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Analysis of Anaphase in Cell Culture: An Adequate Test System for the Distinction between Compounds Which Selectively Alter the Chromosome Structure or the Mitotic Apparatus

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A system of anaphase analysis is presented as an adequate test to detect aberrations in chromosome structure and alterations in the mitotic apparatus. Particular emphasis is placed on those data which suggest that proteins may be the preferred targets of chemical compounds that induce aneuploidy.

Introduction

It has been reported that anaphase analysis is less sensitive in the detection of chromosome rearrangements than the cytogenetic examination of metaphase (*1-3*); however, our results show that this system has a high degree of sensitivity in the identification of compounds that induce not only chromosomal lesions but also those alterations which lead to defective chromosomal segregation. It is even possible to determine in the same material the percent of polynucleated cells, a useful indicator of chromosome damage (4). This system of anaphase analysis also has the advantages in comparison with the study of metaphase of being more rapid and economic and of not requiring previous experience in cytogenetics.

The types and possible origin of the anomalies detected in this system are bridges (dicentric chromosomes); lag chromosomes (centromere loss, centromere malfunction, microtubule alterations); sticky chromosomes (telomeric region damage, modification of chromosomal proteins); multipolar spindle (centriole anomalies, microtubule modifications).

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It has been shown that compounds that act directly on DNA can produce dicentric chromosomes, acentric chromosomes, or the loss of the telomere, which results respectively in the formation of bridges, lag chromosomes, and sticky chromosomes (*5-10*). The interaction of chemical agents with chromosomal proteins or with the mitotic apparatus proteins also appear to contribute to the induction of lag chromosomes, sticky chromosomes, and the formation of multipolar spindles.

Characteristics of the Test

The biological material employed to carry out this test was the Chinese hamster ovary (CHO) cell line; as it is easy to manipulate and to synchronize, can grow indefinitely, has high plating efficiency, a short generation time (12 hr), a relatively stable modal chromosomal number ($2n=22$), β-glucoronidase and aryl sulfatase activity (*11*), but does not have inducible microsomal enzymes.

The experimental method used is the following. The cells maintained in T_{75} flasks, in logarithmic growth with MacCoy's modified medium containing 10% fetal calf serum, are rinsed with PBS (free of Ca^{2+} and Mg^{2+}) and a 1% solution of trypsin is added in order to detach the cells. A cellular suspension is made with 1×10^6 cells/ml (T_{75} confluent flasks have

around 10-14 × 10^6 cells).

The suspended cells are plated on coverslips which had been placed in sterile 60 mm Petri dishes; the number of cells, depend on the size of the coverslips (20 × 20 mm or 20 × 30 mm) and vary between 2.5 and 5.0 × 10^5 cells/0.5 ml to give a semiconfluent culture. The number of Petri dishes is conditioned to the range of doses to be tested and the number of anaphases to be analyzed. The cells are left to rest for approximately 15 min to allow the cells to attach to the surface of the coverslips. Once the cells have adhered to the glass the compound under study, dissolved in 0.2 ml PBS (free of Ca^{2+} and Mg^{2+}), is added with medium to obtain a final volume of 5 ml. The Petri dishes are divided into groups as follows: (a) controls (two dishes) to which only buffer is added; (b) unknowns (two dishes/dose) that receive the drug in PBS or alone (in logarithmic or geometric progressions of concentrations); (c) in the case of drugs that must be metabolized, body fluids (urine) from treated mice, or the drug in the presence of liver homogenate are used. For last, other controls are needed as normal urine (from untreated mice) or liver homogenate without the drug. The Petri dishes are incubated for about 24 hr at 37°C in a humid atmosphere containing 5% of CO_2. All the experiments are done in duplicate.

Periodic microscopic examination is carried out to determine the optimum time for fixation with adequate number anaphases. The cells are fixed for 15 min by the direct addition of 1 ml of acetic acid to the culture medium; thereafter this medium is removed and 5 ml fixative (acetic acid; methanol, 1:3) is added, after which the cells are stained with 2% of acetic-orcein, washed with destilled water and mounted.

Five hundred anaphases are analyzed per dose and cytotoxic effects are measured indirectly in 2000 cells by means of the mitotic index for each experiment. The type of anomalies detected in the anaphase system are shown in Figure 1.

CHO cells have approximately 12.97% ± 3.88 of spontaneous abnormalities. The relative proportions of each type on the population are: bridges, 4.27 ± 1.19%; sticky chromosomes, 0.80 ± 0.40%; chromosome lag, 5.57 ± 1.57%; multipolar spindles, 2.27 ± 1.61%.

