No increased DNA damage in peripheral lymphocytes of sewage workers as evaluated by alkaline single cell gel electrophoresis

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Abstract

Objectives—To study whether sewage workers are exposed to genotoxic substances. An increased risk of cancers among sewage workers has been noted. If this increased risk is due to an exposure to genotoxic agents, primary DNA damage could be used as a biological marker of exposure.

Methods—In a cross sectional study, DNA damage in peripheral lymphocytes from 35 sewage workers and 30 controls was compared with alkaline single cell gel electrophoresis, a technique for detecting single strand breaks and alkali labile sites in DNA. The controls were selected from among municipal workers matched for age and smoking habit. Information about occupational exposures and possible confounders was collected by means of a questionnaire.

Results—No increase in DNA damage was found among the sewage workers when compared with the unexposed controls.

Conclusion—The failure to detect increased damage to DNA in peripheral lymphocytes by alkaline single cell gel electrophoresis suggests that the sewage workers studied here were not exposed to genotoxic agents to a greater extent than other municipal workers. It may be, however, that the lymphocyte is not the appropriate target cell to study, or that sewage workers are exposed to carcinogens which do not damage the genetic material.

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An increased occurrence of various cancers, especially of the stomach, kidneys, and brain, has been found among sewage workers.¹⁻³ No specific occupational exposure has been identified which might cause this excess of tumours although mutagenic activity has been shown in waste water by the Ames test,⁴ and increased mutagenic activity has been reported in the urine of sewage workers.⁵ Sludge from waste water treatment plants has been analysed for mutagenicity with varying results.^{6,7}

The exposures which take place during sewage treatment may vary at different locations within one plant.⁸ The content of municipal waste water may also vary between communities. Industries that use the municipal waste water treatment plant for disposal of industrial waste may sometimes be an important source of specific exposures.⁹ The many and varied exposures of sewage work make it difficult to find specific carcinogens that could be related to an observed increased risk of cancers and instead of searching for specific genotoxic carcinogens in the environment, the biological markers of genotoxicity could act as a proxy for exposure to carcinogenic substances. In the present study we used alkaline single cell gel electrophoresis, also known as the comet assay, to measure DNA damage among sewage workers. Alkaline single cell gel electrophoresis is a sensitive technique for detecting single strand breaks and alkali labile sites in DNA induced by various types of genotoxic agents, as well as ionising radiation.¹⁰⁻¹² Although there is variation both within and between people when following up people over time, this method gives stable measures of DNA damage at a group level when studying unexposed subjects.¹³

The hypothesis tested in this study was whether sewage workers had increased DNA damage due to occupational exposure to genotoxic agents as measured by the comet assay on peripheral lymphocytes. This study was also our first test of the comet assay in active service conditions in an epidemiological study. The Ethics committee of the Faculty of Medicine, Uppsala University approved the study and all subjects gave their informed consent to participate.

Subjects and methods

Thirty five (92%) of 38 sewage workers employed at the municipal sewage plant in Uppsala and 30 (79%) of 38 invited controls participated in the investigation. The controls were selected from among municipal construction workers not working in the sewage treatment process, and were matched for age (five years) and present smoking habits (tobacco smoker or non-smoker). Matching for sex was not entirely possible due to limited numbers, thus five female employees at the sewage plant but only one female control participated in the study. The mean (SD) age of the 65 subjects was 45.7 (8.7) years, and 18

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were smokers. Each sewage worker and his or her matched control were invited on the same day for blood sampling.

