

³²P-Postlabeling Detection of DNA Adducts in Mice Treated With the Herbicide Roundup

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Roundup is a postemergence herbicide acting on the synthesis of amino acids and other important endogenous chemicals in plants. Roundup is commonly used in agriculture, forestry, and nurseries for the control or destruction of most herbaceous plants. The present study shows that Roundup is able to induce a dose-dependent formation of DNA adducts in the kidneys and liver of mice. The levels of Roundup-related DNA adducts observed in mouse kidneys and liver at the highest dose of herbicide tested (600 mg/kg) were 3.0 ± 0.1 (SE) and

1.7 ± 0.1 (SE) adducts/ 10^8 nucleotides, respectively. The Roundup DNA adducts were not related to the active ingredient, the isopropylammonium salt of glyphosate, but to another, unknown component of the herbicide mixture. Additional experiments are needed to identify the chemical specie(s) of Roundup mixture involved in DNA adduct formation. Findings of this study may help to protect agricultural workers from health hazards and provide a basis for risk assessment. *Environ. Mol. Mutagen.* 31:55–59, 1998 © 1998 Wiley-Liss, Inc.

Key words: Roundup; herbicide; DNA adducts; ³²P-postlabeling

INTRODUCTION

Roundup is a nonselective systemic, broad spectrum, postemergence herbicide which is absorbed by the foliage and non-woody system and rapidly translocated throughout the plants. Roundup acts on various enzyme systems, thus interfering with the formation of amino acids and other important endogenous chemicals. It is very effective for the control of a great variety of annual, biennial, and perennial species of grasses, sedges, and broadleaf weeds. Roundup is used in many plantation crops (e.g., coffee, tea, bananas), in a wide range of crops (e.g., vegetables, cereals, kiwi fruits, cotton), on non-crop areas (e.g., public areas, parks, gardens, roadside applications, as well as home uses), and in water systems for aquatic weed control. The means of application today vary from hand-held sprayers to specialized large spray equipment. The increasing use of Roundup has led to a growing public awareness and concern about its possible toxicity. Roundup is a complex technical formulation, which contains 14.4–75% of glyphosate-isopropylammonium (CAS No.: 38641-94-0), glyphosate-trimesium (CAS No.: 81591-81-3), or glyphosate-sesquiosodium (CAS No.: 70393-85-0), as active ingredient and a large amount of different additives, coformulants, and surfactants that enhance the herbicidal activity of glyphosate salt [Kidd and James, 1991; Worthing and Hance, 1991].

The acute toxicity of Roundup has been evaluated in short-term feeding tests (13 weeks) and no effects have

been observed at 20,000 mg/kg diet in rats, while growth retardation and an increase of organ weights occur at 50,000 mg/kg diet in mice [WHO, 1994]. In long-term feeding studies, growth retardation, a hepatocyte hypertrophy or a necrosis, a urinary bladder epithelial hyperplasia, and a gastric inflammation have been shown at 20,000–30,000 mg/kg diet in rodents [WHO, 1994]. In multigeneration studies, a decrease of body weights and a renal tubular dilation have been found at 30,000 mg/kg diet in rodents [WHO, 1994]. The genetic activity of Roundup has been investigated in prokaryotes, green plants, insects, mammalian cells (in vitro), and rodents (in vivo). In in vitro tests, Roundup induces reverse mutations in *Salmonella typhimurium* TA100 and TA98, with and without metabolic activation, respectively; sex-linked recessive lethal mutations in *Drosophila* [Kale et al., 1995]; chromosomal damage in root-tips cells of *Allium cepa* [Rank et al., 1993], and in human lymphocytes [Vig-

