

Comparison of Aryl Hydrocarbon Hydroxylase Induction in Cultured Blood Lymphocytes and Pulmonary Macrophages

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ABSTRACT Aryl hydrocarbon hydroxylase induction was studied in cultured peripheral blood lymphocytes and pulmonary alveolar macrophages from 15 smokers and 8 nonsmokers with a variety of pulmonary diseases. Enzyme levels in lymphocytes from cigarette smokers cultured in medium without an inducing agent were 57 ± 6 mU/ 10^6 cells (mean \pm SEM), while enzyme levels in lymphocytes from nonsmokers were 20 ± 2 mU/ 10^6 cells ($P < 0.001$). When lymphocytes were cultured in the presence of the inducing agent, benzo(a)anthracene, enzyme activity was increased to 168 ± 23 mU/ 10^6 cells in smokers' cells and 99 ± 22 mU/ 10^6 cells in lymphocytes from nonsmokers ($P < 0.04$). When noninduced enzyme values in cultured macrophages were compared, smokers' cells had enzyme levels of 45 ± 5 mU/ 10^6 cells, whereas nonsmokers had enzyme activity of 24 ± 2 mU/ 10^6 cells ($P < 0.002$). However, pulmonary macrophages from smokers or nonsmokers, cultured in the presence of benzo(a)anthracene, had similar levels of induced enzyme activity ($P > 0.1$). A positive correlation was observed for nonsmokers ($r = 0.596$, $P > 0.1$ < 0.2) or smokers ($r = 0.640$, $P < 0.04$), when enzyme values for noninduced cultures of macrophages and lymphocytes from individual patients were simultaneously compared. Enzyme values for macrophages and lymphocytes cultured in the presence of an inducer also revealed a positive correlation for individual smokers ($r = 0.801$, $P < 0.001$) or nonsmokers ($r = 0.785$, $P < 0.01$). Inducibility (expressed as fold-induction) for macrophages

and lymphocytes from individual patients was also positively correlated ($r = 0.889$, $P < 0.001$ for nonsmokers and $r = 0.942$, $P < 0.001$ for smokers). These results indicate that the capacity for aryl hydrocarbon hydroxylase induction is similar whether tested in lymphocytes or pulmonary macrophages from this group of pulmonary disease patients.

INTRODUCTION

Aryl hydrocarbon hydroxylase (AHH)¹ is an inducible, monooxygenase complex localized in the endoplasmic reticulum of many body tissues (1-5). By hydroxylation, AHH converts lipophilic polycyclic aromatic hydrocarbons (e.g. benzo(a)pyrene and benzo(a)anthracene [BA]) into more polar, water-soluble compounds that are eliminated from the body. During hydroxylation, intermediate compounds with enhanced mutagenic (6-10) and carcinogenic (11, 12) activities are formed. Large quantities of AHH may be harmful to cigarette smokers through increased production of these potent intermediate compounds from the polycyclic hydrocarbons present in cigarette smoke. Since capacity for induction of AHH might be genetically determined in both animals (13-15) and man (16, 17), individual variation in the production of this enzyme could be important in modifying an individual's risk of developing lung cancer.

When the cultured peripheral blood lymphocyte has been used as the test cell, variability and intra-individual fluctuation of AHH inducibility (17) have hindered attempts to investigate the possible associa-

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¹Abbreviations used in this paper: AHH, aryl hydrocarbon hydroxylase; BA, benzo(a)anthracene; PAM, pulmonary alveolar macrophage.

tion previously reported between levels of AHH and the development of lung cancer (18). Comparison of AHH activity in more than one tissue from an individual has proved more satisfactory for the investigation of the capacity for AHH induction (19, 20). In addition to lymphocytes, we have selected the pulmonary alveolar macrophage (PAM) as another tissue that can be used to measure AHH inducibility. These cells, directly exposed to cigarette smoke in the lungs of smokers, are obtainable by broncho-alveolar lavage (5). Since AHH is inducible in cultured human PAMs by either cigarette smoke extracts (21) or BA (22), these cells are suitable for determining *in vitro* AHH inducibility.

