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Haemoglobin adducts of acrylonitrile and ethylene oxide in acrylonitrile workers, dependent on polymorphisms of the glutathione transferases GSTT1 and GSTM1

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Abstract Fifty-nine persons with industrial handling of low levels of acrylonitrile (AN) were studied. As part of a medical surveillance programme an extended haemoglobin adduct monitoring [N-(cyanoethyl)valine, CEV; N-(methyl)valine, MV; N-(hydroxyethyl)valine, HEV] was performed. Moreover, the genetic states of the polymorphic glutathione transferases GSTM1 and GSTT1 were assayed by polymerase chain reaction (PCR). Repetitive analyses of CEV and MV in subsequent years resulted in comparable values (means, 59.8 and 70.3 μg CEV/l blood; 6.7 and 6.7 μg MV/l blood). Hence, the industrial AN exposures were well below current official standards. Monitoring the haemoglobin adduct CEV appears as a suitable means of biomonitoring and medical surveillance under such exposure conditions. There was also no apparent correlation between the CEV and HEV or CEV and MV adduct levels. The MV and HEV values observed represented background levels, which apparently are not related to any occupational chemical exposure. There was no consistent effect of the genetic GSTM1 or GSTT1 state on CEV adduct levels induced by acrylonitrile exposure. Therefore, neither GSTM1 nor GSTT1 appears as a major AN metabolizing isoenzyme in humans. The low and physiological background levels of MV were also not influenced by the genetic GSTM1 state, but the MV adduct levels tended to be higher in GSTT1− individuals compared to GSTT1+ persons. With respect to the background levels of HEV adducts observed, there was no major influence of the GSTM1 state, but GST− individuals displayed adduct levels that were about 1/3 higher than those of GSTT1+ individuals. The coincidence with known differences in rates of background sister chromatid exchange between GSTT1− and GSTT1+ persons suggests that the lower ethylene oxide (EO) detoxification rate in GSTT1− persons, indicated by elevated blood protein hydroxyethyl adduct levels, leads to an increased genotoxic effect of the physiological EO background.

Key words Acrylonitrile · Ethylene oxide · Haemoglobin adducts · Glutathione transferase (GST) polymorphisms

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Introduction

Acrylonitrile (AN) and ethylene oxide (EO) are industrially important carcinogenic C_2-compounds; their genotoxicity is viewed in connection with their chemical reactivity and ability to bind to macromolecular targets (Peter et al. 1983; Farmer 1994; Kirkovsky et al. 1998). Moreover, elevated adduct levels of AN and EO in haemoglobin have been reported in smokers (Farmer 1994; Tavares et al. 1996). EO is also a normal body constituent being formed as a metabolite of physiological ethylene (Bolt 1996, 1998). Both compounds, AN and EO, are reactive towards glutathione and are detoxified via glutathione transferases (GST; Peter et al. 1983; Hallier et al. 1993).

Recently, much interest has been focused on toxicological implications of genetic polymorphisms of GST, in particular the isoenzymes GSTT1 and GSTM1 (Chen et al. 1996; To-Figueras et al. 1997). Ethylene oxide and some other C_1 and C_2 compounds are preferred substrates of human GSTT1 (Hallier et al. 1993; Schröder et al. 1996), which is expressed in human liver, kidneys and erythrocytes (Thier et al. 1997). The role of human polymorphic GSTT1 or GSTM1 in metabolism of acrylonitrile is not known.

The frequency of the GSTM1 null genotype in European or US white populations (Chen et al. 1996; To-Figueras et al. 1997), as well as in Chinese (Shen et al. 1998) amounts to ~50%, in US blacks ~28% (Chen et al. 1996). By contrast, frequencies of the GSTT1 null genotype and/or the equivalent 'non-conjugator' phenotype have been reported to range between 15 and 20% in Germany (Kempkes et al. 1996), US whites (Chen et al. 1996), Spain (To-Figueras et al. 1997) and Turkey (Oke et al. 1998). Higher frequencies have been recorded among US blacks (24%; Chen et al. 1996) and Chinese (49% in Shanghai; Shen et al. 1998). This pinpoints the potential impact of GST polymorphisms for individual human susceptibilities to reactive low-molecular-weight toxicants, both of exogenous and of endogenous nature.