ASSESSMENT OF EXPOSURE AND PERSONAL FACTORS

Data about workplace exposures, medical history, and lifestyle factors were collected through a questionnaire and supplemented by a personal interview conducted by a physician. The data showed that some controls were occasionally exposed to waste water in their present job—for instance when repairing leaking sewers. Therefore, three models for allocation into exposure groups were used for the statistical analyses, to minimise any bias due to misclassification of exposure. For all three exposure classifications we required those considered to be exposed to have had at least eight hours of occupational exposure to waste water or sludge each week during the two weeks preceding the blood sampling. This cut off distinguished between the workers with regular tasks close to the sewage treatment process and the administrative personnel at the plant. In the first exposure classification (A) all the sewage workers and all the construction workers who had worked in sewage contaminated environments, and had been exposed for at least eight hours in two weeks, were considered to be exposed. The remaining workers were all classified as controls. For the second classification (B), only sewage workers who had been exposed for at least eight hours in two weeks were classified as exposed, whereas all others were used as controls. For the third exposure classification (C) the controls were restricted to those who had absolutely no occupational exposure to sewage, whereas the exposed group were the same as in B. All six women were considered as controls for classifications A and B and they were rejected in C. Information was also collected about possible confounding exposures during the previous week (tobacco smoking, alcohol consumption, occupational exposure to organic solvents, ongoing infections, heavy physical exertion, intake of vitamins, and medical procedures causing genotoxic exposure, such as ionising radiation or cytostatic drugs).

BLOOD SAMPLING AND ISOLATION OF LYMPHOCYTES

Venous blood (5 ml) was collected by routine venepuncture into a sterile tube with heparin (Vacutainer, Becton Dickinson, NJ, USA). The blood samples (one to eight subjects on each occasion) were collected between 8 00 and 10 00 am, coded, and kept on ice until the lymphocytes were isolated (usually within two hours after the blood sampling) on a density gradient of Percoll (Pharmacia LKB, Uppsala, Sweden) by a procedure described previously.¹³ The isolated single cell suspension mainly consisted of viable lymphocytes (>99% viability).

ANIMALS AND CHEMICALS

To ensure the validity of the assay, one mouse exposed to a control mutagen (cyclophosphamide) and one unexposed mouse were included each day; 24 mice were used in total. The animals, 18–22 g female C57BL/6 mice (B and K, Uppsala, Sweden), were allowed free access to tap water and a standard pellet diet (Ewos AB, Södertälje, Sweden) and kept at 23°C with a 12/12 hour light/dark cycle. Sixteen hours before each blood sample was taken, one mouse was given an intraperitoneal injection of physiological saline (10 µl/g body wt) and at the same time another was injected with a freshly prepared solution of cyclophosphamide in physiological saline (150 mg/kg body wt). On the day of the blood sampling, the animals were killed by CO₂ asphyxiation and 0.5 ml blood was collected as described previously.¹⁴ Unless stated otherwise, all chemicals were of analytical quality and purchased from Sigma Chemical, St Louis, MO, USA.

ALKALINE SINGLE CELL GEL ELECTROPHORESIS AND EVALUATION OF DNA DAMAGE

The comet assay was performed under alkaline conditions by a slightly modified procedure described in detail elsewhere.^{10–14} Briefly, a mixture of an aliquot of 10 µl of a freshly prepared suspension of lymphocytes and 75 µl 0.6% low melting point agarose (International Biotechnologies, New Haven, USA) in Dulbecco's phosphate buffered saline (PBS) (pH 7.4) was layered on top of a microscope slide (Menzel, Germany) precoated with low melting point agarose. After lysis for one hour at 4°C in 2.5 M NaCl: 100 mM Na₂-EDTA: 10 mM Trizma base: 1% sodium lauryl sarcosinate (pH adjusted to 10 with NaOH), adding 1% Triton X-100 and 10% dimethyl sulphoxide (DMSO) just before use, the slides were transferred to a separate tank containing electrophoresis buffer (1 mM Na₂-EDTA and 300 mM NaOH, pH > 13). After unwinding of DNA for 30 minutes at 15°C, excess buffer was carefully removed from the slides before they were transferred to the electrophoresis unit. Single cell gel electrophoresis was performed in freshly prepared electrophoresis buffer for 15 minutes at 15°C, with a field strength of 0.7 V/cm. After electrophoresis, the slides were neutralised with 0.4 M Trizma buffer (pH 7.5), dried at room temperature, and kept in a sealed container before analysis.

