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Aryl Hydrocarbon Hydroxylase Induction in Mouse Peritoneal Macrophages and Blood-Derived Human Macrophages[1,2] (38152)

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(Introduced by J. S. Remington)

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Microsomes isolated from mammalian liver contain a series of hydroxylases and other enzymes involved in the metabolism of carcinogenic hydrocarbons (1). Although *in vitro* and *in vivo* induction of aryl hydrocarbon hydroxylases has been studied in animals, investigation of the effects of inducing agents in humans has been limited by a general unavailability of human hepatic microsomes, and by the toxicity of many of the compounds used to accomplish induction.

Therefore, it was of interest to determine if a system for studying microsomal enzymes could be developed which used a more readily available cell type. Circulating blood-derived mononuclear phagocytic cells could be particularly useful for such studies since they can be obtained as a homogeneous population and maintained *in vitro* without proliferation for long periods of time (2–4). During cultivation they differentiate into structurally more complex and functionally more active macrophages which are actively engaged in protein synthesis and which contain a highly developed lysosomal system and an abundant endoplasmic reticulum (5–8).

The experiments described in this communication were designed to examine the activity and inducibility of aryl hydrocarbon hydroxylase in blood-derived human macrophages and mouse peritoneal macrophages.

Materials and Methods. Cultivation of Mouse Mononuclear Phagocytes. Cells from the peritoneal cavity of male mice aged 6 weeks (strain C57B1/6, Simonsen Laboratories, Inc., Gilroy, California) were harvested by techniques previously described (9). An 8 ml sample of cells (3.0×10^6/ml) in Medium 199 (Schwarz-Mann, Division of Becton-Dickinson, Orangeburg, New York) containing 20% newborn calf serum (NBCS) (Grand Island Biological Company, Santa Clara, California) and 1000 units/ml penicillin G was dispensed to each 30 cm^2 glass T-flask and incubated for 60 min at 37°. The cells were then washed twice in Medium 199 and the cultures incubated in fresh Medium 199 containing 30% NBCS. Cells in culture were harvested in 2 ml saline per flask and centrifuged at 480 *g* for 5 min at 12°. The cells were resuspended in 0.3 ml of Tris buffer pH 7.0 containing 0.25 *M* sucrose and homogenized for immediate use in enzyme assay.

In vivo induction of aryl hydrocarbon hydroxylase in mouse liver. Sibling male mice were given 2 intraperitoneal injections of 0.25 ml of a 3-methylcholanthrene solution in corn oil (10 mg/ml) at 24 hr intervals. Control mice were injected with an equal volume of corn oil. Mice were sacrificed 24 hr after the last injection and the livers removed, homoge-

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[2] Kindly supplied by the Stanford Red Cross Blood Center. All blood donors were healthy adults with no current drug intake and with WBC within normal range (6000–8000 WBC/mm^3 of whole blood).

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nized in 4 parts Tris buffer pH 7.0 containing 0.25 *M* sucrose, and assayed immediately for aryl hydrocarbon hydroxylase activity.

Preparation of human blood-derived macrophages. Buffy coat cells from 4 pints of blood [2] were pooled using sterile AE-7 plasma transfer sets (Fenwal Laboratories, Morton Grove, Illinois) and transferred to sterile plastic bottles containing heparin. An equal volume of Medium 199 containing 20% NBCS and 1,000 units/ml penicillin was added to each flask to reconstitute the buffy coat cells. Erythrocytes were sedimented by addition of a sterile solution of 3% Dextran (MW 250,000, Sigma Chemical Company, St. Louis, Missouri) at room temperature for 1 hr. The leukocyte-rich top layer was removed and centrifuged at 150 *g* for 10 min at 12°. The supernatant was discarded and the packed cells suspended in sterile 35% albumin (Fraction V, Sigma Chemical Company, St. Louis, Missouri) and diluted with phosphate buffered saline (PBS) to a final albumin concentration of 27% according to the procedure described by Bennett and Cohn (6). The albumin gradients were centrifuged at 12° for 40 min at 2400 *g*. The surface layer containing monocytes and lymphocytes was collected, suspended in 5 ml PBS, and the cells counted in a hemocytometer. The cells were resuspended in warm Medium 199 containing 20% NBCS and 1,000 units/ml penicillin to a density of 4×10^6 cells/ml. An 8 ml aliquot was added to each 30 cm^2 glass T-flask and incubated in a 5%CO_2–95% air environment. Lymphocytes were removed by change of medium and one washing procedure with PBS as described above for mouse cell cultures on the second day of cultivation. The cells were incubated an additional 13 days in an equal volume of complete medium containing 20% NBCS.

