3-Methylcholanthrene-induced Monoxygenase (O-Deethylation) Activity of Human Lymphocytes

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SUMMARY

With a direct fluorescence assay, the levels of mixed-function oxidase activity were determined in mitogen-activated human lymphocytes. The O-deethylation of ethoxyresorufin to resorufin was used to quantitate mixed-function oxidase activity. Ethoxyresorufin O-deethylase activity was low to nondetectable in noninduced, mitogen-activated cells, but it was readily detected in 3-methylcholanthrene, mitogen-activated lymphocytes. The activity was: (a) dependent on assay time and number of lymphocytes; (b) dependent on the presence of reduced nicotinamide adenine dinucleotide phosphate; (c) stable to freezing at —80° for at least 2 weeks; (d) reproducibly detected in duplicate samples of blood from one individual when cultured and assayed at the same time; and (e) quite variable in samples of blood from one individual at different times. Since in hepatic and pulmonary tissue of model animal systems ethoxyresorufin is a specific substrate for cytochrome P-448-associated monooxygenases, the use of this chemical could proffer an assay that specifically measures human cytochrome P-448-associated activity.

INTRODUCTION

It has been suggested that a correlation exists (11) in man between susceptibility to bronchogenic cancer (cigarette smoke associated) and the inducibility by chemical carcinogens of monooxygenase enzymes in cultured blood lymphocytes that hydroxylate such carcinogens (2, 6, 8–11, 13, 26). This observation seems to be analogous to the situation in model systems using inbred strains of mice in which the activity of a specific monooxygenase, AHH is (a) host-gene regulated (7, 17, 19, 20, 24, 25), (b) genetically linked to cancer susceptibility caused by MC and benzo(a)pyrene (12, 15, 16, 18), and (c) specifically associated with the presence of cytochrome P-448 (see review, Refs. 19 and 20).

Ethoxyresorufin can serve as a substrate for mixed-function oxidases in rat (3–5), mouse, or hamster (5) tissue, and its metabolism seems to be intimately associated with the level of cytochrome P-448 (4, 5). Recent evidence suggests that the metabolism of ethoxyresorufin in inbred strains of mice is under the same genetic control as is the metabolism of benzo(a)pyrene via the AHH enzyme complex. Since the assay for O-deethylation of ethoxyresorufin (3) is direct (requiring no extraction steps), sensitive, and amenable to automation and since ethoxyresorufin may be specifically metabolized by only cytochrome P-448, this chemical may serve as an effective alternate substrate [rather than benzo(a)pyrene] for determination of mixed-function oxidase activity in human tissue. This report describes such an assay.

MATERIALS AND METHODS

Ethoxyresorufin and resorufin were prepared as previously described (3). Lymphocytes were isolated from apparently healthy human volunteers (ages 24 to 39) who were under no current drug regimen. All were nonsmokers except for Subject T. R. Lymphocytes were isolated in Ficoll-Hypaque gradients (specific gravity, 1.080) (Microbiological Associates, Bethesda, Md.), washed once with Hanks' balanced salt solution, and cultured in 25-cm² plastic flasks in 8.0-ml aliquots at a density of 0.5 × 10⁸ cells/ml. The culture medium was Roswell Park Memorial Institute Medium 1640 supplemented with 17% fetal calf serum, 0.025 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer, 1% phytohemagglutinin M (Burroughs-Wellcome, Greenville, N. C.), 50 j. g streptomycin per ml, and 50 units penicillin per ml. Mitogen stimulation is a prerequisite for the development of monooxygenase activity in lymphocytes (6, 26). After 72 hr of exposure to the mitogens, lymphocyte cultures were treated with either 1.5 nmoles MC per ml medium or an equivalent amount of the solvent acetone. Acetone concentration was 0.2%. Cells were collected 24 hr later and either assayed immediately or stored as frozen pellets in 0.2 ml 0.1 M Tris-HCl, pH 8.5, at —80° for up to 4 weeks. Approximately 4 × 10⁶ cells were frozen per tube.

For assay of frozen material, each cell pellet was thawed to 4° in the presence of 0.2 ml of 0.01 M ethoxyresorufin (dissolved in 0.1 M Tris-HCl, pH 8.5), sonically disrupted (three 2-sec bursts), and transferred to a 0.5- × 0.5-cm microfluorometer cuvet. The fluorescence of the suspension was measured at an emission wavelength of 586 nm with an excitation wavelength of 520 nm, using an Aminco-Bowman spectrophotofluorometer with a sample compartment thermostatically regulated to 25°. After the fluores-
cidence was monitored for 5 to 10 min, a 10-μl aliquot of a 1 μM solution of authentic resorufin in ethanol was added to the cuvet to standardize the reaction. Data are given in terms of pmoles of resorufin formed per 10⁶ cells per min. The number of viable cells was determined by trypan blue exclusion using a hemocytometer. Total cellular DNA was also determined in some cultures as previously described (13, 21). For the purpose of this presentation, all data are given "per 10⁶ cells" using the correction factor 9.8 μg DNA per 10⁶ cells (R. E. Kouri, unpublished observation).

