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Single Cell Gel/Comet Assay: Guidelines for In Vitro and In Vivo Genetic Toxicology Testing

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At the International Workshop on Genotoxicity Test Procedures (IWGTP) held in Washington, DC, March 25–26, 1999, an expert panel met to develop guidelines for the use of the single-cell gel (SCG)/Comet assay in genetic toxicology. The expert panel reached a consensus that the optimal version of the Comet assay for identifying agents with genotoxic activity was the alkaline (pH > 13) version of the assay developed by Singh et al. [1988]. The pH > 13 version is capable of detecting DNA single-strand breaks (SSB), alkali-labile sites (ALS), DNA-DNA/DNA-protein cross-linking, and SSB associated with incomplete excision repair sites. Relative to other genotoxicity tests, the advantages of the SCG assay include its demonstrated sensitivity for detecting low levels of DNA damage, the requirement for small numbers of cells per sample, its flexibility, its low costs, its ease of application, and the short time needed to complete a study. The expert panel decided that no single version of the alkaline (pH > 13) Comet assay was clearly superior. However, critical technical steps within the assay were discussed and guidelines developed for preparing slides with agarose gels, lysing cells to liberate DNA, exposing the liberated DNA to alkali to produce single-stranded DNA and to express ALS as SSB, electrophoresing the DNA using pH > 13 alkaline conditions, alkali neutralization, DNA staining, comet visualization, and data collection. Based on the current state of knowledge, the expert panel developed guidelines for conducting in vitro or in vivo Comet assays. The goal of the expert panel was to identify minimal standards for obtaining reproducible and reliable Comet data deemed suitable for regulatory submission. The expert panel used the current Organization for Economic Co-operation and Development (OECD) guidelines for in vitro and in vivo genetic toxicological studies as guides during the development of the corresponding in vitro and in vivo SCG assay guidelines. Guideline topics considered included initial considerations, principles of the test method, description of the test method, procedure, results, data analysis and reporting. Special consideration was given by the expert panel to the potential adverse effect of DNA degradation associated with cytotoxicity on the interpretation of Comet assay results. The expert panel also discussed related SCG methodologies that might be useful in the interpretation of positive Comet data. The related methodologies discussed included: (1) the use of different pH conditions during electrophoreses to discriminate between DNA strand breaks and ALS; (2) the use of repair enzymes or antibodies to detect specific classes of DNA damage; (3) the use of a neutral diffusion assay to identify apoptotic/necrotic cells; and (4) the use of the acellular SCG assay to evaluate the ability of a test substance to interact directly with DNA. The alkaline (pH > 13) Comet assay guidelines developed by the expert panel represent a work in progress. Additional information is needed before the assay can be critically evaluated for its utility in genetic toxicology. The information needed includes comprehensive data on the different sources of variability (e.g., cell to cell, gel to gel, run to run, culture to culture, animal to animal, experiment to experiment) intrinsic to the alkaline (pH > 3) SCG assay, the generation of a large database based on in vitro

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and in vivo testing using these guidelines, and the results of appropriately designed multilaboratory international validation studies. Environ. Mol. Mutagen. 35:206–221, 2000. © 2000 Wiley-Liss, Inc.

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INTRODUCTION

A number of techniques for detecting DNA damage, as opposed to the biological effects (e.g., micronuclei, mutations, structural chromosomal aberrations) that result from DNA damage, have been used to identify substances with genotoxic activity. Until recently, the most frequently used methods involved either the detection of DNA repair synthesis (so-called unscheduled DNA synthesis or UDS) in individual cells, or the detection of DNA SSB and ALS in pooled cell populations using the alkaline elution assay. The UDS technique is based on the replication of DNA during the excision repair of certain types of DNA lesions, as demonstrated by the incorporation of tritiated thymidine into the DNA repair sites. While providing information at the level of the individual cell, the technique is technically cumbersome, requires the use of radioactivity, and is limited in sensitivity. The alkaline elution assay ignores the critical importance of intercellular differences in DNA damage and requires relatively large numbers of cells. A more useful approach for assessing DNA damage is the single-cell gel (SCG) or Comet assay. The terms "SCG" or "Comet" are used interchangeable throughout this report; the term "comet" is used to identify the individual cell DNA migration patterns produced by this assay (Fig. 1).

Östling and Johanson [1984] were the first to develop a microgel electrophoresis technique for detecting DNA damage at the level of the single cell. In their technique, cells embedded in agarose were placed on a microscope slide, the cells were lysed by detergents and high salt, and the liberated DNA electrophoresed under neutral conditions. Cells with an increased frequency of DNA double-strand breaks (DSB) displayed increased migration of DNA toward the anode. The migrating DNA was quantitated by staining with ethidium bromide and by measuring the intensity of fluorescence at two fixed positions within the migration pattern using a microscope photometer. The neutral conditions used greatly limited the general utility of the assay.

Subsequently, Singh et al. [1988] introduced a microgel technique involving electrophoresis under alkaline (pH $>$ 13) conditions for detecting DNA damage in single cells. At this pH, increased DNA migration is associated with increased levels of frank SSB, SSB associated with incomplete excision repair sites, and ALS. Because almost all genotoxic agents induce orders of magnitude more SSB and/or ALS than DSB, this version of the assay offered greatly increased sensitivity for identifying genotoxic agents. Two years later, Olive et al. (1990a) introduced another alkaline version of this assay in which DNA is electrophoresed at a pH of ~12.3. Since the introduction of the alkaline (pH $>$ 13) Comet assay in 1988, the breadth of applications and the number of investigators using this technique have increased almost exponentially. Compared with other genotoxicity assays, the advantages of the technique include: (1) its demonstrated sensitivity for detecting low levels of DNA damage; (2) the requirement for small numbers of cells per sample; (3) flexibility; (4) low costs; (5) ease of application; (6) the ability to conduct studies using relatively small amounts of a test substance; and (7) the relatively short time period (a few days) needed to complete an experiment. During the last decade, this assay has developed into a basic tool for use by investigators interested in research areas ranging from human and environmental biomonitoring to DNA repair processes to genetic toxicology. General reviews on this technique that have been published include Tice et al. [1991], McKelvey-Martin et al. [1993], Tice [1995], Fairbairn et al. [1995], Anderson et al. [1998], Rojas et al. [1999], and Speit and Hartmann [1999]. Unless otherwise noted, information supporting the conclusions of the expert panel can be found in these reviews.

Attractive uses of this assay in genetic toxicology include: (1) as a potentially high-throughput screening assay; (2) in mechanistic studies to distinguish between genotoxicity versus cytotoxicity induced chromosomal damage; (3) in mechanistic in vivo studies to distinguish between genotoxic versus non-genotoxic carcinogens; and (4) potentially, as part of a battery of in vitro/in vivo assays used for regulatory submissions. However, the SCG assay has yet to undergo appropriate multilaboratory, international validation studies to demonstrate its interlaboratory and intralaboratory reproducibility and reliability and the adequacy of its performance against currently accepted methods [see ICCVAM, 1997]. As a test for genotoxicity, the Comet assay can be used to identify possible human mutagens and carcinogens [Anderson et al., 1998]. However, a perfect correlation between chemicals positive in this test and carcinogenicity is not expected. The correlation would be expected to depend on chemical class and on the mechanism of carcinogenicity involved.

