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Single Cell Gel Electrophoresis Assay in the Earthworm for the Detection of Genotoxic Compounds in Soils

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The single cell gel electrophoresis assay or comet test is a new, rapid, simple, sensitive and inexpensive technique for measuring and analysing DNA breakage in mammalian cells (Singh et al., 1988; Olive et al., 1990; Tice et al., 1990b). The mechanisms on which the technique relies are still not well understood but it is clear that essentially single strand breaks and alkali labile sites in DNA are detected. An important advantage of the technique is that it is applicable to any eukaryotic organism and cell type. Although most studies performed to date have utilized in vitro (mutagen) exposed (human) peripheral blood lymphocytes, other cell types (and organisms) have been investigated. Thus far, only a few in vivo studies have been performed or suggested (Singh et al., 1988; Tice et al., 1990a,b; McKelvey-Martin et al., 1993). In this paper we describe some preliminary results obtained in coelomocytes of earthworms residing in polluted soil samples or being directly exposed to a mutagen. Earthworms have already proved to be a good animal system for monitoring pollution in terrestrial ecosystems (e.g. Morgan and Morgan, 1991; Fitzpatrick et al. 1992). Furthermore, they are easy to keep in the laboratory with minimal costs and care. Our results show that the earthworm system may be very valuable for the monitoring and detection of genotoxic compounds in terrestrial ecosystems.

MATERIALS AND METHODS

Adult earthworms (Lumbricus terrestris, approx. weight = 1.25g) were allowed to acclimate to laboratory conditions for several weeks before the test. They were kept in a "standard" black earth. The effect of X-rays and mitomycin C was tested whereas some tests were also performed in samples from soils obtained near an illegal dumping ground (table 1). In the latter case Eisenia fetida (approx. weight = 0.38g) were used instead of Lumbricus terrestris.
After exposure of the earthworms, their coelomocytes were obtained according to the non-invasive extrusion method described by Eyambe et al. (1991). Individual earthworms were rinsed in saline (4°C), and the content from the lower gut was expelled by massage to reduce fecal contamination of extrusion

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fluid in which the worms were placed for three minutes. This extrusion medium consists of 5% ethanol + 95% saline + 2.5 mg/ml EDTA + 10 mg/ml guaiacol glycerol ether (pH=7.3).
Coelomocytes are "spontaneously" secreted in the medium and washed prior to the SCGE-assay. Most coelomocytes are basophils, presumably because of their presence in and around the integumental pores through which mucus is secreted (Eyambe et al., 1991).
The SCGE-assay was performed as described by Singh et al. (1988). Essential steps involve at least a 1 hour lysis of the cells by detergent at high salt concentrations and electrophoresis under alkaline conditions (20 minutes alkali treatment followed by 30 minutes electrophoresis at 300mA and 25V). Under fluorescence microscopy, ethidium bromide stained nuclei with DNA damage display increased migration of single-stranded DNA from the nucleus towards the anode. This provides the "comet" appearance of cells with DNA damage. The length of migration is directly related to the magnitude of the damage present in each cell. Comet tail lengths (nuclear region + tail) were measured with a calibrated scale in the ocular of the microscope. Tail lengths are given in arbitrary units; one unit being approximatively 10µm. Two worms were used for each dose or sample. In this preliminary study cells from the two worms were pooled and distributed over two microscope slides. From each slide 25 cells were analysed (50 cells per sample).

Table 1. Experimental design.

	MODES OF EXPOSURE	
A	B	C
X-irradiation of animals kept on ice	Animals kept in 20ml PBS + mitomycin C on ice	Animals kept in 50g humidified (65% PBS; pH = 7.2) "polluted" or "control" soil samples
Testing immediately after irradiation or 1.5 hours later	Exposure time = 2 hrs.	Exposure time = 2 days
Doses applied: 0, 5, 10, 15 cGy	Doses applied: 12.5 ng/ml 25.0 ng/ml 50.0 ng/ml	Pollution from an illegal dumping ground controls are standard black earth or earth from "ordinary environments" (considered unpolluted)

RESULTS AND DISCUSSION

Mean comet tail lengths measured in coelomocytes from X-ray exposed earthworms are given in figures 1 and 2. Figure 1 shows how the X-irradiation results in a clear linear dose-response relationship, whereas figure 2 shows how significant DNA repair can be prevented by keeping the worms on ice during a