RESULTS AND DISCUSSION

The O-deethylation of ethoxyresorufin to resorufin was monitored directly as an increase in the fluorescence of the sample (see Charts 1 and 2). The reaction rate was linear for at least 35 min following an initial lag phase of 1 to 2 min (the significance of which is under investigation). The deethylation rate was directly proportional to the lymphocyte concentration over the range studied, 2 to 8 × 10⁶ cells (r = 0.983) and could be extrapolated through zero. The addition of NADPH at a final concentration of 0.125 to 0.25 mM always ensured optimum reaction rates, but NADH (2.5 μM to 2.5 mM) could not substitute for NADPH. Neither Mg²⁺, nor Mn²⁺, nor an NADPH-regenerating system (isocitric acid plus isocitrate dehydrogenase) increased the rate of O-deethylation.

The optimum pH for the reaction was 8.5 to 8.75. Ethoxyresorufin concentrations of 2.5 to 5.0 μM produced maximum deethylation rates. Although the lymphocytes were routinely sonically disrupted to give a more homogenous sample, the reaction could be readily monitored with suspensions of whole, unsonically disrupted lymphocytes; the reaction rates were identical for both types of samples. Results quoted in this paragraph apply to the mitogen-stimulated lymphocytes cultured with MC or in those 2 lymphocyte preparations (see Table 1) in which a definite reaction rate was observed in the non-MC-treated cultures.

The presence or formation of 0.3 pmol resorufin in the cuvet yields a signal:noise ratio of approximately 10. To determine the ultimate sensitivity of this assay, we assume 10 × 10⁶ cells/cuvet as the upper limit for cell concentration (because at higher cell concentrations, turbidity becomes a problem) and a linear reaction rate limit of 30 min. Thus, the formation of 0.3 pmol resorufin per 10 × 10⁶ cells per 30 min can be detected. This corresponds to 0.001 pmol resorufin formed per min per 10⁶ cells. Thus, the ultimate sensitivity of this assay compares very favorably to the sensitivity of the assay for AHH activity recently described by Gurtoo et al. (8) using benzo(a)pyrene as a substrate for this mixed-function oxidase. For the results reported in this paper, the assay was routinely run for 5 to 10 min and at a concentration of 4 × 10⁶ cells/tube. Therefore under these conditions the smallest rate that can be detected is ~0.015 pmol/min/10⁶ cells.

Levels of ethoxyresorufin deethylation activity in lympho-
lymphocytes from 10 normal human volunteers are given in Table 1. MC-induced activity was easily detected in every lymphocyte preparation, and the average intrassay (between duplicate samples of blood) variation was 0.12 (coefficient of variation). Under the conditions of this assay (4 x 10⁶ cells assay for ~5 min), the level of control or non-MC-treated activity was very low and could be detected in only 2 subjects (C. M. 2nd week, and T. R.). For the remaining individuals, deethylase activity was less than could be detected in a 5-min incubation (~0.015 pmole resorufin formed per min per 10⁶ cells). For certain lymphocyte cultures, incubation was carried out for 30 min, and still no distinct reaction rate could be detected. Thus, the level of O-deethylase activity in at least some of these noninduced mitogen-activated lymphocytes is less than 0.003 pmole/min/10⁶ cells. This low control activity could possibly result from the action of 2 different parameters: (a) the lot of fetal calf serum (Lot 86629; Microbiological Associates) used in this study; and (b) in these uninduced lymphocyte preparations there may be an absence of those particular classes of terminal oxidases, the P-448 cytochromes, which seem to be specifically involved in the O-deethylase of ethoxyresorufin. The lot of fetal calf serum definitely affects control AHH activity in mitogen-activated human lymphocytes (13, 14), and this lot of fetal calf serum was specifically used because of the low control AHH activity observed in lymphocytes grown in medium supplemented with this lot of serum (data not shown). It has been suggested that there is little or no constitutive AHH activity in human lymphocytes (13, 14) and that the "noninduced" AHH activity levels observed in these lymphocytes probably result from their response to unidentified inducers in the culture medium, mitogens, etc.