Based on the current state of knowledge, the expert panel reached a consensus as to the most appropriate methodology to use when applying this assay to an in vitro or in vivo evaluation of genotoxic activity. The expert panel identified the minimal experimental and methodological standards needed to ensure that the results of Comet studies would be accepted as valid by knowledgeable scientists and by regulatory agencies. Due to the considerable variability in SCG

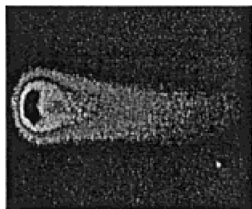

Fig. 1. Cell DNA migration pattern produced by the Single Cell Gel/Comet assay.

methodology used by different scientists and because of the lack of robust data for some technical aspects of the assay, the expert panel erred on the side of caution in establishing these minimal conditions. In the future, it should be possible to better define these minimal standards using the results of appropriate multilaboratory, international validation studies and based on greater information on chemical/product class-specific responses.

The first consensus decision of the expert panel was that, in terms of a testing strategy for genetic toxicology, the alkaline (pH > 13) version of the Comet assay is the methodology of choice. Generally, DNA is denatured and unwound at pH values above 12.0 because of the disruption of hydrogen bonds between double-stranded DNA [Kohn, 1991]. At pH conditions of 12.6 or higher, ALS (e.g., apurinic sites) are quickly transformed to strand breaks [Kohn, 1991]. A pH of > 13 would be expected to maximize the expression of ALS as SSB. Preference for the pH > 13 Comet assay does not mean that positive data obtained using other versions of the assay are not acceptable for identifying genotoxic agents. However, negative data may need to be considered with more caution. There was no attempt to identify the optimal methods to use when using the Comet assay for any other purpose (e.g., human biomonitoring), although the guidelines established by the expert panel may be useful in developing relevant guidelines.

METHODOLOGY

All methodological steps associated with the alkaline (pH > 13) SCG assay are equally important for obtaining reproducible and reliable results. In general, best results are obtained if sample processing, solution preparation and usage, and equipment utilization and maintenance are conducted using the strict quality control (QC) criteria considered appropriate for techniques in molecular biology. Once a suspension of cells is obtained, the basic steps of the assay (Fig. 2) include (1) preparation of microscope slides layered with cells in agarose; (2) lysis of cells to liberate DNA; (3) exposure to alkali (pH > 13) to obtain single-stranded DNA and to express ALS as SSB; (4) electrophoresis under alkaline (pH > 13) conditions; (5) neutralization of alkali; (6) DNA staining and comet visualization; and (7) comet scoring. There is no single correct method for conducting these steps; however, the expert panel agreed on general guidance.

Slide Preparation

The ultimate goal of slide preparation is to obtain uniform gels sufficiently stable to survive through to data collection, as well as to ensure easily visualized comets with minimal background noise. A number of different techniques have been used to prepare Comet slides. Generally, but not exclusively, microscope slides are used, with each slide containing one or two independent gels. Initially, fully frosted slides were used most commonly because they offered increased gel bonding and thus stability. However, within the last few years, either conventional microscope slides [Klaude et al., 1996] or slides specifically modified to increase gel stability have been used increasingly. The number of agarose layers used per gel range from one to three. In the single-layer procedure, cells are suspended in low-melting point (LMP) agarose (generally at 37°C) and placed directly on a slide. In the three-layer procedure, the cells contained in agarose are placed on a slide precoated with a layer of regular agarose. After adding the cell-containing layer, another layer of LMP agarose is added to fill in any residual holes in the second agarose layer and to increase the distance between cells and the gel surface. In the two-layer method, the top layer of LMP agarose in the three-layer method is omitted. An appropriately sized coverslip is used to flatten out each molten agarose layer, and the slides are often chilled during the process to enhance gelling of the agarose. Precoated slides developed specifically for this assay can be obtained commercially. One generally successful method for generating stable gels is to dip conventional microscope slides (avoiding slides designated as super clean) in molten regular agarose (generally 1%–1.5%), and then allowing the gel to dry at room temperature or by a brief exposure to 40°–50°C. Within a few days, the second cell-containing layer of agarose (generally 0.5% to 1.0%) is then added, followed by a third layer of agarose at the same concentration. The concentration of cells in agarose, as well as the concentration of agarose, are important parameters for ensuring a successful analysis. The optimal number of cells (at least for image analysis) is typically not more than a few per visual field. Higher cell densities can result in a significant proportion of overlapping comets, especially at high levels of DNA migration. Higher agarose concentrations can affect the extent of DNA migration, as well as the accessibility of the DNA to other manipulations (see section on Related Methodologies).

Lysis

After the agarose gel has solidified, the slides are placed, generally for at least 1 hr, in a lysis solution consisting of high salts and detergents. The original lysing solution de-