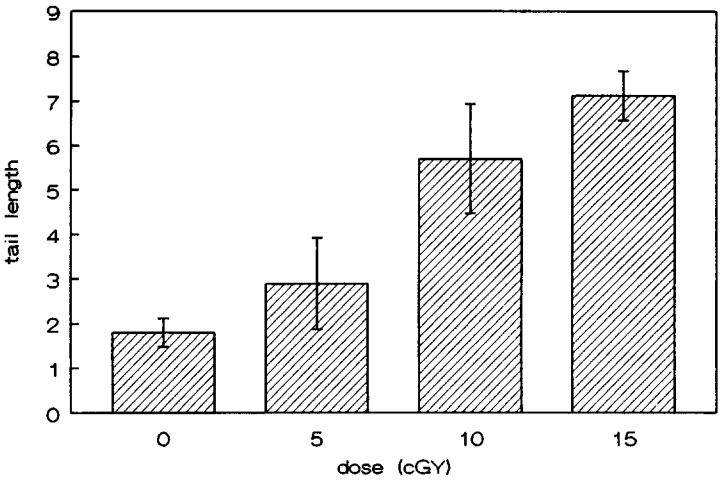

Figure 1. Mean comet tail lengths in coelomocytes from earthworms exposed to X-rays at 4°C (bars = standard deviation)

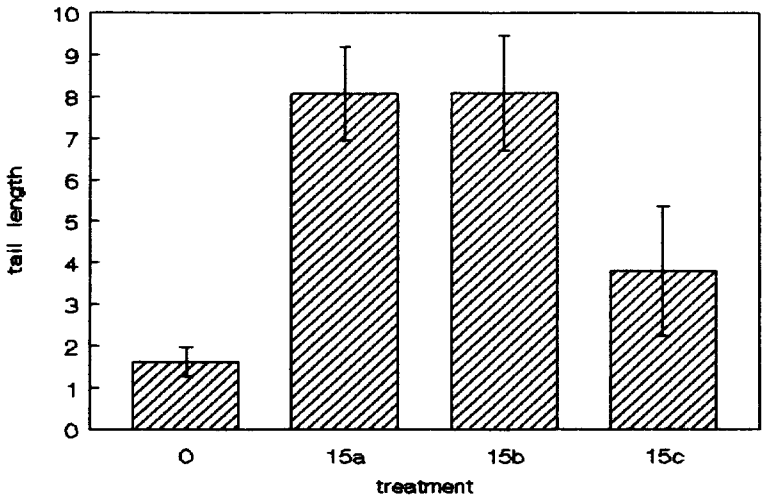

Figure 2. Mean comet tail lengths in coelomocytes from X-ray exposed earthworms; 15a = SCGE-test performed immediately after a 15cGy exposure of the animals, 15b = same exposure conditions but earthworms remained on ice (4°C) for 1.5 hrs prior to the SCGE-assay, 15c = same as 15b except that the worms were kept at 10°C following exposure and prior to the test. Bars = standard deviation.

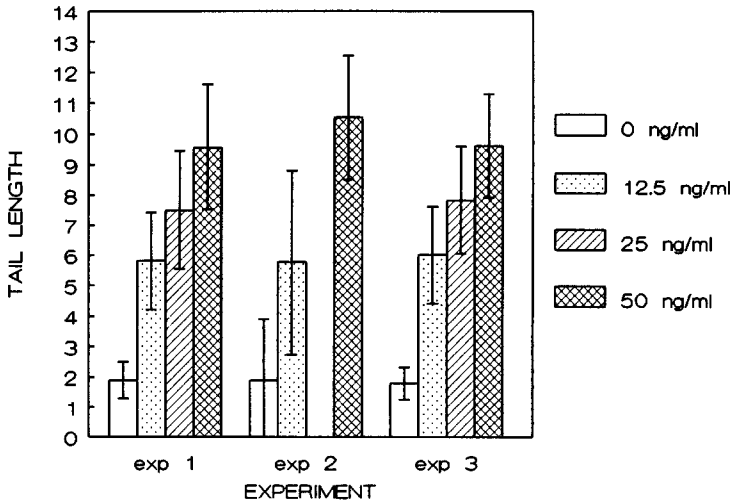

Figure 3. Mean comet tail lengths in coelomocytes from earthworms exposed to mitomycin C at 4°C. The figure gives the dose-response relationship for 3 independent experiments. Bars = standard deviation.

