Evaluation of biomarkers in plasma, blood, and urine samples from coke oven workers: significance of exposure to polycyclic aromatic hydrocarbons

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Abstract

Objective—**The aim was to assess the significance of two biomarkers; antibody to benzo(a)pyrene DNA adducts and concentration of hydroxyethylvaline haemoglobin adducts in samples from a well studied group of coke oven workers. As a measure of exposure we have used 1-hydroxypyrene in urine.**

Methods—**Urine and blood samples were collected from coke oven workers and a control group. Samples from coke oven plant workers were collected in January and June. 1-Hydroxypyrene was measured in urine by high performance liquid chromatography (HPLC), antibodies to benzo(a)pyrene DNA adducts were measured by ELISA and hydroxyethylvaline haemoglobin adducts were measured by gas chromatography-mass spectrometry (GC-MS).**

Results—**Mean urinary 1-hydroxypyrene in samples from coke oven workers varied from 1·11 to 5·53 umol/mol creatinine and 0·14 umol/mol creatinine in the control group. Workers at the top side had the highest values of urinary 1-hydroxypyrene. Antibody to benzo(a)pyrene DNA adducts did not correlate with either 1-hydroxypyrene nor length of work at the coke oven plant. But antibody concentration in samples collected in January was predictive of the concentration in samples collected in June. A small non-significant increase in hydroxyethylvaline haemoglobin adducts was found in samples from coke oven workers relative to the control group when comparing smokers and non-smokers separately.**

Conclusion—**1-Hydroxypyrene correlates well with exposure groups based on job description. Antibodies to benzo(a)pyrene DNA adducts was related to people and not exposure. Work at a coke oven plant might lead to increased hydroxyethylvaline haemoglobin adducts.**

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Occupational exposure to polycyclic aromatic hydrocarbons (PAHs) is encountered in coke oven plants, electrochemical industry, electrode factories, and in various manufacturing plants with combustion processes. Several PAH compounds are potent carcinogens and working in coke production, aluminum production, and other processes with PAH exposure is thought to carry an increased risk of cancer.[1 2] There is a general interest in development and evaluation monitoring methods for exposure and effects of exposure to ensure reliable health surveillance at work places with PAH exposure.[3 4]

Measurement of 1-hydroxypyrene in urine has been shown to be a reasonably reliable method for monitoring of PAH exposure,[5-8] and has been used as an exposure estimate for evaluating other methods.[9-11]

There is considerable interest in studying associations between exposure markers or effect indicators and cancer in workers occupationally exposed to PAHs.[12-14] Benzo(a)-pyrene is metabolically activated to benzo(a)pyrenediolepoxide (BPDE), which can react with DNA resulting in benzo(a)-pyrene DNA adducts. It has been shown in humans who are occupationally exposed to high concentrations of PAHs,[15 16] or moderate amounts of PAH in the environment,[17] that about 40% have antibodies to benzo(a)pyrene DNA adducts. Antibodies to other PAH DNA adducts have also been found,[17] and in experiments after chronic PAH exposure in mice antibodies to benzo(a)pyrene DNA adducts have been detected.[18] Occupational exposure to antigenic compounds has been extensively studied mainly for hypersensitivity.[19 20] But the far reaching implications of antibodies to carcinogenic compounds have not yet been established, although a general association between carcinogenesis and immunoresponses has been discussed.[21]

Exposure to ethylene oxide leads to formation of hydroxyethylvaline haemoglobin adducts[22] and smoking increases the concentration of these adducts.[23] A background level of hydroxyethylvaline adducts in haemoglobin has been established and smoking and occupational exposure to ethylene oxide have been shown to increase this background level by up to about 10-fold and 300-fold respectively.[24 25] Studies have also been conducted in several polluted areas in search of variations due to environmental pollution.[26] In coke oven plants many compounds are formed, which gives a harmful working environment. Methylating agents have been detected as a result of combustion processes[27] and therefore it is likely that other alkylating agents will be present at coke oven plants, although we are not aware of

any reports that show this.

We have measured 1-hydroxypyrene in urine from coke oven workers and a control group and analysed the association between urinary 1-hydroxypyrene and antibodies to benzo(a)pyrene DNA adducts and measured hydroxyethylvaline haemoglobin adducts. We found a possible association between PAH exposure and hydroxyethylvaline adducts in haemoglobin. Antibodies to benzo(a)pyrene DNA adducts did not correlate with exposure, but were person related judged from samples collected at two time periods.