The slides were stained with ethidium bromide in water (20 µg/ml; 50 µl/slide) and examined at 500 × magnification with a fluorescence microscope attached to a black and white CCD video camera connected to a computer based image analysis system.¹⁵ In each individual experiment, 50 "comets" per slide (three to four slides per human subject, two slides per mouse) were randomly captured at a constant depth in the gel, avoiding the edges of the gel, occasional dead cells (<1% of all cells), and superimposed comets. The image analysis program Aequitas (1A, version 1.2, Dynamic Data Links, Cambridge, UK) and the

special application for the comet assay Auto-Cell (version 8A, Reppalon AB, Hågersten, Sweden) were used for automatic analysis of digitised images. Tail moment, tail inertia, and tail length were used as indicators of DNA damage.¹⁵ All calculations were based on absolute intensities, and units for length are given as number of pixels.

STATISTICAL METHODS

The one tailed Kolmogorov-Smirnov two sample test was used to compare the cell populations from the cyclophosphamide exposed mouse and the unexposed mouse respectively in the laboratory validation procedure each day. After decoding the samples, the potential DNA damaging effect of sewage work was analysed. The mean and median values of each subject's tail moment, tail inertia, and tail length were standardised by division by the corresponding value for the untreated mouse on the same day. Based on the pooled group data from the 12 different sampling occasions, the standardised mean tail moment, tail inertia, and tail length were compared with a *t* test for independent samples. Pairs of exposed and unexposed subjects were matched for age, smoking habit, and day of blood sampling, for comparison of the standardised mean tail moments with a *t* test for paired data. After testing the normality of the data (Kolmogorov-Smirnov and Shapiro-Wilk tests), normality was rejected in each exposure class for the standardised mean tail moment as recorded, but not in natural logarithmic (ln) units. Thus, the paired *t* tests were performed for ln of standardised mean tail moment. Two sample *t* tests and linear regression were used to assess potential confounding. The level of significance was set at 5% in all tests, and all *P* values were two tailed, unless stated otherwise.

ASSESSMENT OF CONFOUNDING

A two sample *t* test performed on ln of standardised mean tail moment grouped on each dichotomous confounder variable resulted in *P* values >0.15 for all variables but

Table 2 Comparison in pairs (paired *t* test) of DNA damage (ln of standardised tail moment computed from the comet assay) in peripheral lymphocytes from exposed sewage workers and controls, matched for age, tobacco smoking, and day of blood sampling (three alternative classifications of exposure were used)

Exposure classification	Pairs n	Paired differences (controls - exposed)		<i>P</i> value
		Mean	(95% CI)	
A	17	-0.009	(-0.57 to 0.55)	0.97
B	11	0.07	(-0.64 to 0.78)	0.83
C	6	0.54	(-0.47 to 1.6)	0.23

intake of vitamins (*P*=0.11). However, those that consumed vitamins had on average more DNA damage, which is contradictory to our original assumption that certain antioxidant vitamins might protect against DNA damage. Linear regression with the dependent variable ln of standardised mean tail moment and the exposure variable (classes A, B, and C respectively in separate analyses) testing one of the potential confounders at a time did not indicate confounding in this study.

Results

After comparison of the positive and the negative mouse samples with the one tailed Kolmogorov-Smirnov two sample test, the comet assay was judged valid for each of the 12 sampling occasions. The total group means (SDs) of tail moment for the entire study period were, for the mice given cyclophosphamide 236 (84), for the mice given physiological saline 70 (44), and for the human subjects (exposed and unexposed combined) 42 (21).

There were no differences in the standardised mean tail moment, mean tail inertia, or mean tail length of the pooled lymphocytes from the exposed workers and their controls (table 1). In a paired comparison of the subjects matched for age, tobacco smoking, and day of blood sampling, with each subject's standardised mean tail moment as the indicator of DNA damage, workers exposed to sewage and controls were found to have the same level of damage (table 2).

Discussion

This study gave no indication that sewage workers have increased damage in the DNA of lymphocytes as measured by the comet assay, but this does not necessarily contradict the previous findings of an increased incidence of cancers.

The validity of the present study was probably not affected by the loss of subjects invited to the study. Nor do we consider the magnitude of the loss critical for the conclusions of the study or that it is likely that there has been any selection of possible genotoxic exposures in either group. Exposure misclassification could have had an effect as several controls had occasional exposure to waste water and this possibility was considered in the data analysis by the use of three different exposure classifications.

