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P53 IMMUNOCYTOCHEMICAL DETECTION AND OCCUPATIONAL EXPOSURE TO ATRAZINE

Carmen Cozmei[1], Brigitte Scutaru[1], Daniela Constantinescu[1], Doina Popa[1], Eugen Carasevici[2]

1. Institute of Public - Health Iasi
2. University of Medicine and Pharmacy - Iasi, Department of Immunology

Abstract. The aims of this study were to evaluate the influence of the herbicide atrazine on peripheral mononuclear cells on occupationally exposed individuals in terms of their possible DNA – damage effect and to determine whether p53 protein can serve as a biomarker for this exposure or not. 19 workers employed by "Chimcomplex" Company – Borzesti (Romania) in the atrazine production unit were examined and p53 immunocytochemistry was performed. The results showed that 68.4% of the workers were positive for p53 protein and these data were interpreted as a proof indicating that immunocytochemical detection of the p53 protein expression is a feasible means for monitoring atrazine exposure.
Key words: p53, immunocytochemistry, monocytes, atrazine

Rezumat. Acest studiu a fost efectuat cu intenția de a evalua influența atrazinului asupra celulelor mononucleare periferice ale muncitorilor expuși la acest erbicid din punct de vedere al acțiunii sale ca presupus agent ce produce alterări la nivelul ADN-ului, şi, de asemenea, de a stabili dacă proteina p53 poate servi ca biomarker pentru monitorizarea expunerii. În acest scop au fost investigați 19 muncitori ai Secției Erbicide a combinatului "Chimcomplex" SA Borzești, iar proteina p53 a fost determinata printr-o metoda imunocitochimică. Rezultatele au indicat o acumulare nucleară a proteinei p53 la 68,4% din muncitori, ceea ce justifică recomandarea determinării imunocitochimice a proteinei p53 ca o foarte utilă metodă de monitorizare a expunerii la atrazin.
Cuvinte cheie: p53, imunocitochimie, monocite, atrazin

INTRODUCTION

The p53 protein is the product of the p53 tumor suppressor gene that is located on chromosome 17 and whose mutations are common features in various human tumors (1,2). This protein assesses DNA damage and acts as a transcription factor regulating genes, which control cell growth, DNA repair and apoptosis (3). When DNA damage is rather low and can be repaired, p53 will arrest the cell in the G1 phase to permit the activity of the DNA repair machinery. In case the DNA damage is very substantiated and unrepaired, p53 induces cells to undergo apoptosis (4). Mutations of the p53 gene lead to the inactivation of the biological properties of the p53 protein so that cells cannot be arrested at the G1 nor can they undergo apoptosis and thus allow the escape of replicating cells with damaged DNA which has as a consequence the acquisition of a malignant phenotype (4). Mutant p53

protein takes an abnormal conformation becoming more stable than the wild-type, it accumulates in the nucleus of DNA-damaged cells, and this intracellular over accumulation can be detected by immunocytochemistry (5,6). This explains the considerable interest in the study of the p53 protein as biomarker for human exposure to chemicals related to some malignancies.

Atrazine, one of the most extensively used herbicides worldwide (7), is classified as a possible human carcinogen by the International Agency for Research on Cancer (8) and the United States Environmental Protection Agency (9). There are some epidemiological studies suggesting the association between the exposure to atrazine and ovarian cancer (10), colon cancer (11) and non-Hodgkin's lymphomas (12). Also, under experimental conditions, atrazine induced the development of uterine adenocarcinomas and lymphomas in female Fisher 344/ LATI rats (13), malignant mammary tumors in female Sprague-Dawley rats (14) and lymphomas in Swiss mice (15).

In a previous paper we reported an increased percentage of peripheral lymphocytes expressing p53 from Wistar rats chronically treated with atrazine (16).

The present study was focused on the p53 protein expression in peripheral mononuclear cells from individuals occupationally exposed to atrazine in order to establish whether it can be considered a biomarker of preneoplastic transformation for the people involved in the manufacturing of this chemical.

