

Case Report

The Salmonella/Micro Some Micro Suspension Assay and Genetic Polymorphism Analysis in a Case-Report of Air Pollution in Rio De Janeiro, Brazil

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Abstract

The use of strains of Salmonella enteric serovar Typhimurium with different metabolic capacities can indicate the class or classes of compounds present in an environmental sample and enable the diagnosis of the mutagenic activity of these pollutants adsorbed on particulate matter (PM) in the air. The sensitivity of Salmonella strains TA98NR, TA98/1, 8-DNP6, YG1021, and YG1024 to detect nitro compounds adsorbed on samples of PM 2.5 was compared from three sites in Rio de Janeiro city. Samples were collected using a high-volume sampler at three sites: one with light traffic and two with heavy traffic. The assays were performed in the presence of 10-50 µg/plate organic extracts with and without exogenous metabolism. The YG1021 and YG1024 strains showed the highest rev/m3 values, confirming their enhanced sensitivity. As YG1024 also demonstrated sensitivity to nitro and amino compounds, we suggest its use in research into environmental air contamination. Individual susceptibility to chemically induced cancer may be partly explained by genetic differences in the activation and detoxification of procarcinogens. Numerous polymorphisms of metabolism genes have been identified and their role in individual susceptibility to cancer has been observed. We evaluated genetic polymorphisms and risk of lung cancer in Rebouças tunnel workers (Rio de Janeiro – Brazil). Deletions of GSTM1 and GSTT1 genes were detected in 100% of samples from control group. In exposure group, single deletions of GSTM1 or GSTT1 genes were detected in 72.74% (n=1) to 86.37% (n=13), respectively. Deletions of both genes were detected in 100% (n = 15). The same individual showed CYP1A1*2B polymorphism and deletions of GSTM1 and GSTT1 genes. These results demonstrate that this individual exposed to occupational pollution at Rebouças tunnel, may have intensified metabolizing pollutants such as PAH, and have difficulties in detoxification of metabolites of these pollutants, increasing the risk of lung cancer development.

INTRODUCTION

The Salmonella/micro some micro suspension assay has been used for large, multisite, and/or time series studies, for bioassay-directed fractionation studies, for identifying the presence of specific classes of mutagens, and for site or source comparisons of relative levels of airborne mutagens [1]. The use of strains of Salmonella enteric serovar Typhimurium with different metabolic capacities can indicate the class or classes of compounds present in an environmental sample [2,3]. The Salmonella/microsome assay identifies the presence of organic compounds and enables diagnosis of the mutagenic activity of these pollutants adsorbed on particulate matter (PM) in the air. The responses are related to the presence of a variety of compounds, including polycyclic aromatic hydrocarbons (PAHs) and nitroarenes [4,5]. Nitroarenes are a class of environmentally hazardous compounds [6]. These nitro compounds have been

detected in the extracts of diesel and gasoline emissions, fly ash, cigarette smoke condensates, and home heater emissions [7,6]. Typical nitroarenes, such as 2-nitrofluorene, 1-nitropyrene (1-NP), and 1,8-dinitropyrene (1,8-DNP), are potent mutagens [8,6]. The contributions of nitroarenes to direct mutagenic activity are commonly investigated through the Salmonella/microsome assay with the following S. enteric serovar Typhimurium strains: TA98 (frame shift strain) and the derivative strains TA98/1.8-DNP6 (O-acetyl transferase-deficient), TA98NR (nitroreductase-deficient) [9], YG1021 (nitroreductase-overproducing), and YG1024 (O-acetyltransferase-overproducing) [10]. Genotoxic evaluation studies of particulate matter using these strains have been conducted previously [11,12,5,3,13-17]. Many studies have shown that variant CYP1A1 alleles combined with GSTM1 and GSTT1 nulls genotype are associated with an increased risk of lung cancer [18-21]. Damage to the genetic material and high

concentrations of PAH metabolites were detected in biological samples taken from Rebouças tunnel workers and can be related to daily exposure to pollutants in the tunnel [22].