One potential source of error in any study may be instability in measurements when they are performed on several different occasions. A

Table 1 Comparison of pooled measures from the comet assay describing DNA damage in lymphocytes from exposed sewage workers and controls by three alternative exposure classifications

Group	Exposure classification	Standardised tail moment mean (SD, median)	Standardised tail inertia mean (SD, median)	Standardised tail length mean (SD, median)
Controls (n=31)	A	0.69 (0.45, 0.53)	0.65 (0.46, 0.52)	0.96 (0.22, 0.96)
Exposed (n=34)		0.69 (0.43, 0.65)	0.66 (0.46, 0.64)	0.95 (0.17, 0.90)
Rejected (n=0)				
Controls (n=42)	B	0.69 (0.42, 0.52)	0.65 (0.44, 0.51)	0.97 (0.20, 0.98)
Exposed (n=23)		0.70 (0.47, 0.67)	0.66 (0.50, 0.66)	0.92 (0.17, 0.88)
Rejected (n=0)				
Controls (n=13)	C	0.69 (0.33, 0.50)	0.64 (0.34, 0.53)	0.97 (0.20, 0.97)
Exposed (n=23)		0.70 (0.47, 0.67)	0.66 (0.50, 0.66)	0.92 (0.18, 0.88)
Rejected (n=29)				

variation in the outcome measures used in the biomonitoring of genotoxic agents may have several causes including the actual effect of the exposure under study, a contribution from background exposures, differences in susceptibility to genotoxic agents, and technical variability in the assay. Potential sources of error in the comet assay have been analysed in a recent study, and the results applied to the present study.¹³ When studying populations with low level exposures, probably close to the normal background level, one has to analyse samples from more people than can be processed simultaneously with techniques presently used. The day to day stability of the method must therefore be ensured, and any possible variability compensated for in the study design, or in the statistical analyses. In the present study we attempted to correct this problem in several ways. Day to day variation in the laboratory procedure was considered by sampling the exposed subject and matched control on the same day, analysing their samples in the same batch, and performing the statistical analysis in pairs. As both groups of workers had jobs requiring occasional emergency work, we lost several pairs as they could not attend for testing on the same day, and we therefore lost power in the paired analyses. Standardisation of the measures of human DNA damage against the negative mouse controls also improved the day to day stability of the method. The rationale for using this standardisation is our assumption that the untreated mouse data, to some extent at least, reflect the drift in laboratory conditions. The inbred and uniformly treated mice are probably homogeneous for possible confounding and exposures of importance for the outcome measures studied.

The lack of increased primary DNA damage may be due to the use of an inappropriate outcome measure. We used lymphocytes as target cells because they are easily obtained but it may be that they are not the best cell type to use for the assessment of genotoxic damage in those organs in which cancer has been reported among sewage workers. The single cell gel assay is insensitive to DNA damage induced in mouse lymphocytes by repeated oral doses of benzo(a)pyrene whereas mouse hepatocytes do show such damage.¹⁴ On the other hand, lymphocytes are highly sensitive to other known genotoxic substances such as cyclophosphamide.^{11 12}

There are several possible explanations as to why our failure to show increased primary DNA damage in the sewage workers is not at odds with previous findings of an increased risk of some types of cancers, besides the possibility that the previously observed risks are chance findings. One may be the difference in time of the exposure in the epidemiological studies and the present study. Thus the cancer incidence studies reflect exposure which took place more than 10 years ago and current exposures may be insufficient to constitute a cancer risk. Improvements in the working conditions at the sewage plants during the past decades may have substantially reduced the level of exposure

to genotoxic substances. It is also possible that the cancers found were caused by exposures specific to certain plants, or by epigenetic carcinogens which are not detected by the comet assay. One of the cancers that has been found in excess among sewage workers is gastric cancer, which has recently been connected with infection by *Helicobacter pylori*. However, in a recent study we found no increase in this infection in sewage workers.¹⁶

This study of genotoxic damage in lymphocyte DNA from sewage workers had two purposes. The first was to discover possible genotoxic exposures among sewage workers, and the second was to test the comet assay in an epidemiological study outside the laboratory. Our primary conclusion was that we found no evidence to suggest that sewage workers have a greater exposure to genotoxic substances than other municipal workers. For the second objective, the comet assay proved to be practicable as an adjunct to an epidemiological study, although demanding. This report has shown that there are several pitfalls in performing epidemiological studies with molecular genetic assays and not just with the comet assay. There is a need to develop and evaluate the comet assay further to make it a standardised routine method for epidemiological studies. As for other presently used molecular bioassays, there is a need to characterise the dose-response relations when using it for exposure assessment, especially at the low level exposures that are of interest in environmental epidemiology. It will also be necessary to describe the relations of the assay to the effect of interest which, in this study, was cancer.