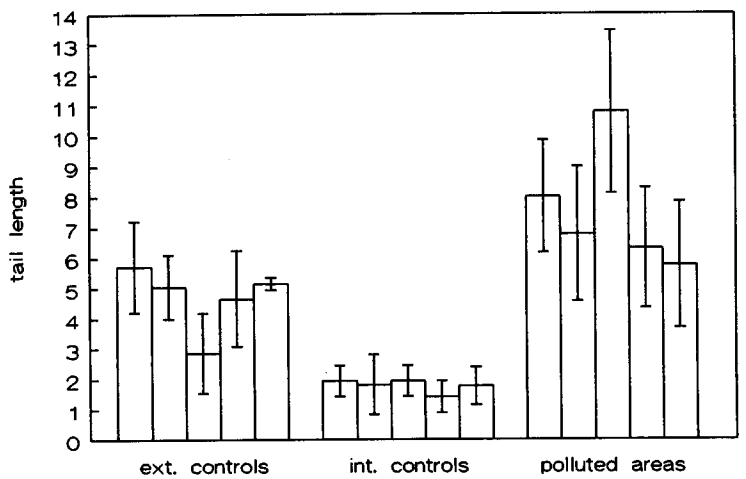

Figure 4. Typical examples of mean comet tail lengths obtained in coelomocytes from non-exposed earthworms kept at 10°C in standard black earth (int. controls), in soil samples from "non-polluted" areas (ext. controls) and in soil samples obtained in well-known polluted areas. Bars = standard deviation.

given period following exposure (here 1.5 hrs) and prior to the test. Figure 3 gives the results obtained for mitomycin C in three independent experiments. It shows that the results are fairly reproducible. We also exposed earthworms to samples of soils obtained from gardens close to an illegal dumping ground that is known to be responsible for the release of toxic substances in the near environment (benzene, aniline and others). A number of samples also came from control areas that are not considered to be polluted. Figure 4 shows that all samples gave higher comet tail lengths than those obtained from worms kept in laboratory conditions (standard black earth = internal controls). Samples from control areas (= external controls) give lower comet tail lengths than from the polluted area but they are higher than from our laboratory controls. This is presumably due to the presence of some (genotoxic) pollutants at background concentrations or it may be inherent to the transfer of the worms from a standard black earth to a soil with different composition and properties. This needs to be clarified in future in order to further optimise the test.

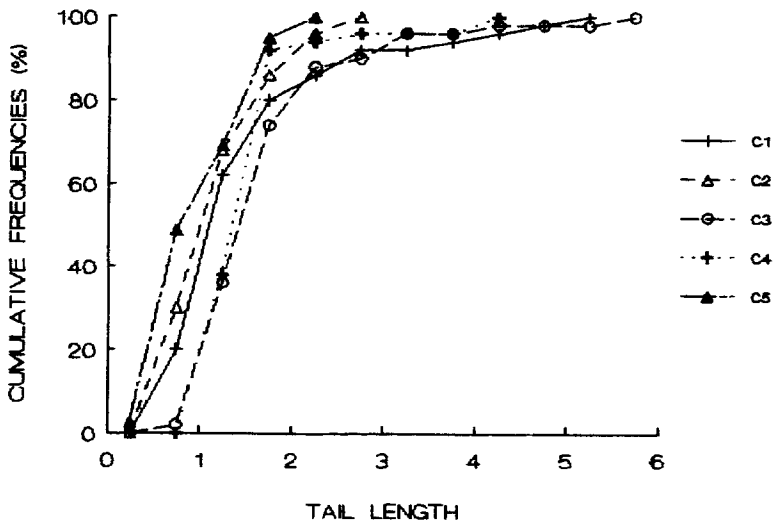

Figure 5. Cumulative mean frequencies of comet tail lengths in coelomocytes from 5 individual control worms.

Figures 5, 6 and 7 give an idea of the comet tail length distributions. Here cumulative distributions are given. It can be seen from figure 5 that curves from 5 individual control worms are very similar; about 80% of the cells display comet tail lengths of less than 20 µm (arbitrary unit=2), whereas only a few cells give substantially longer tail lengths. Figures 6 and 7 give cumulative frequencies for the X-ray experiment (cf. figure 1) and one of the MMC experiments (cf. fig. 3). A clear shift to longer tail lengths is observed with increasing doses of the mutagens. This indicates that most cells are affected by the (mutagen) exposure.

