Quantitation of Polycyclic Aromatic Hydrocarbons, 1-Hydroxypyrene, and Mutagenicity in Urine of Coal Tar-treated Psoriasis Patients and Untreated Volunteers

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Abstract

Coal tar-treated psoriasis patients were used as a model population to test a newly developed enzyme-linked immunosorbent assay (ELISA) for urinary excretion of benzo(a)pyrene and related polycyclic aromatic hydrocarbons (PAHs). The ability of the ELISA to detect exposure was also compared with that of two previously established biomonitoring methods, measurement of urinary 1-hydroxypyrene by high performance liquid chromatography with fluorescence detection and mutagenicity measured by the Salmonella typhimurium mutagenesis assay. Urine samples were collected from 57 patients and 53 untreated volunteers. Urinary excretion of PAH metabolites, measured by competitive ELISA with a monoclonal antibody (4D5), was elevated in patients (mean, 730 ± 1370 pmol/mol creatinine) compared with untreated volunteers (110 ± 90 pmol/mol creatinine; P < 0.0001). 1-Hydroxypyrene also was elevated in patients (mean, 547 ± 928 pmol/mol creatinine) compared with volunteers (mean, 0.14 ± 0.17 pmol/mol creatinine; P < 0.0001). Much larger differences between mean values in patients and volunteers were observed with the 1-hydroxypyrene assay compared with the PAH metabolite ELISA. No significant effect of smoking could be detected by either assay. Analysis by the Salmonella typhimurium mutagenesis assay indicated elevated mutagenicity in urine from patients (1410 ± 2750 revertants/mmol creatinine) compared with volunteers (715 ± 846 revertants/mmol creatinine; P = 0.072). In all subjects, there was a good correlation between the PAH metabolites and both 1-hydroxypyrene (r = 0.717; P < 0.0001) and urinary mutagenicity (r = 0.317; P = 0.004). These results suggest that the ELISA, which easily can be carried out on large numbers of samples, can be used for monitoring urinary excretion of PAHs in a high exposure population. Ongoing studies are designed to determine its applicability to lower exposure populations.

Introduction

Analysis of urinary excretion of chemical carcinogens has been used extensively to monitor environmental and occupational exposure to PAHs [reviewed in (1)]. Methods of analysis include those for specific chemicals, such as 1-hydroxypyrene, by a highly sensitive HPLC method with fluorescence detection (2, 3), or for mutagenic agents in general with the Salmonella typhimurium mutagenesis assay (4, 5). Although both of these methods have been used extensively in human studies, immunoassays can be used much more easily to analyze the large number of samples routinely collected in epidemiological studies.

To develop an ELISA for monitoring exposure to BP by measurement of urinary excretion, we generated a monoclonal antibody (4D5) from mice immunized with BP coupled to carrier protein (6). The antiserum recognizes BP (50% inhibition at 4 pmol) and a number of hydroxylated BP metabolites (50% inhibition at 1–90 pmol). In addition, there is cross-reactivity with several other PAHs tested, including 1-hydroxypyrene (50% inhibition at 5.2 pmol) and 7,12-dimethylbenz(a)anthracene (50% inhibition at 67 pmol). Because of this cross-reactivity, the ELISA will detect multiple PAH metabolites, which may be present in urine samples from humans who are exposed to complex PAH mixtures. Thus, the assay serves as a marker of exposure to this general class of chemicals.

The present study tests the applicability of the ELISA for use on human samples in coal tar-treated psoriasis patients as a model PAH-exposed population and untreated volunteers. Results from the ELISA are compared with those from the established methods for measurement of urinary excretion of 1-hydroxypyrene by HPLC and measurement of mutagenicity by the Salmonella typhimurium mutagenesis assay (4).