Enzyme assay. The assay for the reaction catalyzed by aryl hydrocarbon hydroxylase was a modification of the procedure of Nebert and Gelboin (10). Reaction mixtures (1.0 ml) contained 50 μmoles $MgCl_2$, 0.6 μg bovine serum albumin, 0.5 μmoles NADPH, 0.1 ml cell homogenate containing 100–500 μg cellular protein, and 80 mμmoles of 3,4 benzopyrene added in 0.04 ml of methanol. The mixture was shaken gently in a 37° water bath for 30 min. The reaction was stopped by adding 1 ml cold acetone followed by 3.0 ml cold hexane. After the addition of acetone-hexane, the mixture was incubated with shaking at 37° for 10 min. A 2.0 ml sample of the organic phase was extracted with 4.0 ml of 1N NaOH by vortex agitation for 10 sec. The phases were separated by short centrifugation and the concentration of extracted hydroxybenzopyrene was measured in a Turner Model 430 spectrophotofluorometer (G. K. Turner Associates, Palo Alto, California) with activation at 396 mμ and fluorescence at 522 mμ. This procedure is sufficiently sensitive to detect 10^{-12} moles per ml of hydroxylated benzopyrene. Quinine sulfate and 3-hydroxybenzopyrene were used as standards for calibration and to check sensitivity and accuracy of the readings. Enzyme activities were determined in duplicate and compared with a blank to which the enzyme source was added after the incubation period. Protein was determined by the Lowry method as modified and reported by Sutherland (11).

One unit of activity of aryl hydrocarbon hydroxylase is defined as that amount of enzyme which catalyzes the formation of one

TABLE I. Time Course of Aryl Hydrocarbon Hydroxylase Activity in Cell Cultures of Mouse Peritoneal Macrophages Induced *In Vitro* With Benzanthracene

	Hours Cells Cultured Before Harvesting				
	1	24	48	72	96
No inducer	.55 ± .10 [a]	.58 ± .03	.52 ± .10	.70 ± .15	.77 ± .03
Benzanthracene [b] added *in vitro*	—	—	1.17 ± .25	—	1.26 ± .36

[a] Values represent specific activity of benzopyrene hydroxylase activity as a mean ± SD of 3 experiments. The assay was performed as described in Materials and Methods.

[b] A 30 μM methanolic solution of benzanthracene was added to cultures 24 hr before harvesting.

TABLE II. Aryl Hydrocarbon Hydroxylase Activity in Mouse Liver[a]

	Benzopyrene Hydroxylase Activity
Liver from control mouse[b]	42[c]
Liver from mouse treated with 3-methylcholanthrene[d]	295

[a] Strain C57Bl/6 male mice (Simonsen Laboratories, Inc., Gilroy, California), aged 6 weeks, fed a diet of Purina Chow and water.

[b] Control mouse received 2 intraperitoneal injections of corn oil at 24 hr intervals.

[c] Values represent the specific activity of benzopyrene hydroxylase as moles of hydroxybenzopyrene formed/30 min/mg liver protein.

[d] Sibling mouse received 2 intraperitoneal injections of 3-methylcholanthrene in corn oil at 24 hr intervals. The treated and control mice were sacrificed 24 hr after the last injection. The assay for benzopyrene hydroxylase is described in Materials and Methods.

$\mu\mu$mole of hydroxybenzopyrene per 30 min per mg of cellular protein.

Results. Aryl hydrocarbon hydroxylase induction in cell cultures of mouse peritoneal macrophages. Preliminary experiments indicated that cultures of mouse peritoneal macrophages exposed to benzanthracene for 24, 48, or 72 hr before harvesting exhibited maximal induction of benzopyrene hydroxylase activity after 24 hr exposure of cell cultures to benzanthracene.

Table I shows the time course of aryl hydrocarbon hydroxylase induction in cell cultures of mouse peritoneal macrophages induced *in vitro* by addition of benzanthracene. Each value reported in Table I represents the specific activity of benzopyrene hydroxylase activity as an average of 3 experiments. For each experiment, peritoneal macrophages from 40 mice were collected, pooled, and dispensed to ten 30 cm^2 glass T-flasks as described in Materials and Methods. Table I, line 1 indicates that cells cultured in the absence of inducer show a slight increase in specific activity of benzopyrene hydroxylase activity after 48 hr of *in vitro* culture when comparing cultures incubated for 1, 24, 48, 72 or 96 hr. Table I, line 2 shows that the addition of benzanthracene to cells cultured for 48 or 96 hr results in an increase in benzopyrene hydroxylase as compared to the activity observed in cell cultures containing no benzanthracene.

