrations.
E	Endoreduplication:	4n cell in which separation of chromosome pairs has failed. Not counted in the cells scored for aberrations.

SIMPLE

TB	Chromatic break:	An achromatic region in one chromatid, larger than the width of a chromatid. The associated fragment may be partially or completely displaced.
SB	Chromosome break:	Chromosome has a clear break, forming an abnormal (deleted) chromosome with an acentric fragment that is dislocated. This classification now includes the acentric fragment (AF). The AF was different from the SB only in that it was not apparently related to any specific chromosome.
DM	"Double Minute " fragment:	These are small double dots, some of which are terminal deletions and some interstitial deletions and probably small rings. Their origins are not distinguishable.

COMPLEX

ID	Interstitial deletion:	Length of chromatid "cut out" from midregion of a chromatid resulting in a small fragment of ring lying beside a shortened chromatid of a gap in the chromatid.
TR	Triradial:	An exchange between two chromosomes, or one chromosome and an acentric fragment, which results in a three-armed configuration.
QR	Quadri radial:	As triradial, but resulting in a four-armed configuration.
D	Dicentric:	An exchange between two chromosomes which results in a chromosome with two centromeres. This is often associated with an acentric fragment in which case it is classified as DF.
DF		Dicentric with fragment.
TC	Tricentric:	An exchange between three chromosomes which results in a chromosome with three centromeres. Often associated with two to three AF. Such exchanges can involve many chromosomes and are named as follows:
QC	Quadricentric:	four centromeres, up to four AF
PC	Pentacentric:	five centromeres, up to five AF
HC	Hexacentric:	six centromeres, up to six AF
R	Ring	A chromosome which forms a circle containing a centromere. This is often associated with an acentric fragment in which case it is classed as RF.
RC	Ring Chromatid:	Single chromatid ring (acentric).
RF		Ring with associated acentric fragment.
CI	Chromosome Intrachange:	Exchange within a chromosome; e.g., a ring that does not include the entire chromosome.

T	Translocation:	Obvious transfer of material between two chromosomes resulting in two abnormal chromosomes. When identifiable, scored as "T" not "2Ab."
AB		Abnormal monocentric chromosome. This is a chromosome whose morphology is abnormal for the karyotype, and often the result of a translocation, pericentric inversion, etc. Classification used if abnormality cannot be ascribed to; e.g., a reciprocal translocation.
Other		
GT/>		A cell which contains more than 10 aberrations. A heavily damaged cell should be analyzed to identify the types of aberrations and may not actually have >10, e.g., multiple fragments such as those found associated with a tricentric.