Materials and Methods

Psoriasis patients were recruited from the inpatient service, Department of Dermatology, The Presbyterian Hospital at Columbia-Presbyterian Medical Center. All patients were diagnosed as having plaque stage psoriasis and were admitted...
for modified Goekerman therapy. Patients applied either an ointment or a gel-based coal tar product, or both, to the entire body surface at least once a day, followed by UVB treatment. Precise dose determinations were not possible because treatments were self applied with variable efficiency. The estimated exposure is 20–100 gm of tars/day. Gas chromatography–mass spectrometry (META Environ, Inc., Boston, MA) of a representative sample of tars used on these patients indicated 140 mg BP/kg and 700 mg pyrene/kg. Thus, patients received approximately 2.8–14 mg BP/day. The number of days of treatment before sample collection was also determined.

Healthy volunteers, comparable with cases in age and sex distribution and smoking status, who had no reported exposures to coal tar shampoos or ointments, were recruited by posted announcements at the Medical Center. Twenty-four-h urine samples were collected from all subjects and frozen at −20°C. A questionnaire was administered by one of three trained interviewers eliciting information on occupation, diet, and smoking history. Urine samples (25 ml) were filtered through Whatman No. 1 filter paper, adjusted to pH 5.0 with HCl, and incubated overnight with β-glucuronidase/aryl sulphatase (200 units/ml, Sigma Chemical Company, St Louis, MO). 1-Hydroxypyrene and PAH metabolites were isolated by Sep-Pak C18 cartridge chromatography (Waters, Milford, MA). Cartridges were prewashed with 10 ml of chloroform, 10 ml of methanol, and 10 ml of water before sample addition. After washing with 10 ml of water and 5 ml of 5% methanol, metabolites were eluted with 5 ml of 80% methanol. The eluent was evaporated under nitrogen and redissolved in 0.5 ml of methanol.

1-Hydroxypyrene was analyzed by HPLC with fluorescence detection essentially as described (2, 3) on a Pharmacia LKB (Pharmacia Fine Chemicals, Piscataway, NJ) instrument with a Spectrovision Model FD-200 fluorometer. Excitation was at 242 nm and emission was measured with a 360-nm blocking filter. A linear gradient from 50–100% methanol in water over 20 min was used for elution of samples on a 5-μm Bondapak C18 column (Waters). A standard curve was generated by measurement of peak height with serial dilutions (1.5 pmol–5 nmol) of a 1-hydroxypyrene standard (Molecular Probes, Eugene, OR).

PAH metabolites were analyzed by competitive ELISA essentially as described (6) on plates coated with BP covalently coupled to bovine serum albumin (BP-BSA, 5 ng) by drying PBS solutions after blocking with 1% fetal calf serum in PBS. Antibody (4D5) was diluted 1:2000 in 1% fetal calf serum in PBS-Tween. A standard curve was generated by addition to the wells of urine extracts equivalent to 2.5 μl of urine in 50 μl of PBS/well. A single sample (control) gave a nondetectable value (<20% inhibition); this individual was assigned a value of 0.1 pmol equivalents of BP/μl, midway between 0 and the lowest detectable value.

Analysis of urinary mutagens by the Salmonella typhimurium mutagenesis assay was carried out as described using strain TA100 (7). Filtered urine samples were extracted by XAD 2 chromatography and duplicate assayed at 6.25–, 12.5–, and 25–ml equivalents/plate with strain TA100 in the presence of 200 units/plate of β-glucuronidase and 0.5 ml of rat liver S9 mix (Aroclor 1254 induced, Organon Teknika, Durham, NC). BP (5 μg/plate) was used as a positive control and resulted in 860 ± 140 revertants/plate while spontaneously revertants were 132 ± 32 revertants/plate. Examination of the background lawn of bacteria indicated that urine extracts from two patients were toxic at all concentrations tested and could not be evaluated. Urine from six controls and five patients were toxic when tested at 25 ml of equivalents/plate. Values are reported as [(revertants in plate)]/[(revertants in blank)]/mmol creatinine for the highest concentration tested without detectable toxicity. Creatinine was analyzed with a kit (Sigma).