Anaphase Anomalies Induced by Chemical Agents

Table 1 shows the results of the studies carried out on a group of chemicals tested in this system in the laboratory of M. Legator (UTMB Galveston Texas). These compounds were divided into alkylating, intercalating, and anesthetic agents. Several other compounds were selected for its study at the University of Mexico. They include: ethylene glycol bis(2-aminoethyl ether-*N,N'*-tetraacetic acid) (EGTA) chosen as a chelating agent of Ca^{2+}, PCMB for its capacity to block thiol groups, glycerol to compare its effects with those of DMSO, KCl for its membrane depolarizing capacity, and H_2O to study the effect of osmolarity changes.

Of the alkylating and intercalating agents only triethylene melamine (TEM) and acetoxy-acetylaminofluorene (acetoxy-AAF), which do not have sulfur- or phosphorus-containing functional groups, did not produce a notable increase in multipolar spindles. This suggests that this type of anomaly, observed with the other compounds, was due fundamentally to the action of electronegative groups such as sulfonates, sulfoxides and phosphamides.

The difference between the effects of volatile and nonvolatile anesthetic chemicals, indicate that both volatility and solubility, as reported by Östergren (*12, 13*) for the production of c-mitosis), are important factors in the induction of mitotic aberrations. As is seen from Table 1, there is a great increase in chromosome lag and multipolar spindles with highly volatile compounds such as CCl_4, (DMSO), or tetrakis(hydroxymethyl)phosphonium sulfate (THPS) in comparison with the effects induced by nonvolatile compounds such as methadone and naltraxone.

The presence of sticky chromosomes has been associated with the action of compounds having intercalating capacity (*9, 10*). We tested only one compound of this type, acetoxy-AAF, and the results obtained appear to support this hypothesis. In the case of Hycanthone, which has both alkylating and intercalating activities (M. Legator, personal communication), a high increase of sticky chromosomes was also observed.

It has been reported that exposure to alkylating agents leads to chromosomal breaks which produce bridges and chromosome lag during anaphase (*5, 6, 8*). In our study, this association was observed principally with TEM. The other alkylating agents, ethyl methanesulfonate (EMS), Hycanthone, and Cytoxan, which possess electronegative groups, as mentioned, produce in addition to chromosome lag and bridges, multipolar spindles and sticky chromosomes. This action has been considered to be brought about by the effect of the drugs on the thiol (-SH) groups of the chromosomal proteins and other proteins that participate in mitosis, as these groups behave as nucleophilic centers like DNA purines and pyrimidines.

A linear dose effect was found for the alkylating and intercalating compounds (L. E. Coutiño and