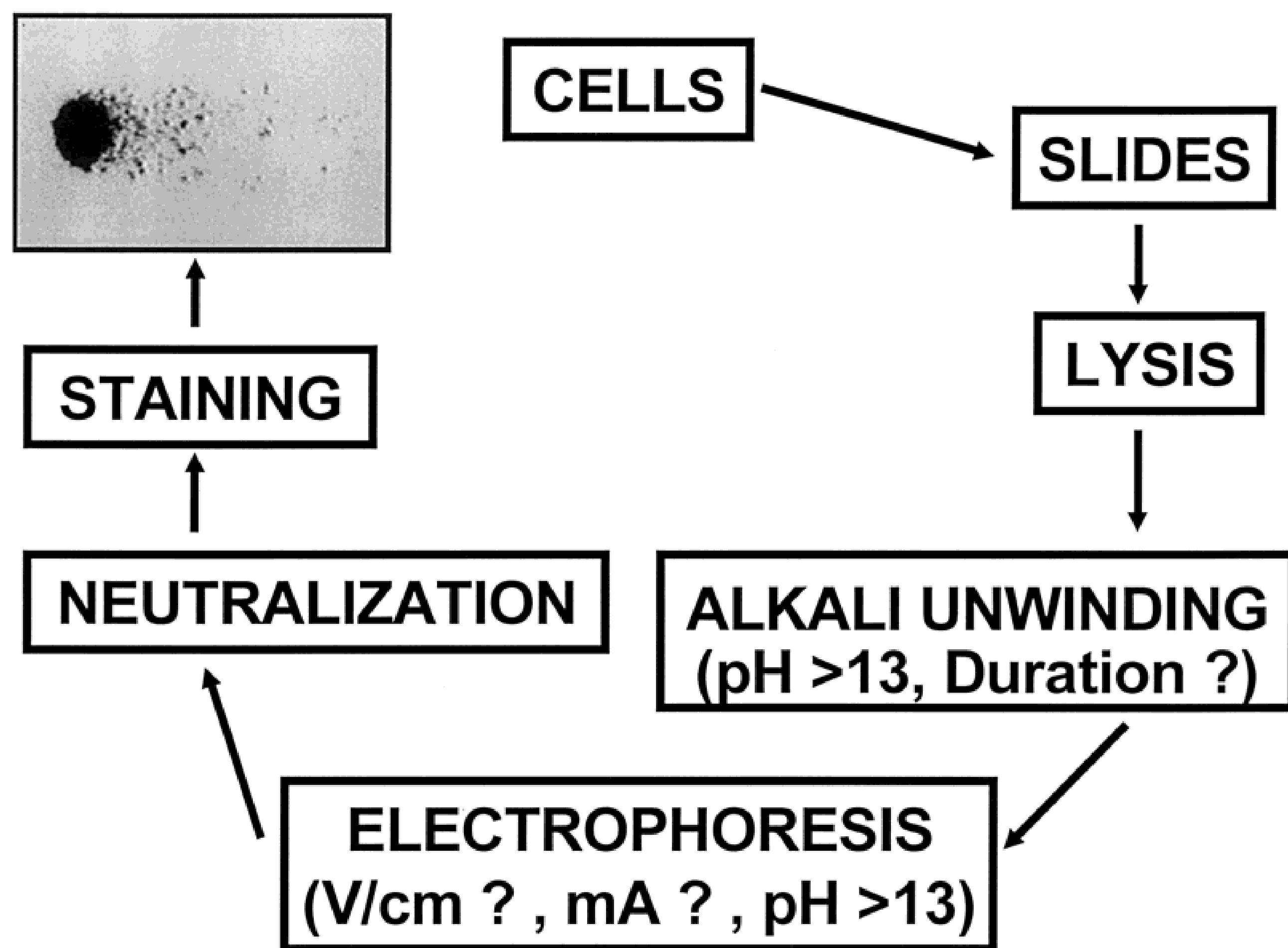

Fig. 2. Schematic representation of critical steps in the alkaline (pH > 13) Comet assay.

veloped by Singh et al. [1988] consisted of 100 mM ethylenediaminetetraacetic acid (EDTA), 2.5 M sodium chloride, 1% N-lauroylsarcosine, 10 mM Trizma base, adjusted to pH 10.0, with 1% Triton X-100 added just prior to use. Subsequently, Tice et al. [1991] added 10% dimethyl sulfoxide (DMSO) to the lysing solution to prevent radical-induced DNA damage associated with the iron released during lysis from erythrocytes present in blood and tissue samples. More recently, McKelvey-Martin et al. [1993] have reported that N-laurylsarcosine is not needed in the lysing solution (i.e., that the inclusion of Triton X-100 is sufficient for cell lysis). However, some cell types may require the presence of the second detergent for lysis to be complete. This may need to be evaluated on a case by case basis. The lysing solution is chilled prior to use, primarily to maintain the stability of the agarose gel. The lysis duration used by different investigators varies considerable, from less than 1 hr to weeks if not months. There is a minimal time needed to appropriately liberate the DNA and this time might vary depending on the cell type. The maximal time slides can be stored in lysis without resulting in an increased background frequency of DNA damage or in the loss of low molecular weight DNA from the gel due to diffusion depends on the technical conditions used by an individual laboratory. At the end of the lysing period, slides are incubated in alkali prior to electrophoresis. It is expected that rinsing the gels to remove residual detergents and salts prior to alkali unwinding would increase assay reproducibility, but formal studies to evaluate this possibility have not been conducted. Between lysis and alkali unwinding, the liberated DNA can be incubated with proteinase K (PK) to remove residual protein or probed with DNA repair enzymes/antibodies to identify specific classes of DNA damage (i.e., oxidative) (see section on Related Methodologies).

Alkali (pH > 13) Unwinding

Prior to electrophoresis, the slides are incubated in alkaline (pH > 13) electrophoresis buffer to produce single-stranded DNA and to express ALS as SSB. The alkaline solution developed by Singh et al. [1988] consists of 1 mM EDTA and 300 mM sodium hydroxide, pH > 13.0. This solution is still used most frequently in Comet studies,

because it maximizes the expression of ALS as SSB. The length of time used for unwinding varies considerable between studies and among investigators. In general, based on the protocol of Singh et al. [1988], an incubation period of 20 min is used. However, in gamma-irradiated human lymphocytes, increasing the alkali unwinding duration from 20 to 60 min greatly enhanced the extent of DNA migration [Vijayalaxmi et al., 1992]. It was hypothesized that this enhancement was due to the presence of radiation-induced ALS that were relatively resistant to pH $>$ 13 alkali. For most purposes, 20 min is considered sufficient for alkali unwinding. Shorter unwinding times need to be demonstrated to be sufficient for the disruption of double-stranded DNA to single-stranded. One method for testing the adequacy of the alkali-unwinding period would be to compare the extent of DNA migration in control and treated cells under different unwinding conditions, keeping all other steps identical.

Electrophoresis

After alkali unwinding, the single-stranded DNA in the gels is electrophoresed under alkaline conditions to produce comets. The alkaline buffer used during electrophoresis is the same pH $>$ 13 buffer used during alkali unwinding. The electrophoretic conditions developed by Singh et al. [1988] were 25 V and 300 mA, with the DNA being electrophoresed for 20 min. Due to large variability in the size of commercially available electrophoresis units, it is more accurate and useful to present the voltage in V/cm. Typical voltages for electrophoresis appear to range from about 0.7 to 1.0 V/cm, although lower and higher voltages have been used. Similarly, studies conducted using different amperage conditions and electrophoresis durations have been reported. Electrophoresis has been conducted in a variety of electrophoresis units, with recirculating units considered more optimal. Similarly, electrophoresis has been conducted at temperatures ranging from 5°C to room temperature; the use of a lower temperature is thought to provide increased reproducibility. Generally, the optimal voltage/amperage and electrophoresis duration depends on the extent of DNA migration desired for the control cells and the range of responses being evaluated for among treated cells. The reported electrophoresis duration has generally ranged from 5 to 40 min, depending on the type of cell being used and the purpose of the experiment. Singh et al. [1994] has stated that increased sensitivity is associated with the use of a modified electrophoresis buffer containing, in addition to the standard ingredients, two radical scavengers (DMSO and 8-hydroxyquinoline), combined with the use of lower voltage/amperage conditions and longer electrophoresis times. Optimal electrophoretic conditions must be defined by the investigator but a consensus among the expert panel was that the conditions used must be such that the DNA from the control cells exhibit, on the average, some migration. The purpose behind having migration among the control cells is to provide information suitable for an evaluation of intralaboratory experiment-to-experiment variability.

The extent of migration among control cells can also be a critical issue in situations where DNA-DNA or DNA-protein cross-linking is induced. DNA cross-linking can be detected by the Comet assay, as demonstrated by retardation in the rate of DNA migration. DNA-DNA cross-linking, such as that induced by mitomycin C [Pfuhler and Wolff, 1996], or DNA-protein cross-linking, such as that induced by formaldehyde [Pfuhler and Wolff, 1996; Merk and Speit, 1998], can be detected in two ways. Control and treated cells can be exposed to a second genotoxic agent (e.g., ionizing radiation, methyl methanesulfonate), and the extent of DNA migration in the presence and absence of this reference agent compared [Olive et al., 1992; Pfuhler and Wolff, 1996; Merk and Speit, 1998]. Alternatively, the presence of DNA cross-linking can be detected directly by increasing the duration of electrophoresis to such an extent that the DNA of control cells exhibit significant migration (i.e., cross-linked DNA will migrate less than the DNA of control cells) [Tice et al., 1997].