CASE PRESENTATION

In the present study, comparisons were made of the sensitivity of strains of *Salmonella* enteric serovar Typhimurium to detect nitro compounds adsorbed on samples of PM 2.5 collected at three sites in the city of Rio de Janeiro: site 1, State University of Rio de Janeiro Capus with light traffic, is located in a residential area of the city northern zone; site 2, Brazil Avenue has heavy traffic (~250,000 vehicles/day) and is the city longest expressway, covering 58 km in length and crossing 27 neighborhoods and site 3, Rebouças Tunnel that also has heavy traffic (~190,000 vehicles/day) and is a 2.8 km-long tunnel that connects the northern and southern zones of the city [15-17]. We also evaluated genetic polymorphisms in CYP1A1 (*2B and *4), GSTM1 and GSTT1 and risk of lung cancer in Rebouças tunnel workers (Rio de Janeiro – Brazil). The exposed group was formed by fifteen male workers tunnel Rebouças with age between 25 and 64 years. Peripheral blood samples were collected in tubes containing EDTA during the working day. After collection, the material were cooled and transported to our laboratory. The same procedures were carried out with volunteers in the control group, formed by eleven servants of the State University of Rio de Janeiro, male with age between 30 and 60 years. All procedures were submitted and approved by the National Research Ethics Committee - CONEP (CAAE N°. 27402014.6.0000.5259).

Salmonella/micro some assay

The organic extracts [15 -17] were assayed for mutagenicity using the microsuspension version [23] of the *Salmonella*/microsome assay [24]. *S. enteric* serovar Typhimurium TA98 (frame shift strain) and the derivative strains TA98/1.8- DNP6 (O-acetyl transferase-deficient) and TA98NR (nitroreductase-deficient) [9] were used, with and without metabolic activation (S9 mix fraction). Five concentrations of each sample (10, 20, 30, 40, and 50 µg/plate) were tested in triplicate. The samples were pre-incubated for 90 min. All assays were carried out under yellow light and in the presence of negative (5 µL/plate DMSO solvent) and positive (0.5 µg/plate 4-nitroquinoline oxide; CASRN. 56-57-5; Sigma Chemical Company; St. Louis, MO, USA) controls. Plates were incubated in the dark at 37°C for 72 h, after which time revertants were counted. The sample was considered to be positive when the mutagenesis value was at least twice the negative value, and when a significant ANOVA ($P < 0.05$) and a positive dose response rate ($P < 0.05$) were observed. The results of the different assays were analyzed using the SALANAL program (*Salmonella* Assay Analysis, version 1.0; Integrated Laboratory Systems; Research Triangle Park, NC, USA). The choice between linear regression and the Bernstein model [25] was made to allow the elimination of data for doses outside the linear portion of the dose-response curve. Positive results were interpreted as presenting significant mutagenicity. Positive responses were expressed as the number revertants per volume of air sampled (rev/m³), i.e., rev/µg multiplied by EOM in µg/m³. In the cytotoxicity test, the solutions containing the sample and

the bacterial culture (100-200 cells) were plated on nutrient agar plates and incubated at 37°C for 24 h, and the surviving colonies were counted. The sample was considered to be cytotoxic if the percentage of surviving cells was less than 60% of the negative control at one or more doses [13].

Genetic polymorphism analysis of CYP1A1*2B and CYP1A1*4

The genetic polymorphism analysis for the CYP1A1*2B and CYP1A1*4 were characterized by RFLP after PCR second Cascorbi et al. [26], DNA was extracted by KIT Pure Link® Genomic. All primers were obtained from New England Biolabs (Uniscience, Brazil); PCR reactions were performed with a Biorad Thermal cycler-MyCycler.

For determination of CYP1A1*2B DNA fragment was amplified using 1 unit Taq polymerase, 10 µ mol/liter of primers a 204-bp fragment with primers M2F 5'-CTGTCTCCCTC'GCITFACAGGAAGC and M2R 5'-TFCCACCCGTTGCAGCAGGATAGCC (reagent concentrations as above; PCR conditions were 35 cycles of 0.5 min at 94°