MATERIALS AND METHODS

Subjects

The subjects studied were distributed in two groups: the target group which comprised 19 workers employed by "Chimcomplex" Company – Borzesti (Romania) in the atrazine production unit and the control group which included the same number of workers non exposed to this chemical matched by sex, age and social-economic characteristics.

Cell preparation

Heparinized fresh whole peripheral blood was diluted 1:1 with RPMI-1640 medium (SIGMA) containing 25 mM HEPES, layered gently onto Ficoll gradient (preheated to 37^0C, density 1077, SIGMA) and centrifuged for 30 min at 1800 rpm, at room temperature. Mononuclear cells accumulated at the interphase were washed twice in the same medium and resuspended at 2 x 10^4 cells/ 0.1 mL.

p53 immunocytologic staining

2 x 10^4 cells were smeared on slides (DAKO ChemMate, BioTek Solutions, USA), three for each individual, by centrifugation at 1000 rpm for 5 min using a cytospin (Hettich, Universal 32R) and were fixed in 95% ethanol. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in phosphate buffer saline (PBS) then nonspecific antibody binding was blocked by incubating the slides for 30 min with fetal calf serum (FCS) (GIBCO) 2% in PBS. The specimens were incubated overnight at 4^0C with a mouse monoclonal anti-p53 protein

antibody (DO-7; DAKO Corporation, Denmark) diluted at 1:50.

The slides were then incubated at room temperature in sequence with biotinilated antimouse immunoglobulin and streptavidin-peroxidase complex for 30 minutes. A 0.5 mg/mL solution of diaminobenzidine tetrachloride and 0.06% hydrogen peroxide was then added to the cells for another 30 minutes. The cells were counterstained with Mayers hematoxylin.

The omission of the specific primary antibody from the staining schedule was chosen as a negative control.

Results were expressed as percentage of monocytes positive for p53 protein and the statistical significance was established by χ^2 test.

Quantitative detection of atrazine

The quantity of atrazine in serum was determined using a competitive ELISA technique (17) in flat-bottom polystyrene microtiter plates (Nunc) pre-coated with 0.3 mL anti-atrazine monoclonal antibody K_4E_7 (Technical University Münich, Germany). Each sample was determined three times on two different plates.

The mean values ± SD resulted from six measures were compared statistically utilizing the "Student's *t* test".

RESULTS

The accumulation of the p53 protein is indicated by the brown color of the nucleus as seen in figure 1. Figure 2 shows a negative staining (normal blue nucleus), which is characteristic of the control group.

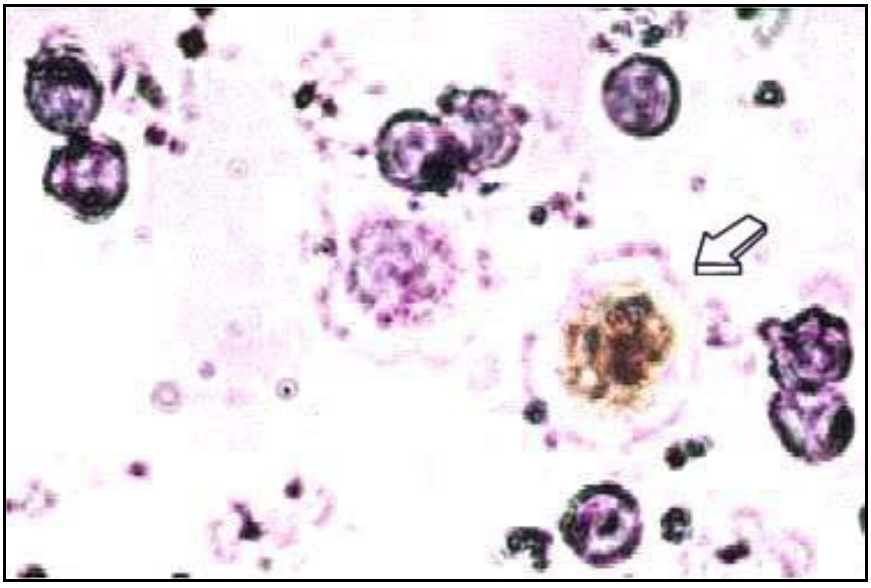

Fig. 1 Monocyte stained positively for p53 protein (x40) identified in a specimen from the target group.