Comparison of AHH levels in freshly lavaged PAMs with the degree of induction of AHH in cultured lymphocytes has revealed a positive correlation between values for these different cells from individual healthy volunteers (19) or patients without cancer (20). In contrast, values for AHH in these cells from cancer patients were dissociated (20). In the present study, we compare *in vitro* inducibility of AHH in PAMs and peripheral blood lymphocytes from patients with a variety of nonneoplastic lung diseases. When studied in cultured cells, similar AHH values were observed for PAMs and lymphocytes from smokers or nonsmokers. Good correlations were noted for individual PAM and lymphocyte AHH values, suggesting that inducibility of the enzyme in these two types of cells is similar.

METHODS

Study subjects. Peripheral blood lymphocytes and PAMs were obtained from 23 patients (6 females and 17 males) ranging in age from 21 to 69 yr (mean 44 yr). Patients included 8 nonsmokers and 15 cigarette smokers. An individual was classified as a nonsmoker if he or she had not smoked tobacco for 6 mo or more before the study, a period selected because previous studies indicated that AHH activity in PAMs elevated by cigarette smoking reverts to lower nonsmoker levels within 3 mo of terminating smoking (5). Only two of the nonsmokers were former smokers; both had stopped smoking 1 yr before study.

None of the patients were diagnosed as having cancer, but they did have a variety of nonneoplastic lung disorders, including four with pulmonary tuberculosis, three with pulmonary abscess, six with chronic pneumonia, two with sarcoidosis, one with interstitial fibrosis, and seven with hemoptysis of undetermined origin. Before the collection of samples, the study was explained to all subjects and written informed consent was obtained on a form approved by the Baylor Institutional Review Board. All patients were on normal institutional diets at the time of this study. Hospital charts were carefully screened to be sure patients were not receiving medications that might be expected to alter their AHH levels.

Preparation of PAMs. PAMs were obtained during diagnostic bronchoscopy as previously described, employing saline lavage with 50–100 ml sterile 0.15 M NaCl through an Olympus 5-B2 fiberoptic bronchoscope

(Olympus Corporation of America, New Hyde Park, N. Y.) (5). PAMs (representing 80–95% of the total number of cells obtained) were separated by centrifugation, washed, and resuspended at a known concentration in Joklick's modified minimum essential medium (GIBCO F-13, Grand Island Biological Co., Grand Island, N. Y.) prepared with ultrapure water (Harleco, Gibbstown, N. J.) and supplemented with 10% fetal calf serum, heparin (10 U/ml), phytohemagglutinin (10 ml/liter), and pokeweed mitogen (10 ml/liter). 10^6 cells were placed in culture vials containing 5 ml of this medium and cultured for 24 h, as previously described, with and without addition of $10\ \mu\text{M}$ of the inducer, BA (22).

$1-2 \times 10^6$ PAMs were placed in culture vials containing 2 ml of the medium and were assayed in triplicate on the day of collection to determine fresh cell AHH activity.

Preparation of lymphocyte cultures. Mononuclear cells were separated from 10 ml venous blood by Ficoll-Hypaque sedimentation (23). Cells were washed with heparinized saline (10 U/ml), resuspended in the medium described above, and quantitated using a model ZBI Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.). Differential counts were made from slides stained with Wright-Giemsa stain (85–92% of the cells obtained were lymphocytes). Cells were then cultured with and without $10\ \mu\text{M}$ BA for 96 h at 37°C in a mixture of CO_2 and air, as previously described (20, 21).

Fluorometric analysis of AHH. The method for fluorometric measurement of AHH in PAMs and lymphocytes has been previously reported (20, 21). AHH activity is expressed in milliunits per 10^6 cells, where 1 U is the amount of fluorescence equivalent to the production of 1 pmol/min of 3-hydroxybenzo(a)pyrene. Values were adjusted for an extraction efficiency of 80%. Values for AHH in cultured PAMs and lymphocytes were expressed as noninduced (cultured without BA), induced (cultured in the presence of BA), delta induction (induced AHH value – noninduced AHH value), or fold-induction [(induced AHH value/noninduced AHH values) – 1]. The coefficients of variation for AHH values in lymphocytes and PAMs were 17% and 13%, respectively.