Materials and method

MATERIALS

1-Hydroxypyrene was from Jansen Chimica, Beerse, Belgium. A mixture of glucuronidase and sulphatase were purchased from Boehringer, Mannheim, Germany. Methanol, high performance liquid chromatography (HPLC) grade, was from Fluka Chimica Buchs, Switzerland. Anti-human immunoglobulin G (IgG) (γ-chain specific) with alkaline phosphatase goat and calf thymus DNA was obtained from Sigma, St Louis, Montana, USA. Benzo(a)pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide (±) (BPDE) was obtained from NCI Chemical Carcinogen Control Standard Repositories, Bethesda, Maryland and Kansas City, Missouri, USA.

STUDY GROUP

All workers participating in this study were recruited on a voluntary basis. The workers were divided into exposure groups based on job category. Top side workers were assigned to the high exposure group, side oven workers to the medium exposure group, and maintenance workers to the low exposure group. Table 1 describes the groups. All biological samples have been stored at −20°C. Blood and urine samples were collected after the shift on working days.

MEASUREMENT OF 1-HYDROXYPYRENE IN WORKERS' URINE

The method used for measurement of 1-hydroxypyrene in urine was performed essentially as described by Jongeneelen *et al.*[5] Urine was diluted with acetic acid, pH adjusted, and incubated for 16 hours after addition of an enzyme mixture of glucuronidase and sulphatase. This mixture was applied on a Sep-Pak C-18 cartridge with the help of a Lab robot, Millilab, Waters, Milford, Massachusetts, USA. The cartridges were washed with water and then eluted with 4 ml of methanol. Of the eluate 20 μl was injected into an HPLC with a Novapak C_{18} column. The elution was with a methanol/water gradient and detection with a fluorescence detector (Perkin-Elmer, Beaconsfield, UK) at 242 nm (λ excitation) and 288 nm (λ emission). Five spiked control urine samples were used as standards with addition of 10, 20, 40, 100 and 250 nmol/l 1-hydroxypyrene. Measurement was performed with Millennium integration software.

Table 1 Distribution of variables in the exposure groups

Groups	Subjects (n) January	Subjects (n) June	Arithmetic mean (SD) age	Smokers (%) (SD)
Control	13	—	48·8 (9·9)	46·1 (51·9)
Low exposure	23	17	38·3 (9·5)	56·1 (50·2)
Medium exposure	26	18	36·7 (11·6)	61·4 (49·3)
High exposure	18	13	33·4 (10·7)	50·0 (50·9)

MEASUREMENT OF ANTIBODIES IN HUMAN PLASMA DIRECTED TO BENZO(A)PYRENE DNA ADDUCTS

As a test for antibodies to benzo(a)pyrene DNA adducts we have measured the binding to benzo(a)pyrenediolepoxide (BPDE) modified calf thymus DNA and in the text we have used the term antibody to benzo(a)pyrene DNA adducts for this activity. The measurements were performed essentially according to the method described by Newman.[17] Microtitre plates of polyvinylchloride Fastbinder 6695 (Costar Europe, Badhoevedorp, the Netherlands) were coated with 80 ng DNA/well of either calf thymus DNA or BPDE modified calf thymus (9,4 fmol BPDE/ng DNA). Calf thymus DNA was modified with BPDE essentially as described by Newman.[17] The DNA was arranged on the plates in triplets of calf thymus DNA and triplets of BPDE modified calf thymus DNA. Coating of plates was done with DNA diluted in 20× standard saline citrate (SSC) which was added to the plates and incubated for 16 hours at 37°C and thereafter stored at −20°C in closed containers until use. The plates were washed with distilled water twice, before the addition of 4% bovine serum albumin in phosphate buffered saline (PBS) at pH 7·4 for one hour to minimise unspecific binding. The plasma samples were diluted 1:25 with 4% bovine serum albumin in PBS and 50 μl was added to triplet wells with BPDE modified calf thymus DNA and triplet wells with calf thymus DNA and incubated for 1·5 hours at 37°C. Then goat anti-human IgG was added (diluted 1:5000) and the plates incubated for two hours. Then the plates were washed twice with 0·2 M diethanolamine before addition of 5 mg p-nitrophenylphosphate dissolved in 10 ml 0·2 M diethanolamine and incubated for one hour. Each sample was added to three wells with BPDE modified calf thymus DNA and three wells with calf thymus DNA. The difference in mean value between the triplicate with modified DNA and triplicate with calf thymus DNA was tested with a *t* test, and if the difference was significant at the 0·05 level the sample was considered positive. In each plate a standard sample was included and all positive values were calculated as percentages of this standard. The samples were assayed three times on different plates, and the mean value was calculated.