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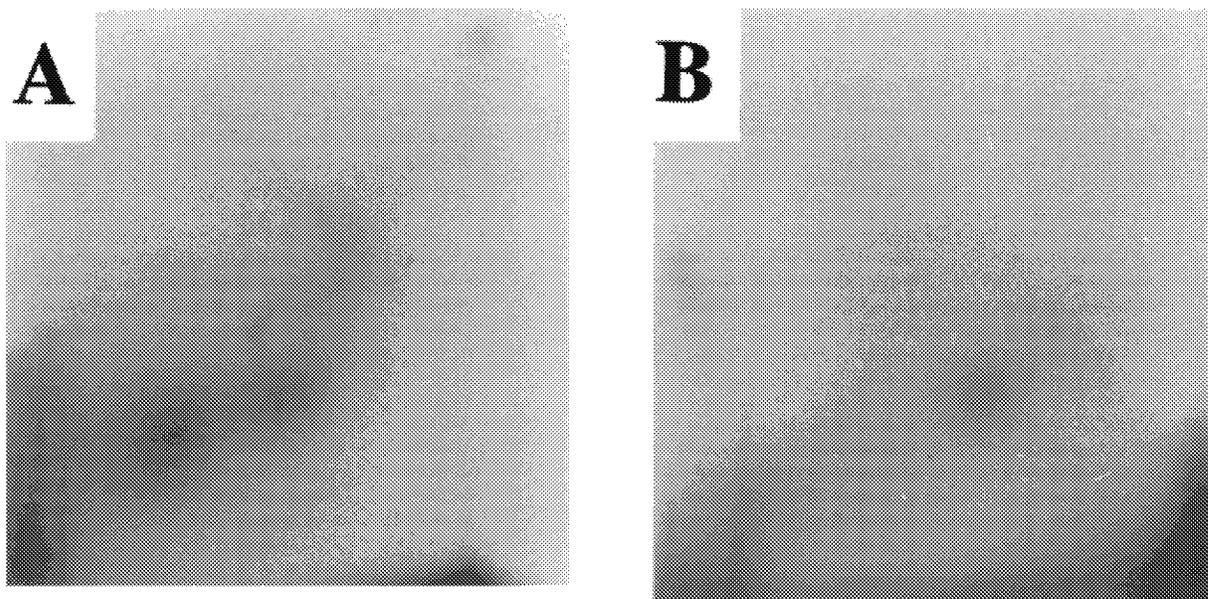


Fig. 1. DNA adduct spots detected in the kidneys (A) and liver (B) of mice treated with 600 mg/kg of Roundup formulation. Autoradiography was performed at -80°C for 72 hr.

fusson and Vyse, 1980; Bolognesi et al., 1997]. In *in vivo* studies, Roundup treatment has been shown to induce micronuclei in bone marrow, DNA single-strand breaks in liver and kidneys, and increased levels of 8-hydroxyguanosine in kidneys [Bolognesi et al., 1997].

Several assays have been developed to evaluate the genotoxic activity of chemical compounds. One of the most-used methods is the ^{32}P -postlabeling technique, which measures the formation of DNA adducts [Beach and Gupta, 1992]. The ^{32}P -postlabeling assay is very sensitive (one adduct for 10^{10} nucleotides), requires a minimum amount of DNA (1–10 μg) for the analysis, and is particularly useful in detecting DNA adducts of unknown nature and in evaluating the possible genotoxic effects of exposure to complex mixtures [Taningher et al., 1997]. The significant association between occupational exposure to pesticide mixtures and DNA adduct formation in peripheral white blood cells of greenhouse floriculturists working in the western part of the Liguria Region, Italy, evidenced a potential genotoxic risk linked to the exposure of these agrochemical compounds [Peluso et al., 1996].

The aim of the present study was to evaluate the capability of Roundup herbicide to induce the formation of DNA adducts using the ^{32}P -postlabeling technique.