Tavares et al. (1996) have experimentally established the dose-dependence of the AN-adduct, N-(2-cyanoethyl)valine (CEV), at the N-terminal valine of haemoglobin in the blood of rats dosed with AN. Furthermore, when investigating small groups of AN-exposed workers they found increased CEV adduct levels, compared to the background in non-exposed persons. As far as smoking was concerned, in persons not occupationally exposed, the observed CEV adduct levels in haemoglobin were dependent on the number of cigarettes smoked per day. There was no correlation between the CEV levels and those of the EO adduct N-(2-hydroxyethyl)valine (HEV). This is interesting as also the EO-related HEV haemoglobin adduct has been connected with smoking. In their study, Tavares et al. (1996) did not discriminate within their groups of persons according to the genetic state of polymorphic GST. This was one reason to perform a study on similar groups of AN-exposed workers and to investigate CEV and HEV adduct levels in haemoglobin, in connection with genotypings of GSTT1 and GSTM1. Another reason was the empirical observation that some persons having occupational contact with AN displayed relatively high levels of cyanide, a product of the oxidative metabolism of AN. This led to the question of participation of polymorphic GST isoenzymes in AN metabolism.

Materials and methods

Subjects and biological monitoring of haemoglobin adducts

Fifty-nine persons with industrial handling of acrylonitrile, at different workplaces and to different extents, were studied in 1994 and 1995. As part of a medical surveillance programme, on different occasions (see Table 1) whole blood was collected from each person by venipuncture using ethylenediaminetetraacetic acid (EDTA) containing syringes (Sarstedt, Germany) and stored at −80 °C. The state of smoker or non-smoker was recorded, as included in Table 1.

The biological adduct monitoring procedures were conducted in accordance with methods and procedures recommended by the Deutsche Forschungsgemeinschaft (DFG 1998). After isolation of erythrocytes and globin the levels of terminal N-alkylvalines [CEV, HEV, N-methyl-valine (MV)] were determined using the method of Lewalter (1996). Accordingly, the resulting data were expressed as μg adduct (CEV, HEV, MV) per litre whole blood (Table 1). For comparison, 10 μg HEV/l blood is considered equivalent to 0.4 nmol HEV/g globin (DFG 1998).

Genotyping

Part of the EDTA blood samples (200 μl each) was used for genotyping of the genetic state of the polymorphic enzymes GSTT1 and GSTM1. Human genomic DNA from nucleated blood cells was prepared by proteinase K digestion and precipitated with sodium chloride according to Miller et al. (1988). GSTT1 genotyping was performed using the polymerase chain reaction (PCR) method described in detail by Pemble et al. (1994) and Kempkes et al. (1996). A 480 bp fragment of the human GSTT1 gene was amplified with the primers (5′-TTC CTT ACT GGT CCT CAC ATC TC-3′ and 5′-TCA CCG GAT CAT GGC CAG CA-3′). As internal control a 268 bp fragment of the human β-globin gene was co-amplified with a second set of primers (5′-CAA CTT CAT CCA CGT TCA CC-3′ and 5′-GAA GAG CCA AGG ACA GGT AC-3′). PCR reactions were performed as follows: initial melting 94 °C for 5 min, followed by 30 cycles each of: melting 94 °C for 1 min, annealing 60 °C for 1 min, extension 72 °C for 1 min. A final extension of, 72 °C for 10 min terminated the procedure.

GSTM1 genotyping was performed using the PCR method described by Shen et al. (1998). A fragment of the 5′-region of exon 4 to the 3′-end of exon 5 (273 bp) was amplified with a set of primers (5′-CTG CCC TAC TTG ATT GAT GGG-3′ and 5′-CTG GAT TGT AGC AGA TCA TGC-3′). The fragment was co-amplified with a fragment of the human actin gene (493 bp) using the primers 5′-GGG CAC GAA GGC TCA TCA TT-3′ and 5′-GGC CCC TCC ATC GTC CAC CG-3′). PCR amplification conditions were the same as those for GSTT1 genotyping, except that the annealing temperature was adjusted to 55 °C. The PCR products were separated electrophoretically on 2% agarose gels stained with ethidium bromide.