MEASUREMENT OF HYDROXYETHYLVALINE IN HAEMOGLOBIN

The method used for the measurement of hydroxyethylvaline in haemoglobin was that of

Bailey *et al*[28] with minor modifications. Globin was prepared from red blood cells by the procedure of Bailey *et al.*[29] Aliquots of globin (50 mg) were mixed with the internal standard (globin that had been reacted with d_4-ethylene oxide). This internal standard contained 25·7 nmol d_4-hydroxyethylvaline/mg protein, and the amount added to each sample contained 77 pmol d_4-hydroxyethylvaline. The globin was dissolved in formamide solution (2 ml) and subjected to an Edman type degradation, with pentafluorophenyl isothiocyanate (10 μl) as reagent. Pyridine (7 μl) was also added and the mixture shaken gently overnight at room temperature and then heated to 45°C for 90 minutes. The product was purified by Sep-Pak C-18 cartridge chromatography and then converted to the trimethylsilyl derivative by reaction with N,O-bis(trimethylsilyl)trifluoroacetamide (20 μl) in acetonitrile (30 μl) at 60°C for 30 minutes. The sample was dried, redissolved in acetonitrile (30 μl), and subjected to GC-MS analysis with electron impact ionisation. Measurement was achieved by selected ion recording of ions m/z 440 and m/z 444 (for d_0-and d_4-hydroxyethylvaline derivatives respectively). The mass spectrometer was a VG Trio 1 quadruple instrument coupled to a Hewlett-Packard 5890 Series II gas chromatograph. The GC column was 25 m × 0·32 mm, coated with SE52 or a similar stationary phase. With each batch of samples analysed a calibration line was established with mixtures of unlabelled and d_4-labelled ethylene oxide treated globin.

STATISTICS

The three main variables in this study were urinary 1-hydroxypyrene, antibodies to benzo(a)pyrene DNA adducts, and hydroxyethylvaline adducts in haemoglobin. All these variables were normally distributed after log transformation. The significance tests were performed on log transformed data, but summary statistics are mainly given for non-transformed data, unless otherwise noted. Analysis of variance of 1-hydroxypyrene in different exposure groups was supplemented with the Schefee test for significance between individual groups. The statistical analysis was performed with Statgraphics' version 5.

Results

COMPARISON WITH VARIABLES MEASURED IN PREVIOUS PUBLICATIONS

The PAH DNA adducts in lymphocytes from these workers have been measured previously with ^{32}P-postlabelling and immunoassay.[30] The urine samples in this study were collected simultaneously. No significant correlation between adducts and urinary 1-hydroxypyrene was found. The urinary PAH metabolite concentration, measured previously on the same samples by a radioimmunoassay,[31] correlated with urinary 1-hydroxypyrene. For samples collected in January and June the Spearman rank correlation coefficient was 0·66. The P value was less than 0·0001 in both analysis.

BIOMONITORING OF URINARY 1-HYDROXYPYRENE

Figure 1 shows box plots of urinary 1-hydroxypyrene divided into exposure groups. In both sample sets (January and June) there was an increase in arithmetic mean and median urinary 1-hydroxypyrene concentrations from low to high exposure. There was a greater diversity in values in the June samples compared with the January samples. All averages (geometric means) of the groups were significantly different ($P < 0{\cdot}05$) based on variance analysis with log transformed data and Scheefe test except between low and

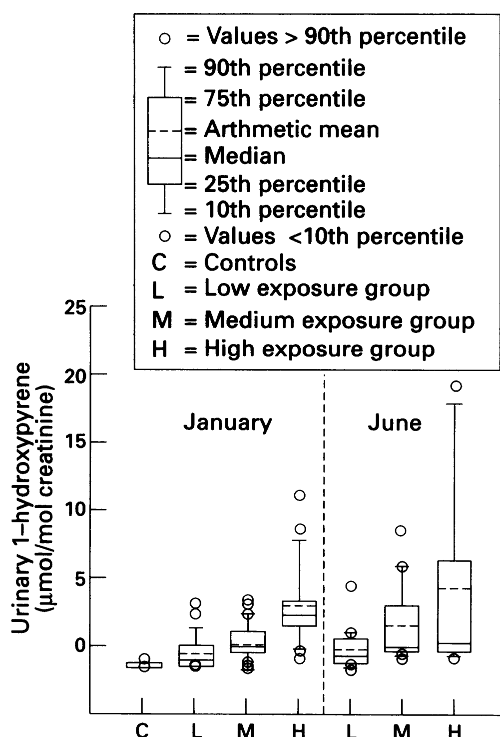

Figure 1 Box plot describing urinary 1-hydroxypyrene from coke oven workers divided into groups based on anticipated PAH exposure. The urine samples were collected in January and in June. For a description of the groups see table 1.