These data only give an example of current results with the test system.

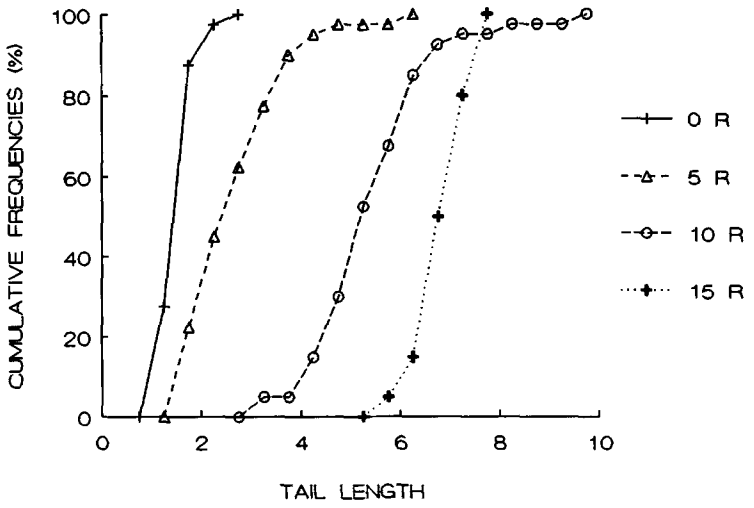

Figure 6. Cumulative frequencies of comet tail lengths for each of the X-ray doses applied (R = cGy)

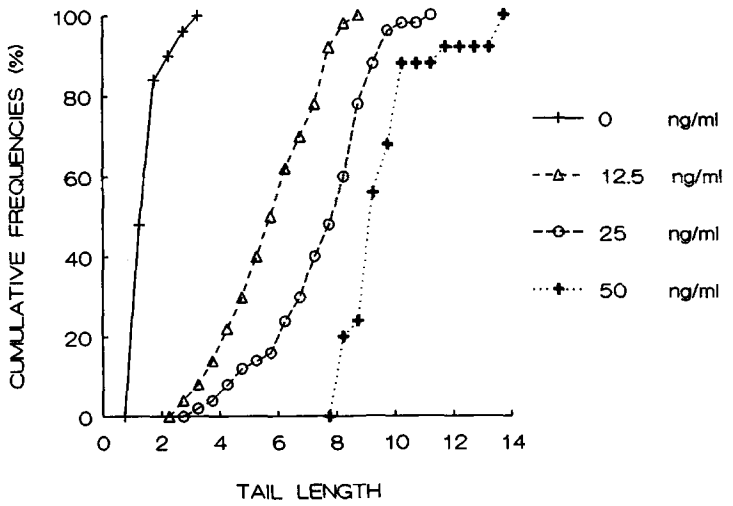

Figure 7. Cumulative frequencies of comet tail lengths for each of the mitomycin C doses applied (example for one of the experiments)

Therefore no extensive statistical analysis was performed. The data demonstrate that the "earthworm SCGE-assay" is a very easy and convenient way to screen chemicals or terrestrial environments for their DNA damaging properties. Combined with analytical analyses that may identify DNA damaging substances it may be of great importance for risk evaluation and eventually also site remediation. However, the mechanisms on which the SCGE-technique relies are not yet well understood and "comets" may apparently not only be induced by direct mutagens but also indirectly. This is suggested, for example, by our experiments with samples from control areas (supposed not to be particularly polluted and hence not mutagenic) or by other results that we obtained with dioxins. Indeed, although dioxins are usually considered to be non mutagenic or at the most weak mutagenic (Wassom et al., 1977) they induce substantial DNA damage as visualised by the comet assay with earthworms (Verschaeve et al., 1993).

A number of studies are certainly required before the test can be fully assessed. There is, for example, an urgent need to obtain a great number of control values (from worms residing in in situ obtained unpolluted earth) so as to better evaluate the extend of the observed DNA damage. As already stressed, also the influence of the composition of the soil samples (pH, chemical and physical characteristics,...) must be extensively investigated together with other methodological factors. We are now performing some of these studies.

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