MATERIAL AND METHODS

Chemicals and Animals

Roundup (30.4% isopropylammonium salt of glyphosate) was purchased from Monsanto, Milan, Italy. Isopropylammonium salt of glyphosate was from Società Italiana Chimici, Rome, Italy. Swiss CD1 male and female mice, 8–10 weeks old, were obtained from Charles

River, Como, Italy. Mice were treated by *i.p.* administration of Roundup (400, 500, and 600 mg/kg, corresponding to 122, 152, and 182 mg/kg of glyphosate salt) or isopropylammonium salt of glyphosate (130 and 270 mg/kg). All the compounds were dissolved in dimethylsulfoxide (DMSO) olive oil. Untreated mouse controls were included in the study. The animals were sacrificed after 24 hr and kidneys and liver were removed, frozen in liquid nitrogen, and stored at -80°C . The organs were separately pooled and DNA was isolated by a procedure involving enzymatic digestion of protein and RNA and solvent extraction [Peluso et al., 1991]. Appropriate animal usage and sacrifice procedures were followed.

^{32}P -DNA Postlabeling Technique

DNA (10 μg) was hydrolyzed with 4 μg of spleen phosphodiesterase (Boehringer) and 0.4 U of micrococcal nuclease (Sigma) for 3.5 hr at 37°C [Bolognesi et al., 1994]. Samples were then treated with 4 μg of nuclease P1 (Sigma) for 40 min at 37°C [Bolognesi et al., 1994]. The hydrolyzed-enriched adducted nucleotides were ^{32}P -labeled by incubation with 50 μCi of carrier free $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3,000 Ci/mmol, Dupont) and 5 U of T4 polynucleotide kinase (Pharmacia) for 40 min at 37°C [Peluso et al., 1997]. Resolution of ^{32}P -labeled DNA digests treated with nuclease P1 was carried out on polyethyleneimine cellulose thin layer chromatography plates (Merck) using the contact-transfer technique [Peluso et al., 1997]. The solvent system selected was: (D1) 1 M sodium phosphate buffer, pH 6.8; (D3) 3 M lithium formate, 7 M urea, pH 3.5; (D4) 0.7 M lithium chloride, 0.45 M tris-HCl, 7.7 M urea, pH 8.0; (D5) 1.7 M sodium phosphate buffer, pH 5.0. The chromatograms were then visualized by autoradiography for 72 hr at -80°C using Kodak XAR-5 films and intensifying screens. The level of DNA adducts was determined by excising areas of chromatograms and measuring the levels of radioactivity present by Cerenkov counting. Quantitation of normal nucleotides was carried out as previously described [Taningher et al., 1995].

RESULTS

In order to investigate the capability of Roundup to induce the formation of DNA adducts, mice were *i.p.*-