Table 2 1-Hydroxypyrene in urine from coke oven workers divided into groups based on exposure and sampling time

Groups	Mean values (µmol/g creatinine)			
	Arithmetic		Geometric	
	January	June	January	June
Control	0·14	—	0·11	—
Low	1·11	1·32	0·67	0·81
Medium	1·80	2·93	1·30	2·17
High	4·26	5·53	3·42	3·07

Table 3 1-Hydroxypyrene in urine from coke oven workers divided into groups based on smoking

Groups	Mean values (µmol/g creatinine)			
	Arithmetic		Geometric	
	January	June	January	June
Control non-smokers	0·08	—	0·07	—
Control smokers	0·22	—	0·17	—
Exposed non-smokers	2·23	2·35	1·22	1·44
Exposed smokers	2·16	3·55	1·40	1·86

Table 4 Antibody to benzo(a)pyrene DNA adducts in plasma from coke oven workers

Groups	Samples (n) All	Samples (n) Positive* n (%)	Arithmetic mean of antibodies (relative units) All	Arithmetic mean of antibodies (relative units) Positive
Samples January:				
Control	13	6 (46·2)	6·44	13·96
Low	23	14 (60·9)	10·19	16·74
Medium	26	12 (46·2)	4·30	9·32
High	18	12 (66·7)	3·68	5·51
Samples June:				
Low	18	9 (50·0)	14·80	29·59
Medium	18	7 (38·9)	3·91	10·06
High	12	4 (33·3)	1·39	4·17
Non-smokers January:				
Control	7	2 (28·6)	0·76	2·67
Low	9	4 (44·4)	2·09	4·71
Medium	10	7 (70·0)	6·85	9·79
High	10	6 (60·0)	3·53	5·89
Non-smokers June:				
Low	9	5 (55·6)	2·74	4·93
Medium	7	3 (42·9)	7·01	16·36
High	5	1 920·0)	0·20	1·00

*All values used were greater than 0.

Table 5 Regression analysis of IgG antibody to benzo(a)pyrene DNA adducts at two time points (data from the June sampling were used as dependent variable)

	Estimate	P value	R^2 for regression (%)	n
All values:				
Intercept	−0·83	0·24		
Slope	1·18	<0·0001	96·8	46
Only positive values:				
Intercept	−1·61	0·58		
Slope	1·19	< 0·0001	97·6	10

*Table 6 Correlation (Pearson) between concentration of antibodies to benzo(a)pyrene DNA adducts and to DNA**

	Anti DNA (January)	Anti DNA (June)
Anti B(a)P DNA (January)	0·55† (43)‡ 0·0002§	—
Anti B(a)P DNA (June)	—	0·69 (20) 0·0008

*Calculation performed on selected positive log transformed data; †correlation coefficient; ‡sample size; §P value.

medium exposure groups in the January sampling and medium and high exposure in the June sampling. Table 2 shows arithmetic and geometric mean. In the control group there was a significant difference between smokers and non-smokers for urinary 1-hydroxypyrene. There was no such significant difference between the exposed smokers and non-smokers. But the arithmetic and geometric means show a larger increase in the exposed group than the differences found in the control group except for the arithmetic mean in the January samples (table 3).

The division into exposure groups explains part of the variation in urinary 1-hydroxypyrene as already shown. The mean values were higher among the workers who used protective masks. There was a negative correlation between urinary 1-hydroxypyrene and age and a positive correlation between exposure groups and age—that is, there was an overrepresentation of young workers in the high exposure group. Regression analysis of 1-hydroxypyrene in urine from workers sampled in June as the dependent variable and January as the independent variable resulted in an intercept of 0·27 and slope of 0·47, P = 0·0002 and R^2 27%. This analysis was performed on log transformed data and the parameters are given directly from the analysis.

ANTIBODY TO BENZO(A)PYRENEDIOLEPOXIDE MODIFIED DNA

Factors likely to influence formation of antibodies to benzo(a)pyrene modified DNA are PAH exposure, duration of work with PAH exposure, smoking, and age. No significant correlations were found between amount of antibody and any of these factors. Table 4 shows a more detailed analysis of the effect of exposure. As there are several values equal to 0 the geometric mean is 0 and is therefore not shown. The only potential dose response of exposure can be seen in the group of non-smokers (January sampling), but the sample size was small. Otherwise no systematic tendency can be seen.

