ENVIRONMENTAL CONTAMINATION. A NEW MOLECULAR TECHNIQUE TO COMPLEMENT CYTOGENETIC ANALYSIS

CONTAMINACIÓN AMBIENTAL. UNA NUEVA TÉCNICA MOLECULAR PARA COMPLEMENTAR EL ANÁLISIS CITOGENÉTICO

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SUMMARY

The effects of environmental pollutants on organisms may be monitored in a number of ways and at different levels. Exposure to genotoxic chemicals results in the formation of covalently bound adducts between the genotoxin and the DNA and these may cause mutations and cytogenetic changes. The primary effects on DNA (i.e. adduct formation) may be monitored using ^{32}P- postlabelling, ELISA or HPLC. Secondary effects on DNA (cytogenetic damage, mutation) may be monitored using a number of biomarker assays capable of detecting DNA strand breaks (e.g. by the alkaline unwinding assay or the Comet assay), unscheduled DNA synthesis, micronuclei, chromosome aberrations, sister chromatid exchanges and phenotypic and genotypic changes due to mutation. The sensitivity and specificity of these assays is variable. Recent developments in molecular biology such as DNA fingerprinting and gene amplification by the polymerase chain reaction (PCR) offer new possibilities for detecting DNA damage. We examined whether an alternative biomarker assay using DNA fingerprinting by arbitrarily primed PCR (AP-PCR) can show differences in the DNA fingerprints of individual animals exposed to benzo(a)pyrene in the laboratory and of animals from control and from polluted areas. The results indicate that DNA fingerprinting by AP-PCR offers a useful alternative biomarker assay for the detection of the genotoxic effects of environmental pollutants.

RESUMEN

El efecto de los contaminantes ambientales sobre los organismos puede ser controlado de diferentes modos y a distintos niveles. La exposición a genotóxicos de tipo químico determina la formación de uniones de tipo covalente entre los genotóxicos y el DNA y ello, puede causar mutaciones y cambios citogenéticos. Los efectos primarios sobre el DNA (por ejemplo formación de acercamientos) pueden ser controlados usando ELISA o HPLC después de marcar con ^{32}P. Los efectos secundarios sobre el DNA (daño citogenético, mutación) pueden ser controlados empleando una variedad de ensayos con biomarcadores capaces de detectar la rotura de los extremos del DNA (por ejemplo mediante ensayos de desenrollado alcalino o el ensayo Comet), síntesis de DNA no catalogada, micronúcleos, aberraciones cromosómicas, cambios entre cromátidas hermanas y

cambios genotípicos y fenotípicos debidos a mutaciones. La sensibilidad y especificidad de esos ensayos es variable. Recientes avances en biología molecular tales como la identificación mediante el DNA y amplificación de genes mediante (PCR) ofrecen nuevas posibilidades para detectar deterioros del DNA. Se ha investigado, si un ensayo con un marcador alternativo usando identificación con DNA mediante PCR arbitrariamente primado, puede mostrar diferencias en las improntas DNA de individuos animales expuestos en el laboratorio al benzo(a)pireno y de animales de áreas control y áreas contaminadas. Los resultados indican que la identificación de DNA mediante AP-PCR ofrece un útil ensayo de biomarcaje alternativo para detectar los efectos genotóxicos de los contaminantes ambientales.

INTRODUCTION

Exposure of an organism to an environmental pollutant triggers a series of events which culminate in an effect on the population and the community. These events start with the binding of the pollutant to a receptor and are followed by a biochemical response, physiological changes, an effect on the individual and finally an effect on the population and community. The early events require only seconds or minutes but the later effects require a much longer time extending to months or years and are of increasing importance. The effects of environmental pollutants may be monitored using a variety of biomarker assays including enzyme inhibition or induction, organ function and immune responses.

Polycyclic aromatic hydrocarbons (PAHs) are widespread in the environment and some act as mutagens and carcinogens in man and in aquatic species (Peakall, 1992) following their enzymatic conversion to electrophilic metabolites; these metabolites form adducts with DNA thus altering the structure of the DNA directly. Benzo[a]pyrene (BP), which has been described as the ultimate carcinogenic PAH, is metabolised to the highly reactive benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE) by the action of monooxygenases and epoxide hydrolases. BPDE is an electrophilic compound that can form both stable covalent adducts with guanines and alkali-labile apurinic sites on DNA, as well as non-covalent adducts that cause DNA strand breaks. If the metabolites of BP are not inactivated or excreted, or if the DNA damage is not repaired, a high dose, or even low but continuous levels of exposure, can lead to severe damage especially in tissues with slow turnover and/or inadequate capacity for recovery.