Consideration was given by the expert panel to the potential effects on assay variability of comet position in the gel, slide position in the gel box, and the scoring of slides electrophoresed at different times. There was general consensus among the expert panel that comets near the edges of the gel should not be scored. However, conflicting data were available on the relative extent of assay variability associated with gel box slide position or multiple electrophoresis runs. The expert panel decided that variability would be reduced by ensuring that coded replicate slides are scored for each sample and that the coded slides are randomly distributed in a gel box in a fully balanced design. A fully balanced design means that, to the extent possible, each electrophoresis run should contain one slide from each sample within an experiment. The expert panel also recognized that the same electrophoresis unit and power supply should be used throughout a study [Vrzoc and Petras, 1996].

Neutralization

After electrophoresis, the alkali in the gels are neutralized by rinsing the slides with a suitable buffer (e.g., trizma at pH 7.5). The protocol of Singh et al. [1988] proposed three washes of trizma buffer for 5 min each. However, increased rinsing may be useful in situations where a high background is seen during scoring [Rojas et al., 1999]. After neutralization, slides can be stained and comets scored, or the gel can be dried, the slides stored, and the comets scored when convenient. In the former case, slides should be scored within a reasonable length of time (e.g., 24 hs) to avoid excessive diffusion of the DNA in the gel. In the latter case, the agarose gels can be dehydrated by immersing the slides in absolute ethanol or methanol for a brief time (e.g., 5 min)

[Gurugunta et al., 1996; Klaude et al., 1996] or by letting the slides dry at room temperature. Treatment of the dried slide with neutral buffered formalin for a few minutes may help to stabilize dried gels during long-term storage (Tice, unpublished data). Dried slides, because they can be rescored at any time, offer an obvious advantage over slides not dried.

DNA Staining and Comet Visualization

The DNA-specific dye and the magnification used for comet visualization depend largely on investigator-specific needs and presumably have little effect on assay sensitivity or reliability. The fluorescent dyes used most frequently are ethidium bromide [e.g., Östling and Johanson, 1984; Singh et al., 1988], propidium iodide [e.g., Olive, 1989], 4,6-diamidino-2-phenylindole (DAPI) [e.g., Gedik et al., 1992], SYBR Green I [e.g., Tice et al., 1998], and YOYO-1 (benzoxazolium-4-quinolinum oxazole yellow homodimer) [e.g., Singh et al., 1994]. For some fluorescent dyes, antifade can be used to greatly reduce the rate of signal quenching [e.g., Tebbs et al., 1999], allowing the same slide to be scored multiple times. Nonfluorescent techniques for visualizing comets based on staining with silver nitrate have also been reported [Kizilian et al., 1999]. Comet image magnification has generally varied from 160× to 600×, with 200× to 400× being used most commonly. Selection as to which magnification is most appropriate depends on the type of cell being evaluated, the range of migration responses to measure, and the constraints of the microscope and/or imaging system.

Comet Scoring

All slides, including those of the positive and negative controls, should be independently coded before microscopic analysis and scored without knowledge of the code. The methods used for quantifying DNA migration by this assay have varied almost as much as the number of scientists using the technique. The most flexible approach for collecting comet data involves the application of image analysis techniques to individual cells, and several dedicated software programs are commercially available. Moreover, a fully automated comet analysis imaging system has been developed [Bocker et al., 1999]. However, methods not based on image analysis systems are as useful. The simplest method for collecting comet data is based on determining the proportion of cells with altered migration. However, this approach is generally limited to electrophoretic conditions where the majority of control cells exhibit no or little DNA migration and fails to provide information about the extent of migration among more damaged cells. A more useful approach classifies comets into several categories (generally four or five), based on the length of migration and/or the perceived relative proportion of the DNA in the tail (e.g., Gedik et al., 1992; Anderson et al., 1994; Kobayashi et al., 1995]. By assigning a numerical value to each migration class, the average extent of DNA migration among cells within a culture or animal can be calculated.

The metric most commonly used in Comet studies is the length of DNA migration, presented generally in microns. Migration length is generally believed to be related directly to fragment size and would be expected to be proportional to the level of SSB and ALS, and inversely proportional to the extent of DNA cross-linking. This metric has been measured using a variety of approaches, including by micrometer in the microscope eyepiece, by ruler on photographic negatives/positives of cell images or on a camera monitor, and by image analysis. The criteria used to identify the trailing and leading edge of the migrating DNA seem to be investigator- and/or software-program–specific. Furthermore, some investigators use the term tail length to describe image length while others apply the term to migrated DNA only. A variant of this metric is to present the ratio of the length to width [Jostes et al., 1993] or width to length [Fairbairn et al., 1993], with cells exhibiting no migration having a ratio of ~ 1.

As the use of computerized image analysis systems to collect comet data has increased, a metric based on the percentage of migrated DNA [Olive et al., 1990b) has become used more frequently. This metric assumes signal linearity in quantifying the amount of DNA ranging over multiple orders of magnitude and that the efficiency of the fluorescent dye in staining migrated and nonmigrated DNA is equal. Neither assumption has been validated for all imaging systems and dyes used.

The concept of tail moment (= a measure of tail length × a measure of DNA in the tail) as a metric for DNA migration was introduced by Olive et al. (1990b). However, a consensus among investigators as to the most appropriate manner in which to calculate tail moment has not been obtained, and the use of this metric eliminates potentially useful information on the relationship between the length of migration and the percentage of migrated DNA.

Which metric of DNA migration is used will depend on the resources of the investigator and the experimental design. The expert panel did not consider any single metric to be without usefulness. However, when using a derived metric such as tail moment, data on tail length and the percentage of migrated DNA should be provided also.

IN VITRO TESTING

There is no limit to the in vitro experimental conditions that can be used to demonstrate the ability a suspect agent to induce DNA damage. Conditions under which suspect agents can be adequately demonstrated to lack genotoxic activity are more limited. The in vitro experimental conditions to be employed using the Comet assay are, in principle, no different from those used for any other in vitro

genetic toxicological assay except that cell sampling would be at the end of the exposure period only. The consensus in vitro guideline developed by the expert panel is based on the structure used for current OECD guidelines for in vitro genetic toxicity testing.

Initial Considerations

Tests conducted in vitro generally require the use of an exogenous source of metabolic activation (e.g., hepatic S9 from an Aroclor 1254-induced male rat). This metabolic activation system cannot mimic completely in vivo conditions. In conducting in vitro Comet studies, care should be taken to avoid conditions that would lead to positive results that do not reflect genotoxicity but arise from DNA damage (i.e., DSB) associated with cytotoxicity [Williams et al., 1974; Elia et al., 1994]. As the in vitro SCG assays can be conducted using microculture techniques, the system is especially valuable when only limited amounts of the test substance are available. The Comet assay is capable of detecting various kind of DNA damages with high sensitivity, if the method is optimized. Because this guideline only describes the basic conditions of the assay, the method should be adjusted scientifically at each laboratory to obtain valid and reproducible results.

DESCRIPTION OF THE METHOD

Cell Types

Any eukaryote cell can theoretically be used for genotoxicity testing in the Comet assay. However, for most purposes, well-characterized cell lines or primary cells used generally in genetic toxicology testing for assessing other types of genetic damage (e.g., chromosomal aberrations, micronuclei, mutations) are preferred. Standard cell lines include mouse lymphoma L5178Y, Chinese hamster ovary, and Chinese hamster lung; standard primary cells include human lymphocytes and rodent hepatocytes. No preference is given to the use of proliferating versus nonproliferating cells. However, nonproliferating cells may be less prone to false-positive responses potentially associated with agents that interfere with DNA synthesis by affecting cellular metabolism. This issue has not been adequately resolved. The use of any cell line or cell type should be justified.