Results

The results of haemoglobin adduct monitoring (CEV, MV, HEV) as well as of GSTM1 and GSTT1 genotyping in the 59 subjects with industrial AN contact are compiled in Table 1. The statistical evaluations of these data are given in Tables 2–4. Repetitive analyses of CEV and MV in subsequent years (1994 and 1995) resulted in comparable values (means, 59.8 and 70.3 μg CEV/l blood; 6.7 and 6.7 μg MV/l blood). Compared with the CEV adduct monitoring data published by Tavares et al. (1996) of workers in an AN polymerization plant, our data are quantitatively comparable. The current occupational exposure limit (Technical Guidance Value, TRK) for acrylonitrile in Germany is set to 7 mg/m^3 ambient air (3 ppm), which has been evaluated to correspond with a CEV adduct level in haemoglobin of 420 μg CEV/l blood (DFG 1998). This means that the actual AN exposures of the study group are definitely well below the current official exposure standards. As recorded in the study of Tavares et al. (1996), there was no apparent correlation between the CEV and HEV adduct levels; the same finding holds for CEV and MV. The MV and HEV values observed in this work represent background levels, which apparently are not related to any occupational chemical exposure.

If the polymorphic GST isoenzymes GSTM1 or GSTT1 had a significant implication in the detoxification

Table 1 Results of haemoglobin adduct monitoring (CEV, MV, HEV), smoking state (0/1), and genetic states of GSTM1 and GSTT1 (+/−) of 59 persons having industrial contact with acrylonitrile [*CEV* N-(cyanoethyl)valine · *MV* N-(methyl)valine · *HEV* N-(hydoxyethyl)valine · *GST* Glutathione transferase]

Smoking habits[a]	No.	GSTM1	GSTT1	CEV (µg/l blood)		MV (µg/l blood)		HEV (µg/l blood) 1995
				1995	1994	1995	1994	
0	567	−	−	4	6.7	4	9.6	19
0	546	+	−	9	6.5	4	3.7	13
1	571	−	+	2	4.7	4	3.8	13
0	590	+	+	3	3.7	1	3.1	14
1	559	+	+	5	6.0	5	4.7	11
1	588	−	+	4	4.5	5	5.5	15
0	541	−	+	5	9.5	3	7.6	11
1	538	+	+	150	186.1	5	10.8	20
1	577	+	−	10	38.6	19	10.4	24
1	536	−	+	70	[b]	7	[b]	10
1	551	−	−	37	42.5	17	14.5	38
0	553	+	+	108	21.5	5	3.9	7
1	560	−	+	36	42.7	10	6.2	20
1	575	−	+	110	33.7	8	6.6	21
0	579	−	+	141	40.0	4	3.2	22
1	582	+	+	227	98.7	6	14.5	18
0	594	+	+	91	53.9	4	4.2	18
0	548	+	+	106	63.2	6	11.0	6
1	565	+	+	86	66.1	3	6.3	9
1	592	+	+	81	92.9	7	7.1	15
0	550	+	+	231	[c]	5	[c]	10
1	596	+	+	17	180.9	3	6.5	11
1	547	+	+	61	77.9	5	n.d.[d]	11
1	539	+	+	20	44.2	4	6.9	19
0	562	−	+	44	68.1	3	4.5	12
1	591	−	+	121	23.7	10	7.4	32
1	595	+	+	96	70.5	10	7.8	15
1	584	−	+	61	21.8	5	2.3	19
1	593	−	+	34	64.0	7	13.3	25
1	568	−	+	128	72.5	8	5.9	19
0	585	+	+	41	152.8	6	11.1	10
1	535	+	−	78	22.5	11	6.7	33
0	540	−	+	15	17.4	9	4.5	16
0	555	+	−	122	83.8	6	4.0	14
0	572	+	+	19	138.1	5	4.8	31
0	583	+	+	5	40.7	4	4.5	17
1	586	−	+	73	35.1	6	3.9	21
0	589	+	+	41	77.8	6	3.9	13
1	543	−	−	38	112.8	9	5.4	23
0	544	+	+	86	156.2	4	5.9	12
1	554	−	+	30	41.5	6	4.8	16
0	580	−	+	52	153.1	2	5.0	9
1	537	−	+	19	67.1	8	5.9	16
1	563	−	+	51	132.1	4	9.8	10
1	570	+	−	54	44.4	12	11.5	23
1	573	−	+	25	151.7	9	5.7	17
1	556	+	+	31	110.6	6	4.8	11
1	558	−	+	34	56.1	4	3.8	15
1	569	−	−	33	232.2	11	18.9	14
0	576	−	+	25	[b]	3	[b]	8
0	542	−	+	130	114.0	7	3.5	7
1	552	+	+	42	[b]	4	[b]	10
1	587	−	−	14	85.4	6	6.6	16
1	564	−	−	63	127.6	16	12.1	37
1	545	+	+	89	[b]	15	[b]	28
1	581	−	+	33	65.0	9	6.0	22
1	557	−	+	15	34.1	7	2.2	21
1	549	−	+	62	61.4	7	5.9	23
0	578	+	+	111	37.6	7	5.1	11