In the case of genotoxic pollutants, the effects on the DNA have so far been monitored using a range of biomarker assays. Primary events (DNA adduct formation) may be monitored using ^{32}P-postlabelling (Lloyd-Jones, 1995), immunoassays with either monoclonal or polyclonal antibodies to BPDE-DNA adducts (Santella, 1988), HPLC combined with fluorescence spectroscopy and gas chromatography combined with mass spectroscopy. Secondary modifications of the DNA may be monitored by detecting DNA strand breaks (for example using the alkaline unwinding assay (Shugart, 1988) or the Comet assay (Ostling and Johanson, 1984)) and by measuring hypomethylation and unscheduled DNA synthesis. Irreversible effects may be monitored using flow cytometry and by detecting cytogenetic changes (such as visible chromosomal abnormalities, sister chromatid exchanges and micronuclei) and mutations (activation of oncogenes

and measurement of mutation rates). Molecular biological procedures such as restriction fragment length polymorphism (RFLP) analysis (Botstein *et al.*, 1980), DNA fingerprinting (Jeffreys, 1987) and gene amplification by the polymerase chain reaction (PCR) (Saiki *et al.*, 1988) offer new possibilities for detecting DNA damage. Recently, the PCR has been used with only one oligonucleotide primer at relatively low stringency to generate DNA fingerprints (Upcroft *et al.*, 1990; Williams *et al.*, 1990; Welsh and McClelland, 1990); such DNA finger-prints have been used to distinguish between species, between different isolates of the same species and between individuals. In this paper we propose that DNA fingerprints obtained using the PCR may be employed reliably to detect damage to the DNA caused by chemicals such as BP.

DNA FINGERPRINTING

Although each individual is genetically unique (except identical twins), most DNA varies very little between individuals but some of the existing variation may result in genetic disease. However, regions of extreme variability are known to occur mainly in *silent* regions of the chromosomes and they have no effect on the individual. Such variable regions are referred to as *minisatellites* (Jeffreys, 1987) which are scattered along chromosomes and their detection provides a means for identifying each individual by DNA fingerprinting; depending on the DNA probe used, these DNA fingerprints may be individual-, population- or species-specific.

DNA fingerprints may be revealed using a variety of methods. Some of these are based on DNA:DNA hybridization either with microsatellite probes or with single locus probes. These procedures involve isolation of the DNA from a sample and using restriction enzymes to generate millions of different DNA fragments some of which contain minisatellites. The DNA fragments are separated by gel electrophoresis and after treatment to separate the two strands of the double helix in each DNA fragment, Southern blotting (Southern, 1975) is used to transfer the single stranded DNA from the gel to a membrane without disrupting the pattern in the gel. The membrane is incubated with a radioactive DNA probe - a segment of single stranded DNA which detects and forms a double helix with any fragments on the membrane containing minisatellites which thus become radioactive. The minisatellites are visualised by exposure of the membrane to x-ray film. Such methods have a number of disadvantages including the requirement for large amounts of very pure and undegraded DNA, the long time necessary, the use of radioactive nucleotides and the requirement for a certain amount of DNA sequence information to enable the choice of the right DNA probe. Most of these problems may be avoided by using methods of fingerprinting based on the PCR (Upcroft *et al.*, 1990; Williams *et al.*, 1990; Welsh and McClelland, 1990).

THE POLYMERASE CHAIN REACTION

The PCR (Saiki *et al.*, 1988) is a fast and efficient cycling procedure for amplifying specific DNA sequences by

millions of times *in vitro*. The PCR requires a target sequence to be amplified in a DNA sample, two synthetic oligonucleotide primers (usually about 20 nucleotides long) complementary to opposite strands of the DNA and flanking the target sequence, the four deoxynucleotide triphosphates and a thermostable DNA polymerase. Once the reaction components have been assembled in the reaction tubes, a number of cycles of PCR (typically 25-45) are performed with each cycle consisting of three basic steps: thermal denaturation of the double stranded DNA at 95°C, annealing of the primers to the complementary sequence on the DNA template at an appropriate temperature depending on the sequence of the primers and DNA synthesis at 72-74°C to extend the primers thus amplifying the target sequence. The PCR products can then be analysed by gel electrophoresis. The amount of starting material required is extremely small (in theory, a single copy of the target sequence) and in cases where there is only one target sequence flanked by two specific oligonucleotide primers there is only one amplification product; if more than one pair of primers is used then more than one PCR product is generated. The drawback of such PCRs is that some DNA sequence information is essential for the design of PCR primers and this may not be available.

DNA FINGERPRINTING USING THE PCR

The requirement for some DNA sequence information may be eliminated by using one of a number of different modifications of the basic PCR procedure which utilise only one short random oligonucleotide primer (usually 10 nucleotides long) instead of two longer ones (Upcroft *et al.*, 1990; Williams *et al.*, 1990; Welsh and McClelland, 1990). Also, in order to maximize the number of PCR products generated, the reaction conditions may be made less stringent by reducing the annealing temperature at least for the first few cycles of the PCR. The modified procedures are commonly referred to as arbitrarily primed PCR (AP-PCR) or random amplified polymorphic DNAs (RAPDs). The result of these modifications is that the reaction generates a number of products of various lengths which can be separated by gel electrophoresis to obtain a *ladder* of PCR products or a DNA fingerprint. Such DNA fingerprints have been used to distinguish between organisms from different species, between different isolates of the same species and between individuals.