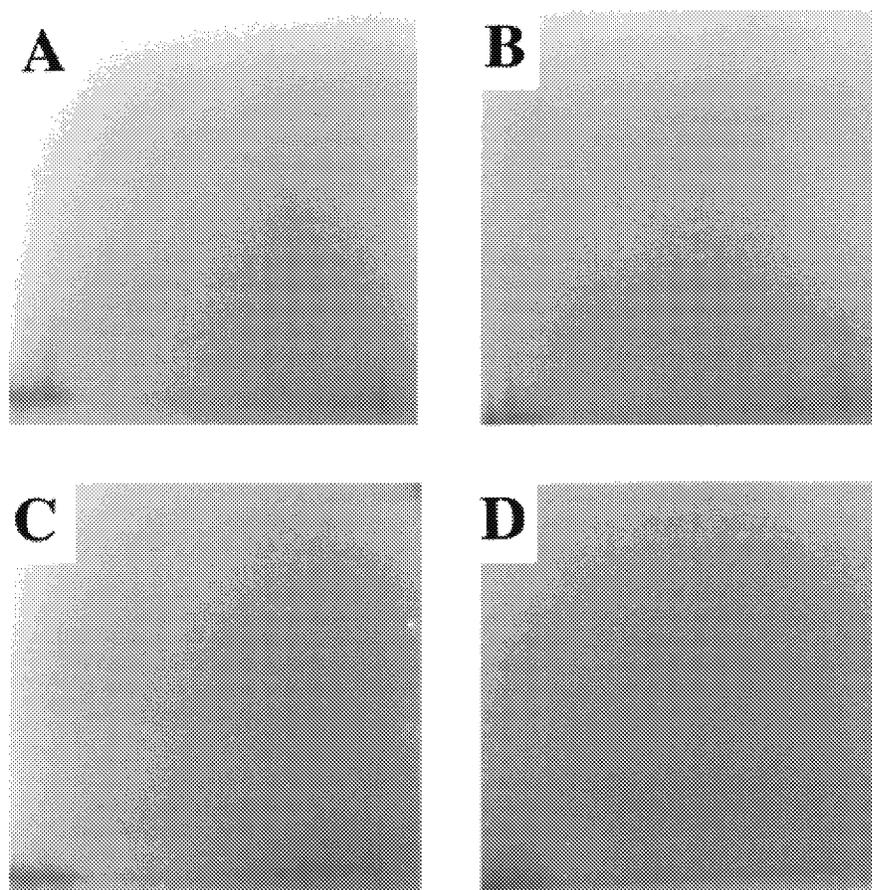


Fig. 2. Autoradiograms of ³²P-labeled DNA from the kidneys (A) and liver (B) of mice treated with 270 mg/kg of the isopropylammonium salt of glyphosate and from kidneys (C) and liver (D) of control mice. Autoradiography was performed at -80°C for 72 hr.

treated with the herbicide formulation and the active ingredient, respectively. The doses used in the present study were lower than the acute oral LD_{50} value of 4.050 mg/kg reported for the isopropylammonium salt of glyphosate in rats [Kidd and James, 1991] and employed in previous *in vivo* studies (450 or 900 mg/kg) [Bolognesi et al., 1997]. DNA adduct formation was detected in the liver and kidneys of mice treated with the Roundup mixture. Three DNA adduct spots were found in kidney DNA (Fig. 1A) and one in hepatic DNA (Fig. 1B). No DNA adducts were observed in the kidneys or liver of mice treated with the isopropylammonium salt of glyphosate (Figs. 2A,B) and in those of controls (Figs. 2C,D). A relationship between the dose of Roundup herbicide and the DNA adduct levels was observed (Table I). The treatment with higher concentrations of Roundup formulation caused a linear increase of the relative adduct levels (RAL) both in the kidneys and liver. The levels of Roundup-related DNA adducts observed in mouse kidneys and liver at the highest dose of herbicide tested (600 mg/kg) were 3.0 ± 0.1 (SE) and 1.7 ± 0.1 (SE) adducts/ 10^8 nucleotides, respectively.

DISCUSSION

Our data show that Roundup, a well-known herbicide which is applied as a foliage spray for the control or destruction of most herbaceous plants, induces a dose-dependent formation of DNA adducts in mice (Fig. 1, Table I) and that Roundup DNA adducts were not related to the active ingredient, the isopropylammonium salt of glyphosate (Fig. 2), but to another, unknown component of the herbicide mixture. Our results are in keeping with previous studies reporting that agrochemical formulations used in agriculture may induce DNA adduct formation [Bolognesi et al., 1994; Peluso et al., 1996; Shah et al., 1997], and that coformulants and surfactants contained in Roundup formulations have an important role in the induction of Roundup toxicity [Sawada et al., 1988; Rank et al., 1993; Kale et al., 1995; Bolognesi et al., 1997]. Our negative ³²P-postlabeling results indicate that the isopropylammonium salt of glyphosate-related DNA adducts, if they exist, have not been detected using our chromatographic system, not necessarily that they have not been formed.