[a] 0, Non-smoker; 1, smoker
[b] No globin (found/available)
[c] No participation on examination
[d] n.d., Not detectable

Table 2 Means ± SD of haemoglobin adducts (CEV MV, HEV in 1995 and 1994; μg adduct/l blood), dependent on the individual genetic states of GSTM1 and GSTT1 (+/−) and on non-smoking/smoking state (0/1) see Table 1 for explanation of abbreviations and notations

GST	*n*	%	Mean ± SD				
			CEV 95	CEV 94	MV 95	MV 94	HEV 95
M1+	28	47.5	72.1 ± 60.4	75.0 ± 53.6	6.4 ± 3.8	6.8 ± 3.1	15.5 ± 7.0
M1−	31	52.5	48.7 ± 39.7	66.2 ± 54.0	7.0 ± 3.5	6.7 ± 3.8	18.3 ± 7.6
T1+	48	81.4	63.9 ± 54.0	69.6 ± 50.5	5.9 ± 2.5	6.1 ± 2.8	15.6 ± 6.1
T1−	11	18.6	42.0 ± 35.7	73.0 ± 66.7	10.5 ± 5.2	9.4 ± 4.7	23.1 ± 9.2
M1+/T1+	23	39.0	76.0 ± 63.1	84.0 ± 55.1	5.5 ± 2.7	6.7 ± 3.1	14.2 ± 6.1
M1+/T1−	5	8.5	54.6 ± 47.9	39.2 ± 29.0	10.4 ± 5.9	7.3 ± 3.6	21.4 ± 8.2
M1−/T1+	25	42.4	52.8 ± 42.3	57.1 ± 43.5	6.2 ± 2.4	5.5 ± 2.4	16.8 ± 6.0
M1−/T1−	6	10.2	31.5 ± 20.6	101.2 ± 78.3	10.5 ± 5.2	11.2 ± 6.1	24.5 ± 10.5
Smoking status							
0	21	35.6	66.1 ± 60.6	65.5 ± 53.7	4.7 ± 1.9	5.4 ± 2.5	13.3 ± 5.8
1	38	64.4	56.3 ± 46.3	72.9 ± 53.9	7.8 ± 3.9	7.5 ± 3.8	19.0 ± 7.4

Table 3 Differences between genetic state of GSTM1, GSTT1 and smoking status[a]; results of *t*-test

	Adduct (see Table 2)	*P*-value	H1[b]
GSTM1	CEV 95	0.0441	+ > −
GSTT1	MV 94	0.0219	+ < −
GSTT1	MV 95	0.0081	+ < −
GSTT1	HEV 95	0.0008	+ < −
Smoking state	MV 94	0.0193	0 < 1
Smoking state	MV 95	0.0001	0 < 1
Smoking state	HEV 95	0.0019	0 < 1

[a] 0, Non-smoker; 1, smoker
[b] H1, Alternative hypothesis

Table 4 Correlations between different groups of haemoglobin adduct data

Adducts	Correlation
CEV 95/MV 95	0.03
CEV 95/HEV 95	−0.02
CEV 95/MV 94	0.15
CEV 94/MV 95	0.03
CEV 94/MV 94	0.40
CEV 94/HEV 95	−0.12
MV 95/HEV 95	0.66
MV 94/HEV 95	0.27

of AN by conjugating this reactive chemical with glutathione, the subgroups of GSTM1− or GSTT1− individuals, respectively, should exhibit consistently higher CEV adduct values, compared to GSTM1+ and GSTT1+ persons. This is obviously not the case. Hence, it must be concluded that neither GSTM1 nor GSTT1 is a major AN metabolizing isoenzyme in humans.

With respect to the very low and physiological background levels of MV observed in this study, there is no influence on these levels of the genetic GSTM1 state. However, such an influence of the GSTT1 state appears possible, as in both years the MV adduct levels are higher in GSTT1− individuals compared to GSTT1+ persons ($P = 0.0219$ in 1994; $P = 0.0081$ in 1995). With respect to the background levels of HEV adducts observed, there seems to be no major influence of the GSTM1 state, but GSTT1− individuals display adduct levels that are about 1/3 higher than those of the GSTT1+ individuals of the study group.