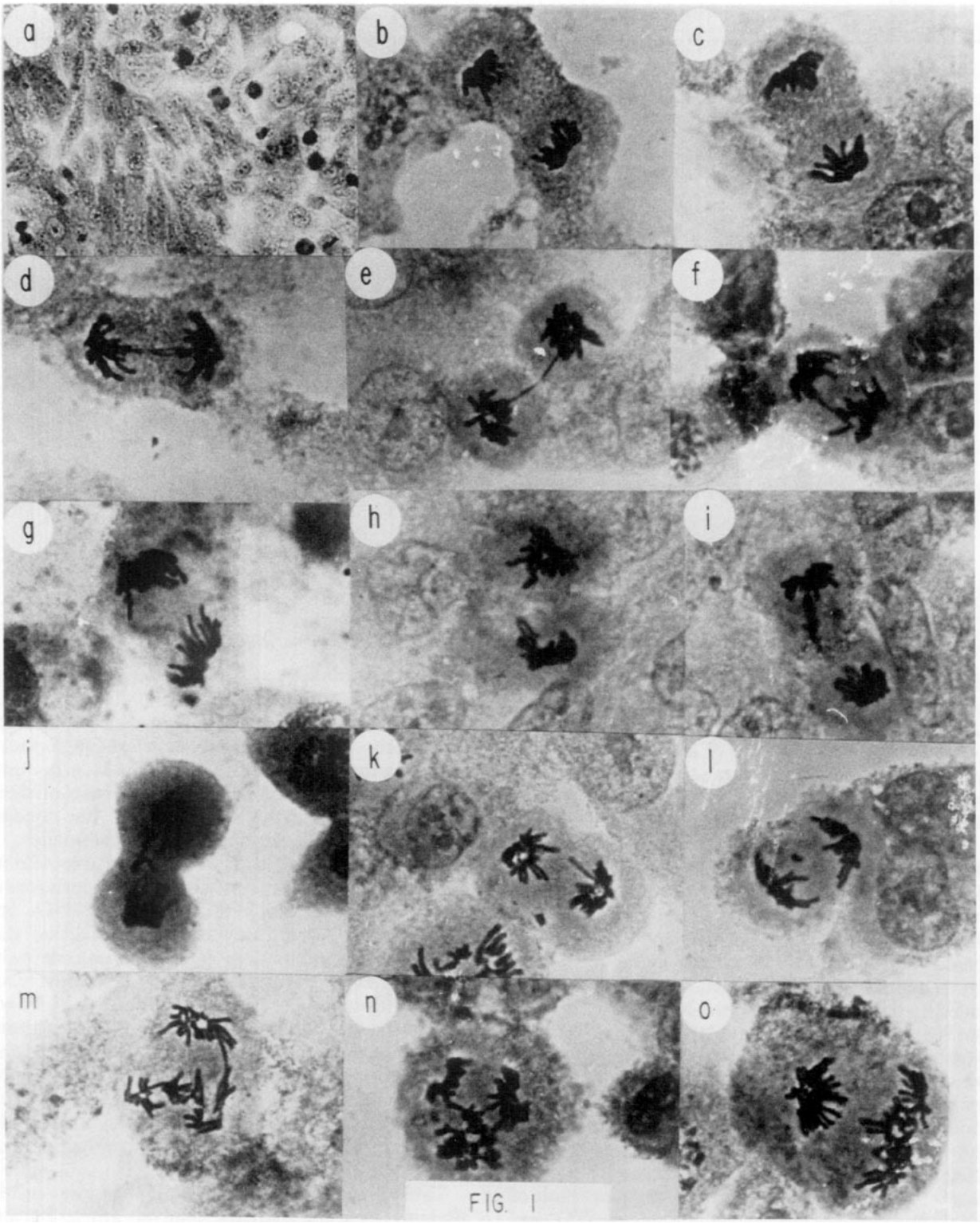

FIGURE 1. Anomalies detected in untreated cells: (*a*) general appearance of the preparations and the different types of anaphases considered; (*b*, *c*) normal anaphases, (*d-o*) abnormal anaphases characterized by the presence of (*d-f*) bridges, (*g-i*) sticky chromosomes, (*j-l*) chromosomal lag, and (*m-o*) multipolar spindles.

Table 1. Comparative frequencies of anomalies detected in anaphase analysis.

Type of drug	mole/e	Ratio of anomalies to control				
		Sticky chromosomes	Bridges	Lag chromosomes	Multipolar spindles	Mitotic index C/T
Alkylating agents						
EMS	4×10^{-4}	3.2	4.5	3.8	5.0	0.4
TEM	1×10^{-6}	3.0	5.0	7.3	1.1	0.12
Hycanthone	1.9×10^{-5}	6.5	5.1	7.6	5.3	0.39
Cytoxan[a] metabolite	N.D.	5.0	4.5	6.3	4.0	0.46
Intercalating agents						
Acetoxyacetylaminofluorene	6.3×10^{-6}	4.5	2.1	1.9	1.0	0.7
Anesthetic agents						
Volatile						
CCl_4	5 μl/ml	2.0	1.7	5.8	6.1	0.75
DMSO	2.5 μl/ml	1.3	1.5	3.7	2.5	1.2
THPS	2.4×10^{-4}	6.6	3.5	9.0	2.3	0.24
Nonvolatile						
Methadone	0.85×10^{-4}	1.3	1.8	1.4	2.0	0.72
Naltraxone	1.6×10^{-4}	1.5	1.6	2.4	1.75	0.62
Others						
EGTA	1×10^{-4}	4.6	2.3	3.5	1.3	0.89
PCMB	2×10^{-5}	3.6	1.67	2.7	1.21	0.74
Glycerol	16 μl; 1 ml	1.6	1.3	2.3	1.39	1.01
KCl	2×10^{-3}	1.3	1.65	2.0	1.85	1.45
H_2O	400 μ/ml	1.5	1.71	2.3	1.0	1.21

[a]Dose that produced the highest frequency of anomalies.
[b]Urine collected during 24 hr after the last dose from animals treated with 80 ml/kl of cytoxan (IP) for 4 days.

M. Legator, in preparation), and a correlation seems to exist between the genetic damage and the cytotoxic effect caused by these compounds, measured by the mitotic index. On the other hand most of the anesthetic drugs, at concentrations which produced missegregation, did not have a cytotoxic effect and also some of them induced mitosis.