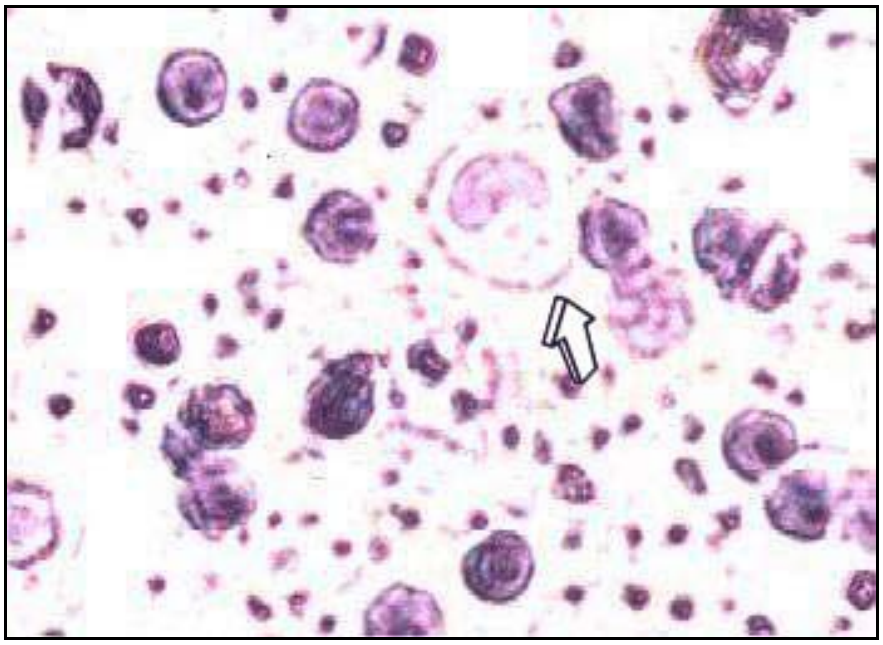

Fig. 2 Monocyte stained negatively for p53 protein (x40) identified in a specimen from the control group

Only the smears that showed more than 3% monocytes with nuclear accumulation were judged as positive for p53 protein. Thus, 68.4% of the workers exposed to atrazine were positive for p53 protein (table 1) while all the persons included in the control group were negative.

Table 1. Percentages of monocytes expressing p53 protein and mean concentrations ±SD of serum atrazine in subjects exposed to atrazine

Subject	p53 positive monocytes (%)	Atrazine (μg/l)	Subject	p53 positive monocytes (%)	Atrazine (μg/l)
1	3.70	52.93 ± 3.00	11	1.50	10.80 ± 0.45
2	0.60	28.36 ± 0.66	12	8.00	42.83 ± 0.15
3	0	18.96 ± 0.66	13	4.20	22.63 ± 1.27
4	3.77	33.76 ± 2.37	14	3.90	6.00 ± 0.70
5	4.66	55.06 ± 1.36	15	4.80	29.23 ± 1.43
6	3.77	35.93 ± 0.49	16	3.77	15.56 ± 1.20
7	0.60	33.93 ± 1.84	17	6.50	9.33 ± 0.46
8	1.75	7.96 ± 0.46	18	8.88	63.46 ± 3.75
9	7.25	13.90 ± 0.80	19	1.94	11.56 ± 0.89
10	3.55	10.13 ± 0.49			

The p53 overexpression was correlated with the serum levels of atrazine determined by ELISA technique (fig. 3).