Discussion

In principle, the present adduct monitoring data in workers with AN contact are consistent with, and hence supportive of, those of Tavares et al. (1996). In both studies the industrial AN exposures range well below current official standards. It may be concluded that monotoring the haemoglobin adduct CEV is a suitable means of biomonitoring and medical surveillance under these exposure conditions. An important initial question of the present study was whether or not, or the extents to which, human polymorphisms of glutathione transferases (GSTM1, GSTT1) would modify the detoxification and thus the toxicity of AN, as is the case for other C_1 and C_2 chemicals, including ethylene oxide (Hallier et al. 1993). Such effects were not visible. In consequence, the genotoxic risk of industrial AN exposure will not be modified by the genetic GSTM1 and GSTT1 state.

Regarding the haemoglobin adducts MV and HEV, which are unrelated to AN exposure, the present study reveals consistent background levels. The MV background is most likely due to physiological methylation mechanisms (via S-adenosyl-methionine). The background of the EO adduct HEV has been related to an endogenous production of ethylene which is partly metabolized to EO (Bolt 1996). This is superimposed by smoking effects as ethylene is considered a constituent of tobacco smoke (Törnqvist et al. 1986). Based on the data of Angerer et al. (1998) an HEV adduct level of 5 μg/l blood (or 0.2 nmol/g globin) would theoretically correspond to an exogenous industrial EO exposure (working conditions of 8 h/days; 5 days/week) of ~50 ppb. Hence, the mean HEV background in

GSTT1+ persons would correspond to an external exposure of 0.15 ppm EO, in GSTT1− persons of 0.2 ppm. These values are somewhat higher compared to those based on animal experimentations (Bolt 1996) and contrast to calculations on the basis of the DNA adduct 7-(2-hydroxyethyl)guanine (Bolt et al. 1997). This phenomenon has been called the 'ethylene oxide paradox' (Bolt 1996, 1998) of which the reason is still not known. As a possible explanation it ought to be considered that the distribution of ethylene oxide within the cells and between diffent compartments is not uniform.

Interestingly, the present data of background HEV levels in haemoglobin differ from previous data of Vollmer et al. (1998) of the physiological backgrounds of S-hydroxyethyl-cysteine (HEC) in albumin. The relative difference of hydroxyethyl adduct concentration depending on GSTT1 genetic states is much higher for albumin (S in cysteine) than for haemoglobin (N in the N-terminal valine; see Table 5). This apparent discrepancy would in fact corroborate the idea of differential binding behaviours of exogenous and endogenous EO towards different targets.

The difference in background EO adduct levels of blood proteins between GSTT1− and GSTT1+ persons has biological consequences. The data of Table 6 summarize previous data of our group (Schröder et al. 1995) and of others (Wiencke et al. 1995) regarding differences in background rates of sister chromatid exchange between GSTT1− and GSTT1+ persons (who had not been occupationally exposed to exogenous ethylene or EO). This coincidence suggests that the low EO detoxification rate in GSTT1− persons leads both to elevated blood protein hydroxyethyl adduct levels and to an increased genotoxic effect of the physiological EO background. Further toxicological consequences of this phenomenon are a matter of present investigations.

Table 5 Ethylene oxide-derived adduct levels in blood proteins, dependent on the genetic state of GSTT1: S-hydroxyethyl-cysteine (HEC) in albumin (Vollmer et al. 1998) and N-hydroxyethyl-valine (HEV) in haemoglobin (this investigation)

	n	nmol HEC (/g albumin) (Mean ± SD)	*n*	µg HEV (/l blood) (Mean ± SD)
GSTT1−	8	31.8 ± 13.9	11	23.0 ± 9.2
GSTT1+	9	9.3 ± 6.9	48	15.6 ± 6.1
GSTM1−	9	16.7 ± 18.1	31	18.3 ± 7.6
GSTM1+	8	23.0 ± 13.0	28	15.5 ± 7.0

Table 6 Background rates of sister chromatid exchange (SCE) in persons of different genetic GSTT1 state

	Background SCE/cell (mean ± SD)	
	Schröder et al. (1995)	Wiencke et al. (1995)
GSTT1−	8.74 ± 1.24 (n = 15)	10.3 ± 1.0 (n = 12)
GSTT1+	7.55 ± 0.77 (n = 15)	8.6 ± 1.1 (n = 66)
P (GSTT1− > GSTT1+)	<0.005	<0.001

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