The identification and characterization of the nature of

TABLE 1. RAL Values in the Kidney and Liver DNA of Mice Treated With Roundup Formulation or the Isopropylammonium Salt of Glyphosate

Compounds	Dose mg/kg	No. of animals	Organs	RAL $\times 10^{-8}$ *
DMSO/olive-oil		6	kidney liver	— —
Glyphosate-salt	130	6	kidney liver	— —
Glyphosate-salt	270	3	kidney liver	— —
Roundup (30.4%)	400 (122)	6	kidney liver	1.9 ± 0.2 0.8 ± 0.1
Roundup (30.4%)	500 (152)	3	kidney liver	2.2 ± 0.3 1.5 ± 0.1
Roundup (30.4%)	600 (182)	3	kidney liver	3.0 ± 0.1 1.7 ± 0.1

*Mean \pm standard error (SE) of four ^{32}P -labeling assays.

Roundup DNA adducts will be very difficult and involve a great deal of effort, which we are now considering in our future plans, since the precise composition of the mixture analyzed in the present study is not available due to protection by patent regulation.

However, we know that Roundup is a complex technical formulation which contains up to 85.6% of so-called "inert ingredients" including additives, coformulants, and surface-active agents [Kidd and James, 1991; Worthing and Hance, 1991], such as sodium lauryl sulfate, Tween 20, polyoxyethylene amines, alkoxyated fatty amines, and long-chain monocarboxylic fatty acids or their salts [Menkes et al., 1991; Sato et al., 1994a,b; Khan and Bonnet, 1994]. Although there are, to our knowledge, no published genotoxicity data on these compounds, it has been reported that the commercial preparations containing fatty amines, such as those used as surfactants in Roundup mixtures, may be contaminated with substantial amounts of long-chain N-nitroso compounds [Kamp and Eisenbrand, 1991]. The N-nitrosoamines are a class of chemical carcinogens [Beland and Poirier, 1993] present as a contaminant in the environment [Preussmann and Eisenbrand, 1984; Sugimira, 1990] which may be metabolically activated to reactive electrophilic species capable of covalent DNA binding, and subsequently to form DNA adducts [Farmer, 1991; Beland and Poirier, 1993]. Moreover, Roundup formulations have been shown to contain several organic solvents, such as cyclohexanone, dipropylene-glycol, propargyl bromide, and dimethylformamide [Sato et al., 1994a,b; Khan and Bonnet, 1994]. Organic solvents are used extensively in agrochemical formulations [Petrelli et al., 1993], and some of them, such as benzene and its metabolites, are carcinogens [Huff et al., 1989; Petrelli et al., 1993] and induce the formation of DNA adducts detectable using a nuclease P1 procedure and a chromatography system similar to that used in the present study [Levay et al., 1993].

Another relevant result is that the induction of DNA damage from the Roundup herbicide was much higher in mouse kidneys than in the liver, both in terms of adduct spot number and adduct levels (Fig. 1, Table 1). Previous studies have shown that in the kidneys Roundup treatment induces oxidative damage, while the active ingredient does not increase the 8-hydroxy guanosine level [Bolognesi et al., 1997]. The genotoxicity of the Roundup formulation toward the induction of oxidative damage and DNA adduct formation, may be due to the fact that the majority of the processes involved in the metabolism of genotoxic pollutants results in the production of highly reactive intermediate products, capable of interacting with DNA and other cellular components. This would imply that the Roundup mixture may act through the formation of electrophilic species capable of DNA binding and hydroxyl radicals responsible for oxidative DNA damage. Clearly, this observation needs confirmatory evidence.

The results of studies on molecular interactions with DNA are extremely important in the definition of a chemical carcinogen and in hazard and risk assessment because the genotoxic carcinogens, for regulatory purposes, are assumed to have no threshold dose for the expression of carcinogenesis [Carpanini, 1996]. Our study provides information on the genotoxic effects of a complex commercial herbicide mixture which is widely available for use by agricultural workers. Additional experiments are needed to identify the chemical specie(s) in the Roundup mixture involved in DNA adduct formation.

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