C 0.5 min at 63°C, and 0.5 min at 72°C). The product was digested with BsrDI (New England Biolabs; 0.5 units). CYP1A1*4 could be determined from the same 204-bp fragment but using BsaI (New England Biolabs; 2.5 units). Both cleavage sites were lost in the case of the mutations and were evaluated on Diamond and 1.5% agarose gel. Genetic polymorphism analysis of GSTM1 and GSTT1 genes. The genetic polymorphism analysis for the GSTM1 and GSTT1 genes was determined simultaneously in a single assay using a multiplex PCR approach [27]. DNA was extracted by KIT Pure Link® Genomic. Briefly, isolated DNA (50ng) was amplified in a 25µl reaction mixture containing 0.5 mmol of each of the following GSTM1 primers: 5' - GAA CTC CCT GAA AAG CTA AAG C-3 and 5' GTT GGG CTC AAA TAT ACG GTG G-3 [15]; and the following GSTT1 primers: 5'-TTCTTACTGGTCTCACATCTC-3 and 5'-TCACCGGATCATGGCCAGCA-3 in the presence of 0.2 mmol/L dNTPs and 1.5 mmol/L de MgCl₂. The PCR conditions consisted of an initial melting temperature of 95° C (5 min) followed by 35 cycles of melting (94°C, 2 min), annealing (59°C, 1 min) and extension (72°C, 1 min). A final extension step (72°C, 10 min) terminates the process. The PCR products from amplification of GSTT1 and GSTM1 genes were then analyzed electrophoretically on Diamond and 1.5% agarose gel. The presence or absence of GSTT1 and GSTM1 genes was detected by the presence or absence of a band at 480 bp (corresponding to GSTT1) and a band at 215 bp (corresponding to GSTM1).

RESULTS AND DISCUSSION

Salmonella/microsome assay

The EOM values ranged from 5.54 to 9.66 µg/m³ at site 1, from 5.48 to 7.76µg/m³ at site 2, and from 20.93 to 25.05 µg/m³ at site 3 in the three months of study, as calculated elsewhere [17]. No cytotoxic effects were detected for any of the samples analyzed. The contribution of nitro compounds to the mutagenic activity of air samples from urban areas is associated with the presence of PAH derivatives. Among these, the mono- and dinitro-PAHs associated with oxygenated PAH have been correlated with direct frame shift mutagenic activity (11; 12; 5). The contribution of mono- and di-nitroarenes to direct mutagenic activity was

investigated though the Salmonella/microsome assay with strains TA98NR and TA98/1,8-DNP6, respectively. Nitroarenes are dependent upon this 'classic' nitroreductase to express their mutagenicity, as evidenced by their greatly decreased activity in the nitroreductase deficient strain [7]. However, some nitroarenes express all or a major fraction of their activity even in the absence of the 'classic' nitroreductase (e.g., 1,8-DNP). The fact that there is residual activity expressed in TA98NR (the microorganism deficient in the 'classic' nitroreductase), and the finding of the full expression of the mutagenicity of other chemicals in TA98NR has led to the conclusion that *S. entericaserovar Typhimurium* may contain additional nitroreductases as well as other specific enzymes. Indeed, it has been possible to construct bacterial strains lacking the enzyme that recognizes 1,8-DNP (e.g., TA98/1,8-DNP6) [7]. The lower mutagenic activity in nitroreductase-deficient and O-acetyltransferase-expressing strains, TA98NR and TA98/1,8-DNP6, compared to the parental strain TA98, indicated the participation of mono- and di-nitroarenes in the total mutagenicity of the extracts. The presence of mono-nitroarenes was detected at site 2 and site 3. A lower mutagenic response was observed for TA98NR (42% at site 2 and 94-95% at site 3) than was observed for TA98. At site 2 and site 3, a lower mutagenic response was observed for the TA98/1,8-DNP6 strain (28-75% at site 2 and 37-73% at site 3) than for TA98, indicating the presence of di-nitroarenes. The extent of the reduction in the mutagenic response detected at sites 2 and 3 can be attributed to the intense traffic in these areas of the city. Studies performed with TA98NR and TA98/1,8-DNP6 in areas contaminated by vehicular emissions and industrial activity in Rio Grande do Sul State of Brazil showed similar results: a reduction of 10-95% for TA98NR and a reduction of 61-85% for TA98/1,8-DNP6 [12,5]. Similar results were also detected in the industrial city of Cubatão, São Paulo [11]. Meanwhile, an absence of mutagenicity was observed at site 1, which is characteristic of residential areas with a low flow of vehicles. Mutagenic results were detected in the presence of metabolic activation for TA98NR and TA98/1,8-DNP6 for all sites. However, the decrease in revertants after metabolization was only observed in few periods. The effects of the S9 mix preparations on the mutagenicity of nitroarenes in the Salmonella strains revealed a broad spectrum of responses ranging from the absence of mutagenicity to an absolute requirement for mutagenic activation. In some instances, the presence of S9 mix permitted the expression of mutagenicity by nitroarenes even in nitroreductase-deficient microorganisms, indicating the presence of nitroreductase activity in the S9 mix [7]. However, it must be noted that, in general, arylamines exhibit much lower mutagenicity in the presence of S9 mix than do corresponding nitroarenes in the absence of S9 mix. Strains YG1021 and YG1024 are also commonly used in environmental studies [13, 14,17]. The higher mutagenic activity seen in over-expressing strains YG1021 and YG1024 compared to the parental strain TA98 indicates the participation of nitro and amine compounds, respectively. The extracts from sites 1, 2, and 3 were therefore also tested with strains YG1021 and YG1024, and showed the following results: increase in reversion for YG1021 (6-14% at site 1; 2-144% at site 2; 12-281% at site 3), and increase in reversion for YG1024 (3-38% at site 1; 105-155% at site 2; 1-145% at site 3) [17]. Two studies conducted in different parts of Rio Grande do Sul also detected the presence of nitro and