Media and Culture Conditions

Appropriate culture media and incubation conditions (culture vessels, CO_2 concentration, temperature, and humidity) should be used in maintaining cultures. Although there are no data to support this requirement, established cell lines should be checked routinely for mycoplasma contamination and should not be used if contaminated.

Culture Preparation

For established cell lines, cells are propagated from stock cultures, and incubated in appropriate culture medium at 37°C. Lymphocytes isolated from the whole blood of healthy subjects are incubated in appropriate culture medium at 37°C. Rodent hepatocytes are isolated using routine procedures and incubated in appropriate culture medium at 37°C.

Metabolic Activation

Cells should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a cofactor-supplemented postmitochondrial fraction (S9) prepared from the livers of male rats treated with enzyme-inducing agents such as Aroclor 1254 [Ames et al., 1975; Natarajan et al., 1976; Matsuoka et al., 1979; Maron and Ames, 1983], or a mixture of phenobarbitone and β-naphthoflavone [Matsushima et al., 1976; Elliot et al., 1992; Galloway et al., 1994]. The postmitochondrial fraction is usually used at concentrations that range from 1% to 10% v/v in the final test medium. The metabolic activation system conditions may depend upon the class of chemical being tested. In some cases, it may be appropriate to utilize more than one S9 concentration. The construction of genetically engineered cell lines expressing specific activating enzymes provides the potential for endogenous activation.

Test Substance/Preparation

Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and, if appropriate, diluted prior to treatment of the cells. Liquid test substances may be added directly to the test systems and/or diluted prior to treatment. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

Test Conditions

The solvent/vehicle should not be suspected of chemical reactivity with the test substance and should be compatible with the survival of the cells and the S9 activity. If other than well-known solvent/vehicles are used, their inclusion should be supported by data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle be considered first. When testing water-unstable substances, the organic solvent used should be free of water.

Exposure Concentrations

The consensus decision of the expert panel was that cells in suspension or monolayer culture should be exposed to the test substance, with and without metabolic activation, for 3 to 6 hr. The use of a different exposure duration should be justified whenever negative data are obtained.

Because DNA damage is associated with cell death, it is critical that the highest dose tested not induce excessive cytotoxicity and that cytotoxicity be evaluated concurrently with each Comet experiment. It may be useful to determine cytotoxicity in a preliminary range-finding experiment. A number of methods for measuring cytotoxicity have been used either at the end of the exposure period (e.g., exclusion of a vital dye) or after a longer culture duration (e.g., cell counts, adenosine triphosphate [ATP] levels) [see Storer et al., 1996 for a comparative study on cytotoxicity methods]. When measuring viability at the end of the exposure period, a general approach has been to avoid the testing of doses that decrease viability, compared to the concurrent control cultures, by more than 30% (e.g., Anderson et al., 1998; Henderson et al., 1998]. The expert panel could not identify the optimal method(s) to use in evaluating cytotoxicity. However, the expert panel consensus was to require the investigator to justify both dose selection and assay results in terms of cytotoxicity. Whether excessive changes in pH or osmolality can lead to a false positive Comet response, as it does for chromosomal damage or mutations [Scott et al., 1991], has not been evaluated for this assay.

Each SCG experiment should include at least three analyzable concentrations. Where cytotoxicity occurs, these concentrations should cover a range from the maximum acceptable toxicity to little or no toxicity. For relatively noncytotoxic compounds, the maximum concentration should be 5 μl/ml, 5 mg/ml, or 0.01 M, whichever is the lowest. For relatively insoluble substances that are not toxic at concentrations lower than the insoluble concentration, the highest dose used should be a concentration above the limit of solubility in the final culture medium at the end of the treatment period. In some cases (e.g., when toxicity occurs only at higher than the lowest insoluble concentration), it is advisable to test at more than one concentration with visible precipitation. It may be useful to assess solubility at the beginning and the end of the treatment, as solubility can change during the course of exposure in the test system due to the presence of cells, S9, serum, etc. Insolubility can be detected using the unaided eye. The precipitate should not interfere with the successful completion of the study.

Controls

Concurrent positive and negative (solvent or vehicle) controls both with and without metabolic activation must be included in each experiment. When metabolic activation is used, the positive control chemical should be the one that requires activation prior to inducing DNA damage. The level of induced DNA migration should not be so obvious that the scorer could easily identify the cultures treated with the positive control agent. Examples of positive control substances to use in experiments without metabolic activation include methyl methanesulphonate (CAS no. 66–27-3), ethyl methanesulphonate (CAS no. 62–50-0), ethylnitrosourea (CAS no. 759–73-9), or 4-nitroquinoline-N-oxide (CAS no. 56–57-5). Examples of positive control substances to use in experiments with metabolic activation include benzo(α)pyrene (CAS no. 50–32-8), 7,12-dimethylbenzanthracene (CAS no. 57–97-6), or cyclophosphamide (monohydrate) (CAS no. 50–18-0 [CAS no. 6055–19-2]). Other appropriate positive control substances may be used. The use of chemical class-related positive control chemicals should be considered, when available. Negative controls, consisting of solvent or vehicle alone in the treatment medium, and treated in the same way as the treatment cultures, should be included. In addition, untreated controls should also be included in experiments where the solvent is not known to be nongenotoxic or noncytotoxic.

PROCEDURE

Treatment with Test Substance

The expert panel consensus was that at least duplicate cultures should be successfully tested at each concentration, including the negative/solvent and positive control dose groups. Gaseous or volatile substances should be tested by appropriate methods, such as in sealed culture vessels (e.g., Krahn et al., 1982].

Analysis

Using independently coded slides and without knowledge of the code, at least 50 cells should be scored per culture with 25 cells scored per each of two replicate slides.

Treatment of Results

The experimental unit of exposure for in vitro studies is the culture, and all statistical analyses should be based on the individual culture response. The mean extent of DNA migration and an associated error term should be calculated for each dose group, as well as for each culture within a dose group. Concurrent measures of cytotoxicity for all cultures and dose groups, including the negative and positive controls, should be included. The consensus of the expert panel was that the results of each experiment should be verified in an independent experiment. Modification of study parameters (e.g., spacing of the test concentrations) to extend the range of conditions assessed should be considered in designing the repeat experiment. In the future, with

increased experience with this assay, this need for independent verification of all results may be modified.

Evaluation and Interpretation of Results

There are several criteria for determining a positive result. These include a concentration-related increase or decrease in DNA migration and a significant corresponding increase or decrease in DNA migration at one or more dose groups. Statistical methods should be used as an aid in evaluating the test results but should not be the only determining factor for identifying a positive response. There was no consensus among the expert panel as to the most appropriate statistical method(s) to use, other than an agreement that the analysis must be based on the individual culture response. However, typical approaches include the use of a trend test for a dose-response relationship and a pairwise comparison of each dose group against the concurrent control to identify significant effects at individual doses. The statistical test should be one-tailed if only an increase in DNA migration or a decrease in DNA migration is being tested for. The statistical test should be two-tailed if either an increase or a decrease in DNA migration could occur.