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- 1 Lafleur J, Vena JE. Retrospective cohort mortality study of cancer among sewage plant workers. *Am J Ind Med* 1991;19:75-86.
- 2 Friis L, Edling C, Hagmar L. Mortality and incidence of cancer among sewage workers: a retrospective cohort study. *Br J Ind Med* 1993;50:653-7.
- 3 Rapiti E, Sperati A, Fano V, Dell'Orco V, Forastiere F. Mortality among municipal workers in garbage handling, waste incinerating and sewage treatment: a retrospective cohort study. *Epidemiology* 1995;6:S135.
- 4 Rappaport SM, Richard MG, Hollstein MC, Talcott RE. Mutagenic activity in organic wastewater concentrates. *Environ Sci Technol* 1979;13:957-61.
- 5 Scarlett-Kranz JM, Babish JG, Strickland D, Goodrich RM, Lisk DJ. Urinary mutagens in municipal sewage workers and water treatment workers. *Am J Epidemiol* 1986;124:884-93.
- 6 Babish JG, Johnson BE, Lisk DJ. Mutagenicity of municipal sewage sludges of American cities. *Environmental Science and Technology* 1983;17:272-7.
- 7 Ottaviani M, Crebelli R, Fuselli S, La Rocca C, Turrio Baldassari LT. Chemical and mutagenic evaluation of sludge from a large wastewater treatment plant. *Ecotoxicol Environ Saf* 1993;26:18-32.
- 8 Laitinen S, Kangas J, Kotimaa M, Liesivuori J, Martikainen PJ, Nevalainen A, et al. Workers' exposure to airborne bacteria and endotoxins at industrial wastewater treatment plants. *Am Ind Hyg Assoc J* 1994;55:1055-60.
- 9 Elia VJ, Clark CS, Majeti VA, Gartside PS, MacDonald T, Richdale N, et al. Hazardous chemical exposure at a municipal wastewater treatment plant. *Environ Res* 1983;32:360-71.
- 10 Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantification of low levels of DNA damage in individual cells. *Exp Cell Res* 1988;175:184-91.
- 11 McKelvey-Martin VJ, Green MHL, Schmezer P, Pool-Zobel BL, De Méo MP, Collins A. The single cell gel electrophoresis assay (comet assay): a European review. *Mutat Res* 1993;288:47-63.

- 12 Fairbairn DW, Olive PL, O'Neill KL. The comet assay: a comprehensive review. *Mutat Res* 1995;339:37-59.
- 13 Hellman B, Vaghef H, Friis L, Edling C. Alkaline single cell gel electrophoresis of DNA fragments in biomonitoring for genotoxicity: an introductory study on healthy human volunteers. *Int Arch Occup Environ Health* 1997;69:185-92.
- 14 Vaghef H, Wisén A-C, Hellman B. Demonstration of benzo(a)pyrene-induced DNA damage in mice by alkaline single cell gel electrophoresis: evidence for strand breaks in liver but not in lymphocytes and bone marrow. *Pharmacol Toxicol* 1996;78:37-43.
- 15 Hellman B, Vaghef H, Boström B. The concepts of tail moment and tail inertia in the single cell gel electrophoresis assay. *Mutat Res* 1995;336:123-31.
- 16 Friis L, Engstrand L, Edling C. Prevalence of *Helicobacter pylori* infection among sewage workers. *Scand J Work Environ Health* 1996;22:364-8.

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- 3 Weinstein L, Swartz MN. Pathogenic properties of invading micro-organisms. In: Sodeman WA Jr, Sodeman WA, eds. *Pathologic physiology, mechanisms of disease*. Philadelphia: W B Saunders, 1974:457-72.