RESULTS

The present study was designed to investigate *in vitro* induction of AHH in cultured peripheral blood lymphocytes and PAMs, to determine whether induction in these two different cell types is comparable. In approaching this question, it was important to tabulate the data separately for nonsmokers and cigarette smokers, since activities of AHH in noninduced cultures of PAMs and lymphocytes were elevated when cells came from cigarette smokers (Table I). In spite of higher AHH activity in freshly obtained PAMs from smokers, the further increment of enzyme induction by BA is similar for cells from smokers and nonsmokers. In lymphocytes from smokers, levels of AHH induction are significantly higher than in cultured lymphocytes from nonsmokers. Because of higher noninduced levels in cultured PAMs and lymphocytes, values for fold-induction for smokers' cells tended to be lower than fold-induction values for nonsmokers' cells, but these differences were not statistically significant. Values for the increment in AHH between

TABLE I
Comparison of AHH Activities in PAMs and Blood Lymphocytes

AHH activity	PAMs			Lymphocytes		
	Nonsmoker (n = 8)	Smoker (n = 15)	P value*	Nonsmoker (n = 8)	Smoker (n = 15)	P value
Noninduced†	24±2§	45±5	<0.002	20±2	57±6	<0.001
BA-induced‡	104±22	166±30	>0.1	99±22	168±23	<0.04
Delta-induction¶	78±19	122±26	>0.1	79±21	112±20	>0.2
Fold-induction**	3.11±60	2.46±0.37	>0.3	3.66±0.83	2.07±37	>0.1

* *t* test for nonpaired data with degrees of freedom adjusted for unequal variances. Values considered significant if *P* < 0.05.

† Cells cultured without the inducer BA. Value expressed as milliunits/10⁶ cells.

§ Means±SEM.

‡ Cells cultured with BA. Values represent milliunits/10⁶ cells.

¶ Represents the BA-induced – noninduced AHH activity expressed as milliunits/10⁶ cells.

** Represents the [(BA-induced AHH/noninduced AHH) – 1].

noninduced and BA-induced cultured cells (delta induction) were higher for cells from smokers than from nonsmokers, but again these differences were not statistically significant.

Values obtained for AHH in PAMs and lymphocytes from patients within the nonsmoker and smoker groups were similar (Fig. 1). Levels of AHH in PAMs freshly obtained by lavage were higher for smokers (96±20 mU/10⁶ cells) than for nonsmokers (20±4) mU/10⁶ cells (*P* < 0.002). Values for freshly obtained peripheral blood lymphocytes were not determined, since these levels are not detectable in appreciable amounts until cells are cultured with mitogens (1). With PAMs from nonsmokers, levels of AHH are virtually identical for cells cultured for 24 h with no inducer in the medium and for cells freshly lavaged from the lung (*P* > 0.3). In contrast, values for PAMs from smokers are reduced to about half the original levels after culture for 24 h with no inducer in the medium (*P* < 0.02).

AHH values were similar when noninduced enzyme levels were compared for nonsmoker or smoker PAMs and lymphocytes (Fig. 1) (*P* > 0.2 for smokers and nonsmokers). Also, when PAMs and lymphocytes from smokers or nonsmokers were cultured in the presence of BA, similar enzyme induction was observed (*P* > 0.2 for both groups). When the AHH induction for PAMs and lymphocytes was calculated by comparing noninduced and BA-induced enzyme levels, both delta induction and fold-induction values were similar for nonsmokers and cigarette smokers (*P* > 0.3 in all instances).

Comparison of AHH values for freshly obtained PAMs with the levels of AHH induced by BA in cultured lymphocytes or cultured PAMs from individual smokers (Fig. 2) demonstrated a good correlation for these two sets of values (*r* = 0.685, *P* < 0.005 for fresh PAM vs. BA-induced PAM AHH, and *r*

= 0.676, *P* < 0.006 for fresh PAM vs. BA-induced lymphocyte AHH). Because AHH levels in non-smokers' cells should be noninduced compared with cells from cigarette smokers, AHH levels were compared in fresh PAMs and in PAMs or lymphocytes from individual nonsmokers. A good correlation was observed for fresh PAM and noninduced PAM AHH values (*r* = 0.795, *p* < 0.01), while the correlation observed between enzyme values for fresh PAMs and noninduced lymphocytes was weaker (*r* = 0.554, *P* > 0.1 < 0.2). Therefore, the induction of AHH in PAMs in the lung (as determined in freshly lavaged cells) was similar to the in vitro induction of the enzyme in cultured PAMs or lymphocytes.