The results obtained with the other group of chemicals indicate that: (a) the appearance of sticky chromosomes, bridges and chromosome lag, in cells treated with EGTA, could be associated to Ca^{2+} chelation; (b) the thiol 1-chloromercuriphenylsulfonic acid (PCMB) may be related to the increase in the frequency of sticky and lag chromosomes produced by that agent; (c) glycerol was less active than DMSO and behaves as nonvolatile anesthetics; this property could be due to the alcohol functional groups (*14*). Depolarization of the cell membrane could play a role in anaphase anomalies, as KCl produced preferentially a moderate increase in chromosome lag and multipolar spindles. Similar effects had been associated with exposure to nonvolatile anesthetics and considered to be a result of membrane depolarization, calcium release (*15*), and alteration of microtubule polymerization (*16*). Calcium protein regulators and the blocking of free thiol groups of tubuline may also be involved in this process (*17-19*). Changes in osmolarity induced a weak increase in almost all the anomalies considered, perhaps as a result of the dilution of the components needed for chromosomal segregation or by high hydrostatic pressure (*20-22*). It has been reported that hypotonic treatments can also induce multinucleated cells (*23*) and c-mitosis (*7*). None of these compounds had cytotoxic effects at the concentrations that produced chromosomal anomalies.

The substances that gave a multipolar spindle increase, were mainly the anesthetics and derivates of sulfonic acid such as THPS, EMS, DMSO, and Hycanthone. There is evidence that indicate that derivates of sulfonic acid affect microtubule polymerization *in vitro* (*24*). This effect could be a result of their electronegativity that contribute to hydrogen bond formation with free thiol groups of the mitotic apparatus, or from the cell membrane. It seems also that the induction of multipolar spindles by anesthetic compounds is mediated by the cell membrane, this suggests that membranes could play a role in the formation and polarity of the mitotic apparatus (*25-27*).

Table 2 shows a comparison between the results obtained by using anaphase analysis and those generated on other test systems (*28-33*) in the case of alkylating and intercalating agents, there is a high degree of agreement among the results of different tests, in that nearly all gave positive results. On the

Table 2. Results showing the sensitivity of anaphase analysis in comparison with other studies.

Type of compound	Activity[a]				
	Anaphase test	Carcinogenic activity[b]	*Salmonella typhimurium*[c]	Cytogenetic studies[c]	Nondisjunction (Drosophila)
Alkylating agents					
EMS	+	+	+	+	+
TEM	+	+	+	+	ND
Hycanthone	+	+	+	+	+
Cytoxan metabolites	+	+	+	+	+
Intercalating agents					
Acetoxy-AAF	+	+	+	+	ND
Anesthetic agents					
Volatile					
CCl_4	+	+	−	−	ND
DMSO	+	−	−	−	ND
THPS	+	ND	−	−	ND
Nonvolatile					
Naltroxone	±	ND	−	−	+
Methadone	±	ND	−	−	+
Others					
PCMB	±	ND	ND	−	ND
EGTA	+	?	ND	+	ND
Glycerol	±	ND	−	−	ND

[a](+) high activity; (±) moderate activity; (−) negative; (ND) not determined.
[b]From a revision made by S. Rinkus.
[c]Data from the laboratory of Dr. Marvin Legator, Dept. of Preventive Medicine and Community Health UTMB. Galveston, Texas 77550, provided by T. Connors, J. Mayne, Dr. S. Zimmering.

contrary, anesthetic compounds were not mutagenic in *Salmonella typhimurium*. A possible explanation of this might be that the anesthetics do not act directly on DNA but on the various proteins involved in cellular division. Since bacteria do not have a mitotic apparatus for the segregation of their genetic material and also do not have chromosomal proteins, it seems quite logical that tests involving bacteria would not be sensitive to chemical agents affecting either chromosomal proteins or the mitotic spindle.

Conclusion

In summary, anaphase analysis has permitted the identification of mutagen which act not only at the chromosome level but also on the mitotic apparatus. The number of compounds studied is small and must be increased in order to validate the results of this test. It is also necessary to corroborate this results in other systems which detect defective chromosomal segregation and to determine if the chromosomal lag and the alterations in the mitotic spindle observed with the anesthetics lead to aneuploidy.

Epidemiological studies done in individuals exposed to anesthetics such as halothene or to chloropropene and vinyl chloride (which also have anesthetic properties) showed that such exposure elevates the incidence of spontaneous abortions by their wives (*34*). This suggests genetic damage to the germ cells, and it remains to be demonstrated if this is a consequence of aneuploidy, which would give a prognostic value to the *in vitro* results obtained with compounds having anesthetic properties. It is also important to remark that other chemicals implied in human pathology (*35*) such as euphorizants, tranquilizers, or hormones, behave as anesthetics (*14*). Several reports had shown that compounds as azaserine, organomercurials, nitrosoureas, divalent cations (As, Hg), antimitotic drugs such as benzimidazoles or malic hydrazine, can bind to free thiol groups or cysteine residues of several proteins as DNA repair enzymes, kinases, glutathione reductase, tubulin etc. modifying their activity (*22, 36-48*). All these data give strong support to the idea that proteins could also be targets for compounds that induce genetic damage.

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