By analysing the dependency of IgG antibody in the June samples on the values found in the January samples we found a high correla-

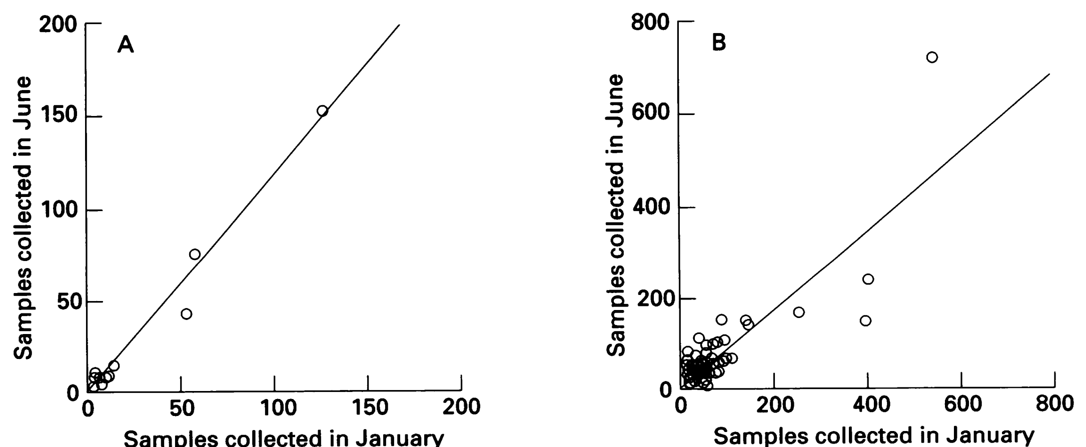

Figure 2 Scatter plots of antibody concentration in June as the dependent variable and the antibody concentration in samples collected in January as the independent variable with regression lines plotted. (A) Antibodies to benzo(a)pyrene DNA adducts. (B) Antibodies to unmodified calf thymus DNA.

Table 7 Hydroxyethylvaline bound to haemoglobin in samples from coke oven workers and a control group

	Hydroxyethylvaline (pmol/g haemoglobin)					
Group	n	Mean (SD)	Median	Interquartile range	1-Hydroxy-pyrene (mean)	Workers' age (mean)
Non-smokers	11	49·1 (25·0)	40·6	30·5	2·18	43·0
Smokers	9	256·8 (128·9)	270·2	185·9	2·36	44·3
Non-exposed non-smokers	6	42·2 (16·2)	39·5	17·0	0·08	52·7
Exposed non-smokers	5	57·3 (32·8)	49·2	29·6	4·69	31·4
Non-exposed smokers	4	211·7 (103·6)	208·0	178·5	0·13	52·0
Exposed smokers	5	293·0 (146·7)	270·2	181·0	4·13	38·2

tion. For this regression analysis about 97% of the variation could be explained both when analysis was done on all measured samples and on selected sample pairs with only positive values (table 5). Figure 2(A) shows a regression plot for samples with positive values of IgG antibody to benzo(a)pyrene modified DNA. A similar regression analysis was done on data from IgG antibodies to calf thymus DNA (fig 2(B)), the regression coefficient was 0·82 and intercept 11·1, P < 0·0001, R^2 73%. If the three highest values in fig 2(A) were deleted, R^2 was reduced to 64%, and if the four highest values in fig 2(B) were deleted R^2 was reduced to 65%. There was also an association between antibodies to benzo(a)pyrene modified DNA and antibodies to DNA (table 6).

HYDROXYETHYLVALINE BOUND TO HAEMOGLOBIN

Hydroxyethylvaline was analysed in 20 haemoglobin samples collected in January, 10 from the control group and 10 from the highly exposed group, 10 were smokers and 10 non-smokers. There was a significant difference between the concentration of hydroxyethylvaline in haemoglobin among the smokers and the non-smokers $z = 3{\cdot}72$ (Mann-Whitney U test, P = 0·0002)

Hydroxyethylvaline data were analysed for correlation with urinary 1-hydroxypyrene, exposure group, smoking, age, and years worked at the plant. Only smoking gave a significant correlation coefficient equal to 0·78 (P = 0·0001, Pearson product moment).