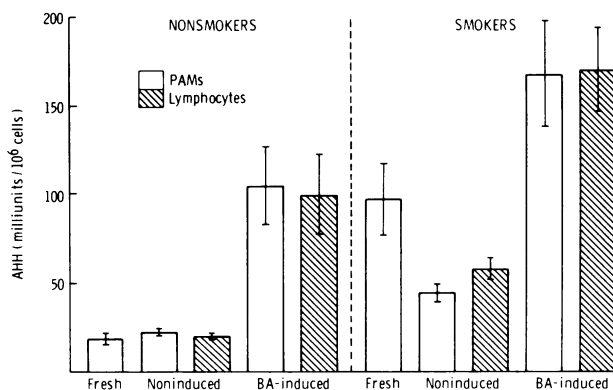


FIGURE 1 AHH activity in PAMs and lymphocytes from smokers and nonsmokers. 1 U of enzyme activity equals the production of 1 pmol of 3-hydroxybenzo(a)pyrene/min. Fresh PAM enzyme activity represents the measurement of AHH in PAMs without further culture after lavage from the lung. Noninduced AHH represents the enzyme levels in cells cultured without an inducer in the medium. Enzyme levels in cells cultured with the inducer BA in the medium are designated as BA-induced. Bars represent the mean ±SEM enzyme values.

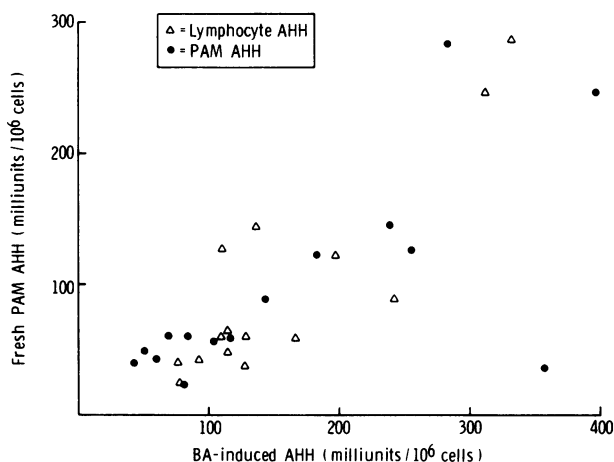


FIGURE 2 Comparison of AHH activity in fresh PAMs and cultured BA-induced PAMs or lymphocytes from individual cigarette smokers ($r = 0.685$, $P < 0.005$ for AHH values in fresh PAMs vs. BA-induced PAMs, and $r = 0.676$, $P < 0.006$ for fresh PAMs vs. BA-induced lymphocytes).

To determine whether a relationship exists between AHH levels in cultured PAMs and lymphocytes from individual smokers or nonsmokers, values for both cell types, cultured without an inducing agent in the medium, were compared (Fig. 3). Values for cells from individual cigarette smokers exhibited a good correlation ($r = 0.640$, $P < 0.04$) as did values for individual nonsmokers ($r = 0.596$, $P > 0.1 < 0.2$). When a similar comparison was performed between levels of AHH in BA-induced PAMs and lymphocytes from individual patients (Fig. 4), a higher degree of correlation was obtained for both nonsmokers ($r = 0.785$, $P < 0.01$)

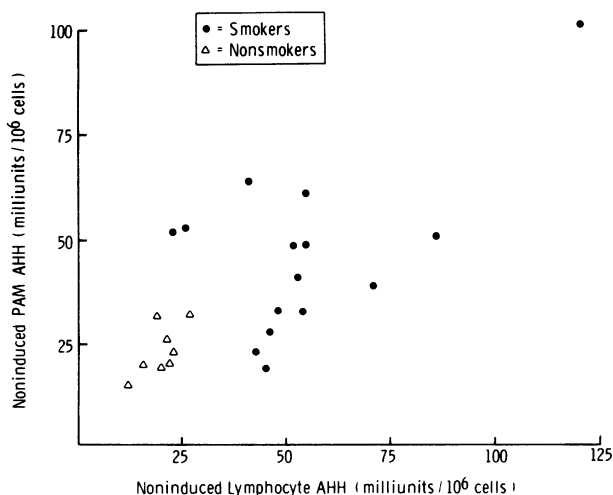


FIGURE 3 Comparison of individual noninduced AHH levels in cultured PAMs and lymphocytes ($r = 0.640$, $P < 0.04$ for smokers and $r = 0.596$, $P > 0.1 < 0.2$ for nonsmokers).