CAN DAMAGE TO THE DNA OF AN ORGANISM AS A RESULT OF EXPOSURE TO GENOTOXIC POLLUTANTS BE DETECTED BY DNA FINGERPRINTING BY PCR?

Exposure of organisms to environmental genotoxic polutants may result in stable damage to the DNA but the currently available methods for detection of such damage do not reveal it all. We asked whether the DNA fingerprints of individuals from control and from polluted areas were different and we investigated the possibility of using DNA fingerprinting by AP-PCR as an alternative biomarker assay (Castellani *et al.*, 1993:

Savva *et al.,* 1994; Walker *et al., 1995).* We hypothesized that DNA adduct formation would alter the DNA fingerprints of individuals due to the fact that the presence of an adduct in a priming site on the DNA would prevent the binding of the primer to that priming site. In addition, the DNA fingerprints will be altered when priming sites are lost or gained as a result of mutations and when certain PCR products are not synthesized as a result of DNA strand breaks.

Briefly, animals from control populations and from those exposed to genotoxic pollutants are collected and DNA is isolated from the tissue of interest using established procedures (Sambrook *et al.,* 1989). An aliquot of the DNA is subjected to AP-PCR and the products are analysed by gel electrophoresis; staining of the gels with ethidium bromide allows visualisation of the DNA under ultraviolet light. The DNA fingerprints of control and exposed animals are then compared and may be analysed statistically.

The potential use of AP-PCR as a biomarker assay was investigated in preliminary studies using rats injected with BP (Castellani *et al.,* 1993: Savva *et al.,* 1994). A blood sample was taken from each animal before exposure to the pollutant; at the end of the exposure period (2-4 weeks) the animals were sacrificed and the livers were removed. DNA isolated from these samples was amplified by AP-PCR using a number of different primers. The DNA fingerprints obtained were found to be reproducible and the results showed that the oligonucleotide primers used could be divided into the following four categories:

i) those which did not yield any visible PCR products either because the PCR was not performed at the correct conditions or because the primers did not anneal to the target DNA;

ii) those which produced DNA fingerprints consisting of only very few bands or, in some cases, a smear of DNA fragments without any clear banding pattern;

iii) those which generated DNA fingerprints consisting of more than 10 clear bands. Although the results showed that the fingerprints of different rats before exposure to BP were different suggesting that these primers were not generating species-specific fingerprints, the results also showed that in some cases there were differences in the fingerprints of individual rats before and after exposure. Therefore, such fingerprints may still be used to show genotoxic effects when individual animals can be sampled at different times before and after exposure to a pollutant (for example, when sequential sampling can be performed on animals exposed to a mutagen under laboratory conditions or animals deployed in the field); and

iv) those which produced identical fingerprints for all the rats before exposure to BP. This indicates that these primers can be used to produce colony-specific, and possibly species-specific, DNA fingerprints. Some primers did not show any differences in fingerprints generated by DNA isolated before and after exposure to BP. However, with some primers the DNA fingerprints obtained before and after exposure to BP showed differences due to the disappearance of some of the PCR products and/or the appearance of new ones.

Thus, this preliminary study suggested that AP-PCR may be a useful procedure

for the detection of the genotoxic effects of environmental chemicals. Both colony-specific and individual specific DNA fingerprints were obtained depending on the oligonucleotide primer used in the PCR and with both types of fingerprints, some differences were observed when fingerprints were compared before and after exposure to BP.

Current studies on crabs confirm these preliminary results. DNA was isolated from the midgut gland of BP-exposed and unexposed crabs; this DNA was used to generate DNA fingerprints comparison of which showed obvious differences between the two groups of animals (Castellani *et al,* manuscript in preparation). In parallel studies on DNA from crabs exposed to BP in the laboratory, we demonstrated the presence of DNA adducts using ELISA techniques and have shown changes in the DNA fingerprints. Recently, RAP-PCR (RNA arbitrarily primed PCR) (Liang and Pardee, 1992) which uses an arbitrary oligonucleotide primer in both the synthesis of single stranded complementary DNA (cDNA) and the PCR has been used to identify PCR products that were present in human fibroblast cells exposed to methyl methane sulphonate (MMS) but not in unexposed cells (McKenzie and Buchner, 1995).

FINAL REMARKS

DNA fingerprinting by AP-PCR offers a useful alternative biomarker assay for the detection of the genotoxic effects of environmental pollutants. The extreme sensitivity of the PCR means that only very small quantities of DNA are required and that radioisotopes are not necessary. Changes observed in the fingerprints of control and exposed animals may be due to the presence of DNA adducts many of which will eventually be repaired and not cause mutations; however, some of the changes may be the result of mutations or DNA strand breaks. Consequently, AP-PCR offers an advantage over most of the currently used techniques which rely on the detection of mutations or gross chromosomal abnormalities; AP-PCR may offer the possibility of a biomarker assay that can be used as an early warning system since it is capable of detecting temporary changes which may not finally manifest themselves as mutations.

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