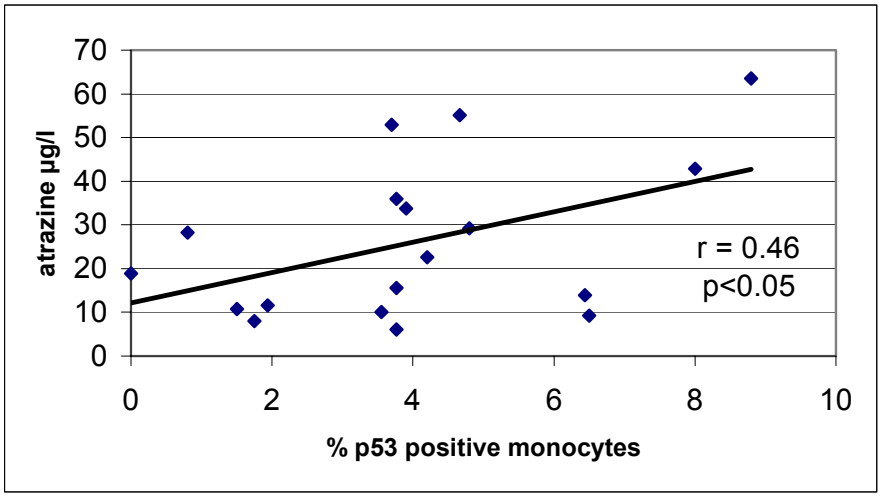

Fig. 3 Correlation between monocytes positive for p53 protein and serum atrazine levels

DISCUSSION

This is the first study, in the existing literature, describing human populations at high exposure for atrazine in conjunction with the p53 protein expression.

There are many controversies concerning the genotoxicity of atrazine and its implication in carcinogenesis. Some authors argue that this pesticide is indeed genotoxic for human peripheral blood lymphocytes (18) and induces whole cell clastogenicity even at very low concentrations in the ground water (19); but for others, this herbicide does not appear to have great potential for in vivo genotoxic/ mutagenic effects (20,21).

We found positive stained monocytes for p53 protein in 13 of the 19 individuals (68.4%), a condition which is clearly correlated with the serum levels of atrazine in the same persons. Consequently, we can assume that this over expression is related to the atrazine exposure.

Also, our results seem to be in consonance with those studies highlighting a relation between atrazine exposure and genotoxic effects, especially as it is already shown that the over expression of the p53 protein correlates with the presence of the mutant p53 proteins (22,23).

But, taking into account the multitude of information concerning the

functions of the p53 gene and its protein product – differentiation, apoptosis, and growth arrest, along with DNA repair (4), the interpretation of the nuclear p53 accumulation determined by us in terms of a cause-effect relation cannot be easily achieved.

We considered the possibility of two different explanations regarding our results. It was shown that freshly isolated monocytes may contain detectable levels of p53 mRNA (24) which disappear after their stimulation by the potent macrophage activation inducer *Staphylococcus aureus* Cowan I (SAC) (4). In this respect, we may speculate that our results point to the fact that atrazine could have a direct toxic effect on monocytes and that the bone marrow tries to reinforce this lineage with new, uncommitted specimens.

The other possibility is that we are dealing with an early myelodysplastic syndrome (MDS), a heterogeneous group of clonal neoplastic hematologic disorders often associated with some risk factors such as ionizing radiations, benzene, cigarette smoke, and chemo-therapeutic drugs (25).

This latter idea is supported by the fact that 46.2% of our subjects with overexpression of p53 protein had also a slight monocytosis. More then that, a decrease in the number of white blood cells and thrombo-cytopenia was present in three of them. The cancer is a multistep process and perhaps this p53 accumulation in the nucleus of the monocytes we have detected in a non-neoplastic state could indicate an early event in an evolution towards a neoplastic condition.

CONCLUSION

Our results enable us to conclude that people occupationally exposed to atrazine have to be closly monitored and the immunocytochemical detection of the p53 protein expression is a handy, feasible means for this purpose.

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