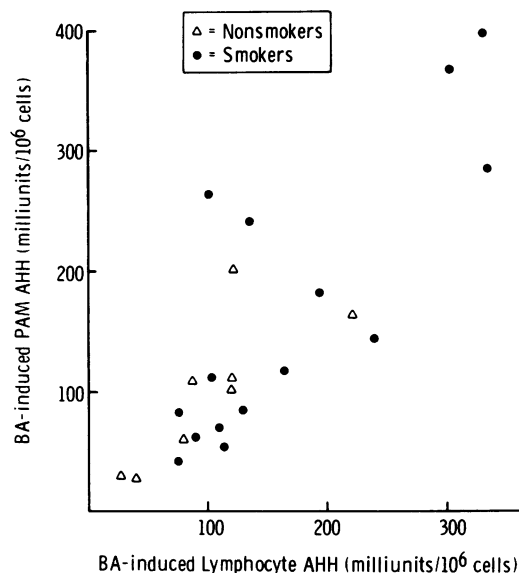


FIGURE 4 Comparison of individual BA-induced AHH activity in cultured PAMs and lymphocytes ($r = 0.801$, $P < 0.001$ for smokers and $r = 0.785$, $P < 0.01$ for nonsmokers).

and smokers ($r = 0.801$, $P < 0.001$). When the values for PAMs and lymphocytes were expressed as delta induction (Fig. 5), the relationship between the two types of cells from nonsmokers and smokers was very similar. The highest levels of enzyme increment after culture with an inducing agent were found with cells from cigarette smokers. When expressed in this manner, the regression lines for nonsmokers ($r = 0.719$, $P < 0.03$) and for cigarette smokers ($r = 0.835$, $P < 0.001$), are almost identical.

The above methods for expressing AHH measurements (in absolute levels of enzyme or in terms of the enzyme increment in cells cultured with and without BA) indicate that AHH levels in one cell type can accurately reflect levels measured in the other cell type. However, cigarette smoking affects the noninduced as well as the BA-induced levels of the enzyme. In approaching the question of whether inherent differences exist in the capacity for AHH induction in different individuals, a more appropriate method might be the use of the ratio of induced to noninduced enzyme values. When the degree of inducibility of AHH (expressed as fold-induction) in PAMs and lymphocytes from individual nonsmokers or cigarette smokers was compared (Fig. 6), values observed for nonsmokers ($r = 0.889$, $P < 0.001$) and for cigarette smokers ($r = 0.948$, $P < 0.001$) were similar. The number of subjects studied is too small to allow any meaningful conclusions to be drawn concerning the distribution within the patient population of individuals with high or low AHH inducibility. However, initial evidence

suggests similarity between AHH inducibility in different tissues in the body of the individual patients.

DISCUSSION

Because of their relative accessibility, studies of AHH induction in man have largely utilized cultured lymphocytes. Studies of AHH induction in other human tissues have been limited to cultured bronchus (24), placental tissue (25), skin fibroblasts (26), and pulmonary alveolar macrophages (5, 19–22). Where values for PAMs and lymphocytes from the same individuals were available, the capacity for AHH induction in these two tissues was similar (19, 20). Previous examinations were not definitive, however, because comparisons were made between AHH values induced in cultured lymphocytes and the degree of AHH activity already present in PAMs freshly obtained by lavage.

Induction of AHH can be measured in cultured PAMs (22). Since the degree of AHH induction in fresh PAMs is positively correlated with the degree of further induction in cultured PAMs from the same individual (22), the relationship between induction of AHH in lymphocytes and PAMs was investigated. Levels of AHH activity in fresh lymphocytes are very low or undetectable, and appreciable amounts of the enzyme are present in these cells only after further culturing with mitogenic agents and inducers of AHH activity (1). When AHH values for freshly obtained PAMs were compared for nonsmokers and cigarette smokers, values were consistently higher in PAMs from smokers. This is consistent with previous reports in which cigarette smoking induced higher levels of AHH activity not only in lung cells, at the portal of

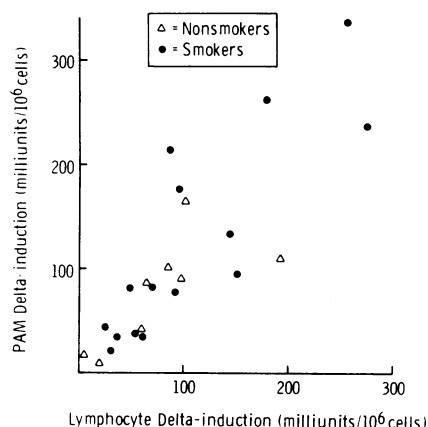


FIGURE 5 Comparison of the increment in AHH between noninduced and BA-induced (delta induction) in cultured PAMs and lymphocytes from the same individual ($r = 0.835$, $P < 0.001$ for smokers and $r = 0.719$, $P < 0.03$ for nonsmokers).