Table 7 shows the actual values. There were higher hydroxyethylvaline adducts in both PAH exposed groups when we divided them into smokers and non-smokers and compared them with control groups of smokers and non-smokers. The differences were not significant. But the sample size was small.

Discussion

The urinary 1-hydroxypyrene results are comparable with previously published results at coke oven plants.[6 11] Our 1-hydroxypyrene data correlates well with expected exposure based on job descriptions, and workers at the top side of the oven have the highest values as expected (also found by others).[6] But it should be noted that even among the workers at the top side several workers were found with low urinary 1-hydroxypyrene (fig 1). It is interesting to see that urinary 1-hydroxypyrene values measured in the June samples can partly be explained by values found in January. This shows that sampling only once gives a fairly good estimate of the exposure at such a plant.

Measurements of 1-hydroxypyrene mirrors the exposure in the short term, about 24 hours,[32] whereas PAH DNA adducts have a longer half life and are therefore likely to be the result of exposure from a much longer time period, at least several weeks.[33] With this background, correlation between adducts and urinary 1-hydroxypyrene would be less likely. Recent reports have found no association,[9] or a weak association[34] between adducts and urinary 1-hydroxypyrene.

Antibodies to benzo(a)pyrene modified DNA were first measured in serum from coke oven workers,[15 16] where about 30% of the workers had such antibodies. Unexposed control groups were not included in these studies. Later Newmann and coworkers studied a non-occupationally exposed group and found antibodies in serum from about 40% of the workers,[17] which is comparable with the data from coke oven workers, and indicates that coke oven workers are not more prone to have high antibody concentrations than non-occupationally exposed people. Our study, with a control group exposed to low concentrations of PAHs, clearly confirms the above indication that high PAH exposure has no effect or only a marginal effect upon formation of antibodies to benzo(a)pyrene DNA adducts. Similar results have been found in a study among foundry workers[35] and patients treated with coal tar.[36] The influence of number of years worked at a coke oven has also been analysed and no significant association has been found,[16] which also supports our findings.

We cannot offer any good explanation why some people have high concentrations of antibody to benzo(a)pyrene DNA adducts, but it is likely to be due to some personal factor, either genetic, or environmental, or both. As we found a high correlation between antibodies to benzo(a)pyrene DNA adducts and antibodies to pure DNA (table 6) a genetic component may be possible. One could dispute this correlation as the analysis for antibodies to DNA and for antibodies to benzo(a)pyrene DNA adducts was done with the same analysis, but the high concentration of antibodies to DNA should decrease the concentration found for antibodies to BPDE modified DNA. Passive smoking and industrial pollution during infancy could induce some lasting formation of antibody. We have no information on these factors, but the studied population does live in an industrialised region. We are planning a follow up of this analysis. The plant has

been closed for several years and it would be interesting to investigate if the same people still have high concentration of these antibodies.

Ethylene oxide has been identified in atmospheric air samples as a result of combustion of hydrocarbon fuels.[27] It is therefore not unlikely that coke oven workers will be exposed to hydroxyethylating agents. The established fact that cigarette smoking increases hydroxyethylvaline in haemoglobin complicates the analysis of the possible effect of the workplace. The increase due to work at the coke oven plant was about 7% to 40% of the difference we found between smokers and non-smokers, and therefore not important as a risk factor compared with the concentrations found among workers occupationally exposed to ethylene oxide.[22 25] To unequivocally analyse the possible contribution from work at a coke oven plant one should study non-smokers in a larger group than we have investigated here.

In summary, we have measured urinary 1-hydroxypyrene concentrations in different working groups at a coke oven plant. We have established new evidence that coke oven workers do not have increased amounts of antibody to benzo(a)pyrene DNA adducts compared with a control group. We have presented evidence that the quantity of antibodies to benzo(a)pyrene DNA adducts are person related, possibly genetic. Hydroxyethylvaline adducts in haemoglobin were increased among coke oven workers, but were not significant.

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1 International Steering Committee of Medical Editors, Uniform requirements for manuscripts submitted to biomedical journals. *Br Med J* 1979;1:532–5.
2 Soter NA, Wasserman SI, Austen KF. Cold urticaria: release into the circulation of histamine and eosinophil chemotactic factor of anaphylaxis during cold challenge. *N Engl J Med* 1976;294:687–90.
3 Weinstein L, Swartz MN. Pathogenic properties of invading micro-organisms. In: Sodeman WA Jr, Sodeman WA, eds. *Pathologic physiology, mechanisms of disease.* Philadelphia: W B Saunders, 1974:457–72.