Table I  Comparison of assay results on coal tar-treated psoriasis patients and untreated volunteers

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Volunteers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>57</td>
<td>53</td>
</tr>
<tr>
<td>Age</td>
<td>42 ± 18</td>
<td>45 ± 15</td>
</tr>
<tr>
<td>Males</td>
<td>31 (54%)</td>
<td>29 (55%)</td>
</tr>
<tr>
<td>Smokers</td>
<td>19 (33%)</td>
<td>17 (32%)</td>
</tr>
<tr>
<td>Cigarettes/day</td>
<td>20 ± 17</td>
<td>22 ± 14</td>
</tr>
<tr>
<td>Range</td>
<td>0.14–70</td>
<td>6–60</td>
</tr>
<tr>
<td>PAH metabolitesa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>730 ± 1370</td>
<td>113 ± 90</td>
</tr>
<tr>
<td>Range</td>
<td>40–9180</td>
<td>10–410</td>
</tr>
<tr>
<td>Mean</td>
<td>546 ± 928</td>
<td>0.14 ± 0.17</td>
</tr>
<tr>
<td>Range</td>
<td>10–5160</td>
<td>0.02–0.98</td>
</tr>
<tr>
<td>Mutagenicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meana</td>
<td>152 ± 134</td>
<td>134 ± 99</td>
</tr>
<tr>
<td>Ranged</td>
<td>0–670</td>
<td>0–481</td>
</tr>
<tr>
<td>Meanb</td>
<td>1400 ± 2700</td>
<td>715 ± 846</td>
</tr>
<tr>
<td>Rangec</td>
<td>0–14600</td>
<td>0–4259</td>
</tr>
<tr>
<td>n</td>
<td>52</td>
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</table>

a micromole equivalents of BP/mmol creatinine
b P values for patient-volunteer differences by t test
c micromole/mol creatinine
d Induced revertants/25 ml urine
e Induced revertants/mmol creatinine

Results

The coal tar-treated psoriasis patient group and the control group were similar in age and sex distribution and smoking status (Table 1). Urinary PAH metabolites, measured by the PAH-ELISA, were elevated in patients (mean 730 ± 1370 micromole equivalents of BP/mmol creatinine) compared with untreated volunteers (110 ± 90 μmol/mol, P < 0.0001) (Table 1, Fig. 1). Urinary levels of 1-hydroxypyrene also were elevated in treated patients (mean 547 ± 928 μmol/mol creatinine) compared with volunteers (mean 0.14 ± 0.17 μmol/
Fig. 1. Concentration of urinary PAH metabolite levels determined by ELISA versus urinary 1-hydroxypyrene levels determined by HPLC with fluorescence detection in 45 volunteers and 51 patients, expressed as pmol/mol creatinine. Data from two volunteers and two patients overlap.

mol, $P < 0.0001$). There was no significant association in patients between the number of days treated and either the level of 1-hydroxypyrene or urinary PAH metabolites. Neither smoking status nor recency of consumption of charcoal-broiled foods influenced levels of PAH metabolites or 1-hydroxypyrene in either patients or volunteers. There was a good correlation between PAH-ELISA data and 1-hydroxypyrene levels in all subjects ($r = 0.717, n = 96, P < 0.0001$) (Table 2, Fig. 1). Correlations also were significant for patients and volunteers analyzed separately (Table 2).

Because the Salmonella typhimurium mutagenesis assay has been used extensively to evaluate human exposure, urines also were tested for mutagenicity and the results were compared with the ELISA and HPLC methods. Induced revertants/25 ml of urine for controls ranged from 0 to 480 (mean, $134 \pm 99$ revertants/25 ml) while in patients the range was 0–670 (mean, $153 \pm 134$ revertants/25 ml). After adjustment for creatinine, mean values were $1413 \pm 2750$ revertants/mmol creatinine in patients and $715 \pm 846$ in controls ($P = 0.072$). There were significant associations between PAH metabolite levels, determined by ELISA, and urinary mutagenicity in all subjects combined and in patients but not in controls (Table 2). There were no significant associations between 1-hydroxypyrene levels and mutagenicity.