In the event that a positive Comet assay response is obtained, it is critical that the investigator(s) assesses the possibility that the increase in migration is not associated with genotoxicity. Information on the extent of cytotoxicity associated with each positive dose group, the nature of the dose-response curve, the intercellular distribution of comet response at each dose, and the presence or absence of DSB breaks in the treated cell population may be useful in this regard. Cytotoxicity is not an issue where cross-linking is induced. In the event that a negative Comet assay response is obtained, it is critical that the investigator(s) assesses the validity of the assay and the dose selection procedure.

Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Reproducibility in independent experiments is considered the strongest evidence for a positive or negative call. However, results may remain equivocal or questionable regardless of the number of times the experiment is repeated. Positive results from the in vitro SCG test indicate that the test substance induces DNA damage in cultured mammalian cells. Negative results indicate that, under the test conditions, the test substance does not induce DNA damage in cultured mammalian cells.

Test Report

The test report must include but is not limited to the following information:

- Test substance: identification and CAS number, if known; physical nature and purity; physicochemical properties relevant to the conduct of the study; and stability of the test substance, if known.
- Solvent/vehicle: justification for choice of solvent/vehicle; solubility and stability of the test substance in solvent/vehicle, if known.
- Cells: type and source of cells; number of cell passages, if applicable; methods for maintenance of cell cultures, if applicable; absence of mycoplasma, if applicable; sex of the blood donor(s), if applicable.
- Test conditions: rationale for selection of test substance concentrations; number of cultures per dose group; cytotoxicity data; solubility limitations; composition of media; CO_2 concentration if applicable; volume of vehicle and test substance added; incubation temperature; incubation duration; duration of treatment; cell density during treatment; type and composition of metabolic activation system, including acceptability criteria; positive and negative controls; methods of slide preparation, including agarose concentration; lysis conditions; alkali conditions and pH, alkali unwinding time; electrophoresis conditions (pH, V/cm, mA); dye and staining procedure; criteria for scoring comets; number of comets analyzed per slide, per culture, per dose group; methods for the measurements of toxicity; and criteria for considering studies as positive, negative or equivocal;
- Results: concurrent toxicity; signs of precipitation; data on pH and osmolality of the treatment medium, if determined; definition for DNA migration parameters; dose-response relationship, where possible; statistical analyses; concurrent negative (solvent/vehicle) and positive control data; historical negative (solvent/vehicle) and positive control data, with ranges, means and standard deviations.
- Discussion of the results.
- Conclusion.

IN VIVO TESTING

A highly significant contribution of the SCG assay to genetic toxicology is in its application to in vivo studies. As only a small numbers of cells are required for analysis, literally any tissue or organ is amenable to investigation. The only requirement is that a sufficient number of single cells (or nuclei) are obtained for analysis and that no or minimal damage is induced during tissue processing. The consensus in vivo guideline developed by the expert panel is based on the structure used for current OECD guidelines for in vivo genetic toxicity testing.

Initial Considerations

It is not appropriate to use this test when there is evidence that the test substance will not reach the target tissue. In conducting in vivo Comet studies, care should be taken to avoid conditions that would lead to positive results that do not reflect genotoxicity but may arise from DNA damage

(i.e., DSB) associated with apoptosis or necrosis. The Comet assay is capable of detecting various kind of DNA damages with high sensitivity, if the method is optimized. Because this guideline only describes the basic conditions of the assay, the method should be adjusted scientifically at each laboratory to obtain valid and reproducible results.

DESCRIPTION OF THE METHOD

Animal Species

Mice or rats are preferred, although any other appropriate mammalian species may be used, if justified. Commonly used laboratory strains of healthy young adult animals should be employed. At the commencement of the study, the weight variation of animals should be minimal and not exceed 20% of the mean weight.

Housing and Feeding Conditions

Appropriate national and international regulations for the use and care of animals must be followed. The temperature in the experimental animal room should be 22ºC ± 3ºC. Although the relative humidity should be at least 30% and preferably not exceed 70%, other than during room cleaning, the optimal range is 50% to 60%. Lighting should be artificial, the sequence being 12-hr light, 12-hr dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test substance when administered by this method. Depending on the experimental design and current laboratory standards, animals may be housed individually or caged in small groups of the same gender.

Preparation of the Animals

Healthy young adult animals are randomly assigned to the control and treatment groups. Cages should be arranged in such a way that possible effects due to cage placement are minimized. The animals are identified uniquely and kept in their cages for at least 5 days prior to the start of the study to allow for acclimatization to the laboratory conditions.

Test Substance Preparation

Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

Test Conditions

The solvent/vehicle should not produce toxic effects at the dose levels used, and should not be suspected of chemical reactivity with the test substance. If other than well-known solvents/vehicles are used, their inclusion should be supported with data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle should be considered first.

Controls

Concurrent negative (solvent/vehicle) and positive controls should be included in each independently performed part of the experiment. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the animals in the treated groups. The positive control should be a substance known to produce DNA migration when administered at exposure levels expected to give a detectable increase over background. The dose(s) should be chosen such that the effects are definitive but do not immediately reveal the identity of the coded slide to the reader. It is acceptable that the positive control may be administered by a route different from the test substance and sampled at only a single time, as long as all animals are treated during the same time period. Negative controls, treated with solvent or vehicle alone should be included for every sampling time, unless acceptable variability in DNA migration among animals is demonstrated using historical control data. Untreated concurrent controls should also be used unless there are historical or published control data demonstrating that no deleterious or genotoxic effects are induced by the chosen solvent/vehicle.

PROCEDURE

Number and Gender of Animals

The expert panel had considerable discussion on the minimal number of animals to test per sex per dose group per sample time. Lacking appropriate interlaboratory and intralaboratory data on the extent of animal-to-animal variability, as a function of species, strain, sex, tissue, sample time, etc., that could be expected for this assay, the expert panel consensus was that a minimum of four scorable animals of a single gender should be included in each dose group at each sample time. In some situations (e.g., an intermediate dose group within a study), a minimum of three scorable animals per gender per dose group might be considered acceptable but should be justified. Where a significant historical database has been generated demonstrating that, under the experimental conditions used, minimal animal-to-animal variability is present, only two animals may be needed for the concurrent negative and positive control groups. However, this needs to be justified and

historical control data must then be used as one of the criteria for considering a study acceptable and in identifying a positive response.

If there are relevant data available from studies in the same species and using the same route of exposure that demonstrate that there are no substantial differences in toxicity between sexes, then testing in a single sex will be sufficient. Currently, there does not appear to be an inherent advantage in preferentially using males or females; choice of which sex to use is left to the discretion of the investigator. Where human exposure to chemicals may be gender-specific, as for example with some pharmaceutical agents, the test should be performed with animals of the appropriate sex.

Treatment Schedule

Test substances are generally administered as a single treatment. However, studies using multiple treatment protocols may be used also [e.g., Tice et al., 1997]. The rationale for the treatment schedule should be justified.

Dose Levels

If a range-finding study is performed because there are no suitable toxicity data available, it should be performed in the same laboratory, using the same species, strain, sex, and treatment regimen used in the main study. The highest dose is defined as the dose that produces signs of toxicity such that based on the same dosing regimen, higher dose levels would be expected to produce mortality, unacceptable signs of animal toxicity, or excessive cytotoxicity in the target tissue by the end of the exposure period defined by the protocol. In general, a minimum of two scorable doses should be present in each experiment. The expert panel recommended that the lower dose should be approximately 50% of the higher dose but that other dose spacing could be used if justified. Substances with specific biological activities at low nontoxic doses (such as hormones and mitogens) may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis.