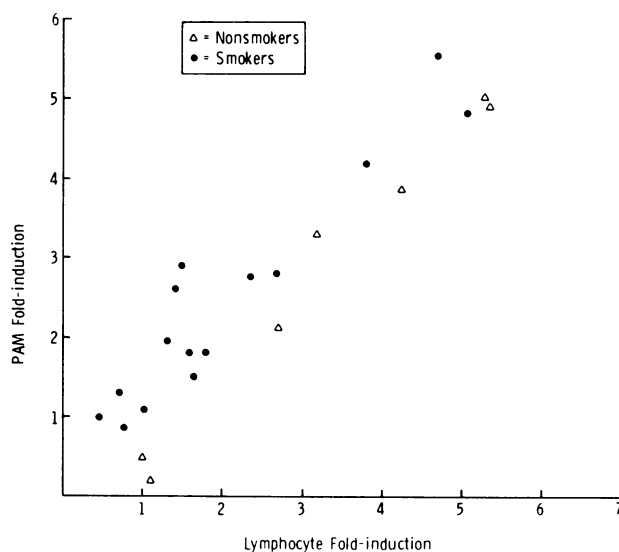


FIGURE 6 Comparison of values for AHH (expressed as fold-induction) in cultured PAMs and lymphocytes from individual patients ($r = 0.948$, $P < 0.001$ for smokers and $r = 0.889$, $P < 0.001$ for nonsmokers).

entry (5, 19, 20), but also in remote tissues, such as placenta (25).

Induction of AHH in cultured cells has been studied with a variety of different inducing agents. The enzyme can be induced in vitro by cigarette tar extracts (21), or by individual polycyclic aromatic hydrocarbons, such as 3-methylcholanthrene (1) or BA (20), components of cigarette smoke. In the present study, when levels of AHH were measured in noninduced cultures of either lymphocytes or PAMs, levels were consistently higher when the cells came from smokers than from nonsmokers. In addition, when lymphocytes were cultured with BA to further induce AHH activity, significantly higher values were obtained for cells from smokers than from nonsmokers. Levels of AHH in BA-induced cultures of PAMs also tended to be higher when cells came from cigarette smokers, but the increases were not statistically significant.

The elevated levels of AHH in cells from smokers might be the result of persistence of polycyclic hydrocarbons in the cells during culture, or could result from the action of other classes of compounds capable of inducing AHH. This supports a report indicating that unidentified compounds present in cigarette smoke can induce the AHH system by a different biochemical pathway from the one utilized by the polycyclic aromatic hydrocarbons (27). In the case of lymphocytes, a clone or more responsive cells may have been stimulated by cigarette smoking. Elevated AHH activity persists in PAMs during short-term culture, perhaps because these are metabolically

active nondividing cells in which continued enzyme induction might be expected.

In determining whether the capacity for AHH induction for cells from individual subjects is reflected by values measured in PAMs, we compared values for fresh PAMs and for BA-induced PAMs or lymphocytes from individual smokers and nonsmokers. In smokers, the most reasonable comparison is between fresh PAMs (which have high levels of AHH induced *in situ*) and BA-induced cultured PAMs or lymphocytes. In nonsmokers, fresh PAMs and noninduced, cultured PAMs or lymphocytes are appropriate. A high degree of positive correlation is present under the specified conditions for PAMs and lymphocytes from individual smokers and nonsmokers. These results indicate that *in vitro* induction of AHH by BA is similar to the *in vivo* induction of the enzyme in PAMs. The individual capacity for AHH induction is measurable even in the presence of alterations produced by cigarette smoking.