amino compounds through the high activity of strains YG1021 (23-264%) and YG1024 (554-1821%) [13,14]. This increase in the percentage of reversal shown by YG1021 and YG1024 is attributed to plasmids pYG216 and pYG219, which increase the sensitivity of these strains to nitro and amino compounds, respectively [6].

Genetic polymorphism analysis

Polymorphism CYP1A1* 2B was detected in all individual's exposure group. No CYP1A1 polymorphism was detected in control group. Deletions of GSTM1 and GSTT1 genes were detected in 100% of samples from control group. In exposure group, single deletions of GSTM1 or GSTT1 genes were detected in 72.74% (n=11) to 86.37% (n=13), respectively. A number of studies have analyzed a possible association between CYP1A1 polymorphisms with the risk to develop lung cancer [28]. In the present study we analyzed samples from 15 workers Rebouças tunnel (total 50 workers), as genetic polymorphisms in CYP1A1 (*2Band*4), GSTM1 and GSTT1. Our results shows apolymorphism CYP1A1*2B in an individual's exposure group. The strong relationship between polymorphism CYP1A1*2B and lung cancer was first found in the Japanese population [19,20]. CYP1A1*2B polymorphism was found in Asians at a high allelic frequency (0.22), followed by Latinos (0.16), and Caucasians (0.09), but not in Africans [29, 30]. In our population the CYP1A1*2B polymorphism is present at the same allelic frequency as in Caucasians [29,30]. Studies performed with Brazilian patients, CYP1A1*2B polymorphism also presented an increased risk of developing lung cancer [29,31]. Our results showed deletions of GSTM1 and GSTT1 genes in 100% of samples from control group. In exposure group, deletions of GSTM1 and GSTT1 genes were detected in 72.74% to 86.37%, respectively. In a study by Rossini et al. [32], with volunteer residents of the city of Rio de Janeiro found that 42.1% of the individuals had the GSTM1 null genotype, whereas 25.4% had the GSTT1 null genotype. The prevalence of the deleted GSTM1 genotype in North Americans was 51% and in Egyptians was 44% [33]. The prevalence of the deleted GSTT1 genotype among North Americans was reported by [33]. They reported that the prevalence of the null genotype was highest among African-Americans (21.8%) and Caucasians (20.4%), whereas the prevalence was lowest among Mexican-Americans (9.7%). As CYP1A1 is a phase I enzyme that is involved in carcinogen activation and GSTM1 and GSTT1 is a predominant phase II enzyme for deactivation, they may be complementary in their modulation of cancer risk [30]. CYP1A1 takes part in the activation of PAHs into diol epoxide metabolites in the lung, and GSTM1 and GSTT1 plays an important role in the detoxification of diol epoxide metabolites [30]. Epidemiological studies on Asian population have shown an association between increased risk of lung cancer and the combination of the GSTM1 null genotype and CYP1A1 variants [27].

CONCLUSION

1 - The Salmonella/microsome assay has been used for large, multi-site and/or time serie studies to identify the presence of specific mutagens and classes of mutagens. All the strains used in the present study are sensitive to nitroarene,

although strains YG1021 and YG1024 showed the highest rev/m3 values, thus confirming their enhanced sensitivity. YG1024 also demonstrated sensitivity to nitro and amino compounds. We recommend the use of strains YG1021 and YG1024 in research into environmental contamination by these pollutants.

2 - In our study, the same individual showed CYP1A1*2B polymorphism and deletions of GSTM1 and GSTT1 gens. These results demonstrate that this individual exposed to occupational pollution at Rebouças tunnel, may have intensified metabolizing pollutants such as PAH, and have difficulties in detoxification of metabolites of these pollutants, increasing the risk of lung cancer development.

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