Limit Dose

Consistent with current relevant OECD guidelines, the limit dose for nontoxic substances is 2,000 mg/kg body weight for single or multiple treatments up to 14 consecutive days and 1,000 mg/kg for treatments that exceed 14 consecutive days. In the absence of animal- or tissue-specific toxicity, it may be acceptable to test only the limit dose. Expected human exposure levels may indicate the need for a higher dose level to be used than these limit doses.

Administration of Doses

The test substance is usually administered orally by gavage using a stomach tube or a suitable intubation cannula, or by intraperitoneal injection. Other routes of exposure may be used if justified. The maximum volume of liquid that can be administered by gavage or injection at one time should be based on the weight of the test animal. Consistent with current relevant OECD guidelines, the maximum volume used should not exceed 20 ml/kg body weight. The use of higher volumes must be justified. Except for irritating or corrosive substances that will normally reveal exacerbated effects with higher concentrations, variability in the test volume should be minimized by adjusting the concentration to ensure a constant volume at all dose levels.

Target Tissue Selection

In principle, the Comet assay can be applied to any tissue. However, the consensus of the expert panel was that in the absence of information on a known target tissue for the class of chemicals being tested (e.g., bladder for some aromatic amines), the most appropriate target tissue is liver, followed by intestine when the liver is negative for genotoxicity. Both tissues can be collected and processed at the same time, with scoring being conducted in a sequential manner. Tissue selection is based on a recent analysis of the results of studies conducted in mice and rats with 163 rodent carcinogens and 30 rodent noncarcinogens [Sasaki et al., 2000]. Rodents were treated once orally at the maximum tolerated dose and bladder, bone marrow, brain, colon, kidney, liver, lung, and stomach sampled at 3, 8, and 24 hr after treatment. The sensitivity of the alkaline (pH > 13) SCG assay for detecting carcinogens was 82%, while the specificity of the test for detecting non-carcinogens was 83%. When data analysis was limited to Ames test-positive carcinogens, sensitivity was increased to 94%. Generally, the Comet assay-positive carcinogens induced a significant increase in DNA migration in cells at the tumor site and also at multiple nontumor sites. Among the Comet assay-positive carcinogens, 73% induced a positive response in at least liver, while 96% induced a positive response in liver and colon. The remaining 4% of the Comet assay-positive carcinogens were positive in stomach or bladder.

Sample Times

In the in vivo data evaluated by Sasaki et al. [2000], 7% of the positive chemicals were uniquely positive at 3 hr, 3% were uniquely positive at 8 hr, and 7% were uniquely positive at 24 hr only. Other in vivo kinetic Comet studies using oral and the intraperitoneal route of treatment are consistent with these findings. Based on these data, the consensus of the expert panel was that two sample times, at 3 to 6 hr and 22 to 26 hr, after a single acute treatment

should be used. The expert panel recommended that data be collected first from a single sample time, as a positive response at that sample time would preclude the need for the second sample time. Which sample time should be tested first is left to the discretion of the investigator. However, to avoid the unnecessary use of animals, the decision should be based on the characteristics of the test substance and on Comet data generated for similar compounds, when available. When the protocol involves multiple daily treatments, a single sample time of 3 to 6 hr after the last treatment is appropriate. Other sampling times may be used if justified.

Preparation of Single Cells from Solid Tissues

Single cell suspensions can be obtained from solid tissue by incubation with collagenase or trypsin [e.g., Pool-Zobel et al., 1992; Betti et al., 1993], mincing with a pair of fine scissors for a few minutes [Tice et al., 1991], or by homogenization to release nuclei [Sasaki et al., 1997; Miyamae et al., 1998]. During mincing or homogenization, EDTA can be added to the processing solution to chelate calcium/magnesium and prevent endonuclease activation, and radical scavengers (e.g., DMSO) can be added to prevent oxidant-induced DNA damage [e.g., Tice et al., 1997]. The consensus of the expert panel was that no single method for obtaining single cells appeared clearly superior to any other method. Any cell dissociation method is acceptable so long as it can be demonstrated that the process is not associated with inappropriate background levels of DNA damage.

Cytotoxicity

A concurrent assessment of cytotoxicity in critically important for data interpretation. An assessment of in vivo cytotoxicity has largely depended on simple dye exclusion techniques (e.g., trypan blue). However, this approach is noninformative if mincing or homogenization is used to provide single cells or nuclei. A dual dye viability assay based on a combination of 5–6 carboxyfluorescein diacetate and ethidium bromide [Strauss, 1991] has been used to identify cells that are metabolically competent and/or have a compromised cellular membrane. Two other approaches have been used for evaluating concurrent cytotoxicity. In the studies conducted by Sasaki et al. [1997], histopathology was used to identify tissues with excessive necrosis or apoptosis in experiments where a positive Comet assay response was obtained. Tice et al. [1998] used a neutral diffusion assay [Vasquez and Tice, 1997a) to detect cells with low molecular weight DNA indicative of apoptosis or necrosis. Although validation studies have not been conducted to identify acceptable cytotoxicity levels for in vivo Comet assays, cell viability in the target tissue that is below 70 to 80% of that in the control animals may be considered excessive.

Analysis

Using independently coded slides and without knowledge of the code, at least 100 cells should be scored per animal with 50 cells scored per each of two replicate slides.

Treatment of Results

The experimental unit of exposure in in vivo studies is the animal, and all statistical analyses should be based on individual animal response data. The mean extent of DNA migration and an associated error term should be calculated for the dose group, as well as for each animal. Concurrent measures of cytotoxicity for each animal in all dose groups, including the solvent and positive control animals, should be presented. The consensus of the expert panel was that the results of each experiment, where not clearly negative or positive, should be verified in an independent experiment. Modification of study parameters (e.g., spacing of the test concentrations) to extend the range of conditions assessed should be considered in designing the repeat experiment.

Evaluation and Interpretation of Results

There are several criteria for determining a positive result. These include a concentration-related increase or decrease in DNA migration and a significant corresponding increase or decrease in DNA migration at one or more dose groups. Statistical methods should be used as an aid in evaluating the test results but should not be the only determining factor for identifying a positive response. There was no consensus among the expert panel as to the most appropriate statistical method(s) to use, other than that the analysis should be based on the individual animal. However, typical approaches include the use of a trend test for a dose-response relationship and a pairwise comparison of each dose group against the concurrent control to identify significant effects at individual doses. The statistical test should be one-tailed if only an increase in DNA migration or a decrease in DNA migration is being tested for. The statistical test should be two-tailed if either an increase or a decrease in DNA migration could occur.

In the event that a positive Comet assay response is obtained, it is critical that the investigator(s) assesses the possibility that the increase in migration is not associated with genotoxicity. Information on the extent of cytotoxicity associated with each positive dose group, the nature of the dose response curve, the intercellular distribution of comet response at each dose, and the presence or absence of DSB breaks in the treated cell population may be useful in this regard. Cytotoxicity is not an issue where cross-linking is induced.

Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test

substance. Reproducibility in independent experiments is considered the strongest evidence for a positive or negative call. However, results may remain equivocal or questionable regardless of the number of times the experiment is repeated. Positive results from the in vivo SCG test indicate that the test substance induces DNA damage in vivo in the target tissue. Negative results indicate that, under the test conditions, the test substance does not induce DNA damage in vivo in the tissues evaluated.