Levels of AHH in PAMs from cigarette smokers are not maximally induced, since enzyme levels can be further induced by culture with BA. The absence of maximal induction in freshly obtained PAMs from smokers might be the result of suboptimal concentrations of polycyclic hydrocarbons in cigarette smoke, or could be a function of variations in the smoking habit immediately before bronchoscopy. Alternatively, AHH levels might decrease during the cell processing, from the time PAMs were obtained from the lung to the time measurements were completed. Values for AHH in PAMs from nonsmokers remain relatively stable, whether measured shortly after they were obtained by lavage or after 24 h in culture medium without an inducer.

Several methods of expressing the group data, whether expressed as absolute values or as induction values, indicated comparable induction of AHH in cultured PAMs and lymphocytes. AHH induction in these cells from individual subjects was compared to determine if values were also similar for individuals. Good correlations were noted between AHH values for PAMs and lymphocytes from both smokers and nonsmokers, whether comparisons involved absolute levels of AHH (Figs. 3 and 4) or whether the data were expressed as induction of AHH (Figs. 5 and 6). The benefit of retaining the concept of fold-induction was accentuated by the observation that values for both smokers and nonsmokers had strikingly similar distribution when AHH inducibilities in cultured PAMs and lymphocytes were compared (Fig. 6).

This study represents the first time that cells from two separate tissues from individual subjects have been shown to possess similar AHH induction capabilities when tested under identical culture conditions.

The observation that the degree of inducibility of AHH in humans varies with the individual and that the capacity for AHH induction can be confirmed by measurement in several different types of cells agrees with animal studies in which AHH induction was similar for different nonhepatic tissues from the same animal (28).

Although the levels of AHH in healthy individuals were not measured in the present study, similar AHH activity should be observed in cultured PAMs or lymphocytes from patients without cancer or healthy volunteers. This is supported by other investigators that have demonstrated that levels of AHH in fresh PAMs and the inducibility of the enzyme in cultured lymphocytes are positively correlated for individual normal healthy volunteers (19) or individual patients without evidence of cancer (20), and the range of AHH values for both groups is similar. Studies designed to investigate AHH activity in cultured PAMs and lymphocytes from healthy volunteers will be necessary, however, before definite conclusions can be drawn.

When induction of AHH was studied in cultured lymphocytes, lung cancer patients exhibited higher lymphocyte AHH inducibility than individuals without lung cancer (18). The postulated mechanisms for a possible increased risk of chemical carcinogenesis in cigarette smokers with high levels of AHH concern the production during metabolism and/or remetabolism of the polycyclic aromatic hydrocarbons in cigarette smoke to intermediate products of high mutagenic (6–10) and carcinogenic (11–12) potential. In addition to differences in the generation of metabolites, another critical factor in determining susceptibility to carcinogens might be individual variation in the affinity of binding of these metabolites to DNA (24).

An additional function of the AHH system in the liver is the metabolism of xenobiotic drugs (29). Although there is poor correlation between inducibility of AHH in hepatic tissue and nonhepatic tissue in mice (13, 30), no similar comparisons have been made with human hepatic and nonhepatic tissues. A test of the capacity of an individual to process drugs would be of clinical importance, especially in those patients whose drug metabolism capabilities are either elevated or depressed.

Investigations examining AHH in PAMs and lymphocytes in the past have compared fresh PAM values and cultured lymphocyte fold-induction values for AHH (19, 20). In the present series, AHH values for cultured as well as fresh PAMs were measured for each individual. This expands the number of comparisons possible on AHH values for PAMs and lymphocytes from individual subjects. By increasing the comparisons for a given individual, some of the day-to-

day variation in AHH values observed when lymphocytes alone are used to measure individual enzyme induction capacity (17) might be eliminated.

The methods employed in the present study for evaluating PAM and lymphocyte AHH might provide a satisfactory approach for distinguishing abnormalities in AHH induction in lung cancer patients (20). In addition, analyzing levels of AHH in separate tissues from the same individual could provide a mechanism for studying the biochemical pathways responsible for the previously reported (but unexplained) dissociation of PAM and lymphocyte AHH values in individuals with lung cancer (20).

By measuring AHH in separate tissues from the same individual, problems previously encountered in using a single tissue for analysis of an individual's AHH characteristics might be resolved. This added dimension for the analysis of AHH in individuals might also provide researchers with another tool for evaluating the relationship of AHH to chemical carcinogenesis in the lungs of cigarette smokers.

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