**Discussion**

All three urine assays, PAH-ELISA, 1-hydroxypyrene, and mutagenicity, detected the high exposure of coal tar-treated patients compared with untreated volunteers. Differences between the two groups were greatest for the 1-hydroxypyrene (3900-fold), which is the most specific assay, intermediate for the PAH-ELISA (6.5-fold), and least for the non-specific mutagenicity assay (2-fold). The large difference with the 1-hydroxypyrene assay is mainly a result of the lower mean level in untreated volunteers ($0.14 \mu mol/mole creatinine$) than for the PAH-ELISA ($113 \mu mol/mole creatinine$), even though the mean values in patients were similar in both assays (730 versus $546 \mu mol/mole creatinine$). The ELISA lacks the specificity of the 1-hydroxypyrene assay because antibody 4D5 cross-reacts with BP and its metabolites, 1-hydroxypyrene, and probably a number of other PAH metabolites. For these reasons, although there is a good correlation between PAH-ELISA and 1-hydroxypyrene data (Fig. 1), it is difficult to compare quantitative values. However, the PAH-ELISA can be used much more easily to analyze large numbers of samples than either the HPLC method for measurement of 1-hydroxypyrene or the mutagenicity assay, and it gave highly significant differences between the two exposure groups. Moreover, the PAH-ELISA may be more relevant to potential cancer risk because pyrene is noncarcinogenic, while BP and a number of other PAHs are carcinogenic.

In this study, neither the PAH-ELISA nor the 1-hydroxypyrene assay detected smoking-related increases in urinary
PAH excretion in patients or volunteers. Although cigarette smoking does not strongly influence urinary 1-hydroxy-pyrene levels, some studies have detected significant effects (8, 9). Other studies have suggested the importance of charcoal food consumption on PAH exposure (10-12). However, no association was found in patients or volunteers between PAH-ELISA or 1-hydroxy-pyrene data and consumption of charcoal-broiled or smoked meat or fish over the past 2 weeks or days since these foods were last eaten.

Coal tar-treated psoriasis patients have been used as a model population for skin exposure to PAH in a number of studies. Percutaneous absorption was demonstrated by increased urinary excretion of mutagens (13-17) and 1-hydroxy-pyrene (3, 14, 15). 1-Hydroxy-pyrene levels in the present study are comparable with those found in previous studies [1.7-14.1 pmol/ml (3) or 25-1565 µg/g creatinine (14, 15)]. These previous studies also determined that the measurement of 1-hydroxy-pyrene was more specific and sensitive than measurement of urinary mutagenicity. 3-Hydroxy-BP also has been detected in the urine of coal tar-treated patients but not controls, and at 10-fold lower levels than 1-hydroxy-pyrene (18). Increased levels of PAH-DNA adducts, sister chromatid exchange, and chromosomal aberrations also have been observed in patients compared with controls (16, 19, 20).

Recently, an radioimmunoassay, using an antiserum with similar specificity to that reported here, was used for the detection of urinary PAH metabolites in coke oven workers (21). Mean levels in controls were 0.44 ng/mmole creatinine and rose to as high as 1.10 ng/mmole in workers. A seasonal difference in metabolite levels was observed with higher values in samples collected in the summer. Smoking also significantly increased metabolite levels.

BP tetroxols also have been measured in human urine from individuals consuming char-broiled foods (22) and from the subjects in the present study (23). Samples were purified by Sep-Pak extraction and immunoaffinity chromatography with antibody 8E11 and analyzed by synchronous fluorescence spectroscopy after HPLC isolation. In those individuals who consumed large quantities of char-broiled foods, levels of BP tetroxols in the range of 0.20-3.12 pmol/ml were measured (22), while in the coal tar-treated patients, values ranged from <0.01-0.3 pmol/ml (23). These data as well as the results presented here suggest that PAH metabolites can be measured sensitively in urine for monitoring human exposure. The use of immunoassays, which easily can be carried out on large numbers of samples, would greatly simplify biological monitoring studies. Ongoing studies are determining the applicability of the PAH-ELISA for detection of occupational exposure.

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References

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