With benzo(a)pyrene as a substrate, some AHH activity was observed in these noninduced lymphocyte preparations in which no deethylase activity was observed (data not shown). This observation could be very important because, if the mixed-function oxidase activity observed in human lymphocytes utilizes the same terminal oxidases (i.e., cytochromes) as do other mammalian tissues like liver or lung, then most of the mixed-function oxidase activity associated with untreated human lymphocytes may result from levels of cytochrome P-450 (not P-448). This seems likely because cytochrome P-450 does support the hydroxylation of benzo(a)pyrene (20, 22, 23) but only poorly supports O-deethylase of ethoxyresorufin (3-5). Thus, the use of ethoxyresorufin as a substrate for human mixed-function oxidase could offer an assay that specifically measures human cytochrome P-448-associated activity.

The interassay variation in levels of deethylase activity for the lymphocytes of 1 individual cultured on separate days and assayed on separate days is quite large (see Table 1). The source of this variation appears to be in the mitogen activation and/or induction steps, since large pools of mitogen-activated lymphocytes from various individuals can be assayed either immediately following the mitogen activation step or after 1 to 4 weeks of storage at ~80° and similar O-deethylase activity is observed (Table 2). It would seem likely that cultured human lymphocytes can be stored frozen for at least 1 to 2 weeks before assay of their deethylase activity. The reason for the apparent increase in control enzyme activity after storing at ~80° for Subject C. M. is not known at this time. Recent information on the level of AHH activity in cultured human lymphocytes also suggests that the major factor influencing interassay variation is the inability to produce a similar level of mitogen stimulation of human lymphocytes from the same individual on separate days (13). The culture conditions that affect degree of mitogen activation and levels of AHH or O-deethylase activity will be discussed at length elsewhere.

The use of ethoxyresorufin as an alternate substrate for the determination of mixed-function oxidase activity in human lymphocytes has some unique advantages. The assay (a) requires very little manipulation and no extraction steps; (b) has comparable sensitivity to existing assay procedures; (c) should be amenable to automation (for the assay of large numbers of samples); (d) results in the formation of only 1 metabolite, resorufin (3-5), and thus, incomplete metabolite extraction is avoided; and, perhaps most importantly, (e) may be a measure of that particular enzyme activity that has been shown in model animal systems to be related to poly-cyclic aromatic hydrocarbon-induced carcinogenesis. This latter advantage pertains to the fact that ethoxyresorufin seems to be preferentially metabolized by enzyme preparations containing cytochrome P-448 (3-5), and the level of enzymes associated with this cytochrome seems to play a major role in MC (12, 15, 16, 18) and benzo(a)pyrene (12) carcinogenesis in inbred strains of mice. Obviously, there is a degree of speculation associated with this particular advantage, because it is not known with certainty that the

<table>
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<th>Control</th>
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Table 2
Ethoxyresorufin deethylation activity of lymphocytes stored at ~80° after culture

Lymphocytes, freshly drawn from 3 people, were cultured with mitogens in the presence or absence (control) of MC, as described in the text, and the cultured lymphocytes were stored at ~80°. Samples of the lymphocytes were assayed for ethoxyresorufin deethylation as described in the text, either immediately or after 1, 2, or 4 weeks of storage. Values are means for 2 samples.

Numbers in parentheses, stored, MC-exposed activities as percentages of the fresh activities.

cytochromes in lymphocytes are, in fact, the same as those obtained by polycyclic aromatic hydrocarbons in other mammalian tissues. A disadvantage of this assay system may be that the parameter, inducibility (the increase of induced activity relative to that observed in control cultures), cannot be used to compare the hydrocarbon-metabolizing activity of various individuals, for little or no constitutive activity can be detected using ethoxyresorufin as substrate. Inducibility has been the parameter of choice for other laboratories (6, 8–11, 16, 26); however, we have previously reported that AHH inducibility can, under certain conditions, be a very unrepeatable method for comparing AHH activities of various individuals (13). 5

The use of this enzyme methodology for the analysis of mixed-function oxidase activity in cultured human lymphocytes from various human populations is limited by the variability in the mitogen activation step. Recent information from our laboratories suggests that the level of another microsomal enzyme, NADH-dependent cytochrome c reductase activity, can be detected in human lymphocytes and that this activity seems to be a good measure of the microsomal content of mitogen-activated lymphocytes (21). Determination of AHH activity per unit cytochrome c reductase activity has resulted in an assay in which much of the inters assay variation is decreased (13). We are presently evaluating the parameter, units of deethylation of ethoxyresorufin per units NADH-dependent cytochrome c reductase activity, as a way of comparing the hydrocarbon-metabolizing activities of different individuals.

REFERENCES

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