Test Report

The test report must include but is not limited to the following information:

- Test Substance: identification and CAS number, if known; physical nature and purity; physicochemical properties relevant to the conduct of the study; and stability of the test substance, if known.
- Solvent/Vehicle*:* justification for choice of solvent/vehicle; solubility and stability of the test substance in solvent/vehicle, if known.
- Test animals: species/strain used; number, age, and sex of animals; source, housing conditions, diet, etc.; individual weight of the animals at the start of the test, including body weight range, mean and standard deviation for each group.
- Test Conditions: details of the administration of the test substance; rationale for route of administration; methods for verifying that test agent reached the general circulation or target tissue, if applicable; conversion from diet/drinking water test substance concentration (ppm) to the actual dose (mg/kg body weight per day), if applicable; details of food and water quality; detailed description of treatment and sampling schedules; methods for measurement of toxicity; detailed methods of single cell preparation; methods of slide preparation, including agarose concentration; lysis conditions; alkali conditions and pH, alkali unwinding time; electrophoresis conditions (pH, V/cm, mA); staining procedure; criteria for scoring comets; number of comets analyzed per slide, per tissue, per animal, per dose group; methods for the measurements of toxicity; and criteria for considering studies as positive, negative or equivocal.
- Results: individual tissue, animal, and group mean values for DNA migration; dose-response relationship, if available; statistical evaluation; signs of toxicity; concurrent negative (solvent/vehicle) and positive control data; historical negative (solvent/vehicle) and positive control data.
- Discussion of the results.
- Conclusion.

RELATED METHODOLOGIES

Specific Classes of DNA Damage

Several investigators have modified the SCG assay to enable the detection of specific classes of DNA damage. At pH > 13, the assay detects SSB, ALS, and DNA cross-linking. The induction of increased levels of SSB and ALS results in an increased ability of the DNA to migrate. In contrast, the presence of DNA cross-linking reduces the ability of the DNA to migrate. Reducing the pH during alkali unwinding and electrophoresis to 12.1 appears to eliminate the expression of ALS as SSB [Miyamae et al., 1997]. Thus, the presence of increased migration at pH $>$ 13 but its absence at pH 12.1 indicates the specific induction of ALS. Equal migration under the two electrophoretic conditions indicates the presence of strand breaks only. In conducting this comparison, it is important that the molarity of the ingredients in the $>$ 13 and 12.1 alkaline buffers remain the same.

The induction of either DNA-DNA or DNA-protein crosslinks reduces the ability of the DNA to migrate in the agarose gel, at any pH. These two types of cross-linking can be distinguished by incubating the lysed DNA in PK prior to electrophoresis. Exposure of cross-linked DNA to PK reduces or eliminates DNA-protein cross-links, while having no effect on the frequency of DNA-DNA cross-links [Merk and Speit, 1998]. The complete or partial loss of DNA-protein cross-links allows the DNA to migrate at the same rate or at a rate more similar to, respectively, the DNA from the control cells.

Two approaches have been developed to detect specific classes of DNA adducts. Gedik et al. [1992] demonstrated that the induction and persistence of ultraviolet (UV) light-induced pyrimidine dimers in HeLa cells could be monitored by incubating lysed DNA with a UV-specific endonuclease. Similarly, the enzyme endonuclease III can be used to detect oxidized pyrimidines (e.g., Collins et al., 1993], while the enzyme FPG can be used to detect 8-OH guanine and other damaged purines [e.g., Collins et al., 1996]. Immunofluorescence techniques can be used to detect specific types of DNA damage, as demonstrated by the detection of UV-induced DNA damage in cultured cells using a lesion-specific antibody [Sauvaigo et al., 1998].

These methods can be used to provide mechanistic information on the types of DNA damage induced by a test substance or, in some situations, to eliminate the possibility that the observed increase in DNA migration is due to cytotoxicity.

Apoptosis/Necrosis

Increased DNA migration accompanies the DNA fragmentation associated with cytotoxicity arising through necrosis or apoptosis. Apoptosis results in the extensive for-

mation of DSB [e.g., Carson et al., 1986; Marks and Fox, 1991]. Similarly, the DNA of necrotic cells also undergoes extensive degradation due to the induction of DSB [Williams et al., 1974; Elia et al., 1994]. Such cells can be detected using either neutral or alkaline electrophoretic conditions [Olive et al., 1993; Vasquez and Tice, 1997a). Several investigators have concluded that based on the characteristic appearance of the comets, apoptotic cells can be readily distinguished from necrotic cells in the alkaline SCG assay [Olive et al., 1993; Fairbairn et al., 1996; Kizilian et al., 1999]. Apoptotic cells were concluded to form comets with large fan-like tails and small heads (i.e., so-called hedgehogs), while necrotic cells were concluded to form comets with relatively large heads and narrow tails of varying lengths (i.e., comets indistinguishable from those resulting from genotoxic damage). However, this characterization may not be completely accurate. For example, comets typical of hedgehogs are induced by high doses of gamma radiation under conditions where apoptosis is not possible (i.e., the cells were processed immediately after irradiation) (Tice, unpublished data). Furthermore, due to the very low molecular weight of the DNA in terminal apoptotic and necrotic cells, the DNA of many of these cells may be lost from the gels under the typical electrophoretic conditions used [Vasquez and Tice, 1997a). In response to this concern, Vasquez and Tice [1997a] developed a low molecular weight diffusion assay for detecting apoptotic/necrotic cells. In this assay, slides are removed from lysis within 2 hr, the slides rinsed free of lysing solution, the DNA stained, and the frequency of cells with an extremely diffuse staining pattern determined. Under the neutral, nonelectrophoretic condition used, extensive DNA diffusion can only occur if the DNA is of very low molecular weight resulting from extensive degradation due to DSB. With further characterization, this approach might prove useful in helping to set the maximum dose of a test substance to test in the Comet assay or in the interpretation of a positive response.

Acellular Assay

Another version of the Comet assay with utility in genetic toxicological studies is an assay based on the treatment of the liberated DNA present in the agarose gels after lysis [Singh et al., 1990; Kasamatsu et al., 1996; Vasquez and Tice, 1997b]. In this acellular assay, slides with gels prepared from untreated cells are removed from lysis and rinsed free of lysing solution. The slides are then exposed, in the absence or presence of metabolic activation, to the test substance of interest for an appropriate period of time. Subsequently, the slides are processed through alkaline unwinding and electrophoresis following the standard protocol. Considering that DNA and not cells are exposed, an alteration in DNA migration under these conditions indicates the ability of the test substance to induce DNA damage independent of cytotoxicity. Theoretically, cells of any type can be used to generate DNA for this assay.

Future Directions

The alkaline (pH $>$ 13) Comet assay guidelines developed by the expert panel represent a work in progress. Several kinds of information are needed before the assay can be critically evaluated for its utility in genetic toxicology. These include information on the different sources of variability (cell to cell, gel to gel, culture to culture, animal to animal, experiment to experiment, etc.) intrinsic to the alkaline (pH $>$ 13) SCG assay, the results of in vitro and in vivo testing based on these guidelines, and the conclusions of appropriately designed multilaboratory international validation studies. It is hoped that the guidelines provided in this report will serve as the basis for further developments with this assay.

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