Aryl hydrocarbon hydroxylase induction in mouse liver. In keeping with the observation that liver is the principal organ for detoxification of numerous carcinogenic hydrocarbons it has been established previously that liver aryl hydrocarbon hydroxylase activity is readily inducible *in vivo* by administration of 3-methylcholanthrene (1). In view of our finding that aryl hydrocarbon hydroxylase was inducible in the cultivated mouse peritoneal macrophage it was of interest to examine specific activity and inducibility of the enzyme in liver from the same animal species. The results of a typical experiment comparing benzopyrene hydroxylase activity in liver from a control mouse to enzyme activity in liver of an animal receiving 2 intraperitoneal injections of 3-methylcholanthrene at 24 hr intervals is shown in Table II. Liver from the control animal exhibited 42 u of benzopyrene hydroxylase activity whereas liver from the 3-methylcholanthrene treated animal contained 295 u of enzyme activity. Identical experiments repeated at least 12 times yielded similar results.

Aryl hydrocarbon hydroxylase induction in blood-derived human macrophages. Human blood monocytes differentiate *in vitro* into macrophages. Within 72 hr these cells become well spread, develop numerous pseudopodal cytoplasmic extensions, and develop numerous large, phase-dense secondary lysosomes (2, 6, 12).

Preliminary experiments (unpublished data) in which the blood-derived human cell cultures were harvested after 3, 6, 12, 15 or 18 days indicated that aryl hydrocarbon hydroxylase activity reaches its maximum level on the 15th day of cell culture. For this reason, 15 day cultures were chosen for assay.

A 30 μM benzanthracene solution in Medium 199 was added to one-half of the cultures on the 14th day of cultivation. The

TABLE III. Aryl Hydrocarbon Hydroxylase Activity in Benzanthracene Treated Cultures of Blood-Derived Human Macrophages

	Benzopyrene Hydroxylase Activity Experiment No. 1	2
Benzanthracene-[a] Treated Cultures	0.580 [b]	2.231
Untreated Cultures	0.212	0.776

[a] Added as a 30 μM methanolic solution on the 14th day of cultivation.

[b] Values represent the specific activity of benzopyrene hydroxylase as μmoles of hydroxybenzopyrene formed/30 min/mg cellular protein.

benzanthracene-treated cultures and the untreated cultures were harvested 24 hr later and assayed immediately for benzopyrene hydroxylase activity as described in Materials and Methods. A total of 26 culture flasks, size 30 cm^2, were used for each experiment reported in Table III. The specific activity of benzopyrene hydroxylase in the treated and untreated cultures is shown in Table III. The untreated human macrophage cultures exhibited 0.212 and 0.776 units of benzopyrene hydroxylase for Experiments No. 1 and 2, respectively, Table III. The specific activity of benzopyrene hydroxylase in benzanthracene-treated cells increased to 0.580 in Experiment No. 1 and to 2.231 in Experiment No. 2.

Discussion. Recent studies of the induction of aryl hydrocarbon hydroxylase activity in cultures of human lymphocytes (13) showed that the addition of phytohemagglutinin or pokeweed mitogen to cell cultures *in vitro* resulted in increased baseline levels of aryl hydrocarbon hydroxylase activity and greater sensitivity to induction by benzanthracene than the cell cultures containing no mitogen. In another study using cell cultures of human leukocyte preparations (14) phytohemagglutinin was added to cell cultures to measure aryl hydrocarbon hydroxylase induction by 3-methylcholanthrene. Mitogen-treated cells in culture show cytoplasmic enlargement and proliferation of endoplasmic reticulum (15) which contains cytochrome P-450 as the substrate binding site (16, 17) for the mixed function oxidase system that metabolizes the aryl hydrocarbons (18).

Since mitogens cause nonspecific leukocyte stimulation, which alters baseline activity of aryl hydrocarbon hydroxylase and the inducibility of this enzyme, the experiments described in this study measuring the activity and inducibility of aryl hydrocarbon hydroxylase in macrophage cell cultures were carried out in the absence of mitogen.

The data shown in Table I indicate that when benzanthracene is added *in vitro* to 48 hr cell cultures of mouse macrophages, there is a 2.25 fold induction of aryl hydrocarbon hydroxylase activity as compared to the activity in cells cultured 48 hours without inducer. Cells cultured 96 hr show a 1.64 fold induction of enzyme activity when compared to the activity in cells cultured 96 hr without inducer. The observed increase in enzyme activity may not be due entirely to substrate-induced cell changes since there is a slight increase in baseline activity of the enzyme in the mouse cell cultures which have no inducer added. The increase in baseline activity may be due to proliferation of endoplasmic reticulum which parallels other non-substrate induced changes in cellular metabolism observed during *in vitro* differentiation of monocytes to macrophages such as increased pyruvate kinase, lysosomal, and mitochondrial enzyme activities (6, 9, 19).

When the levels of aryl hydrocarbon hydroxylase activity in mouse peritoneal macrophages were compared to the enzyme levels in mouse liver, the liver exhibited 70 times more activity. Further, the enzyme levels in mouse liver increased 7 fold when induced *in vivo* with 3-methylcholanthrene.

The data in Table III, Experiment No. 1, show that the addition of benzanthracene to cultures of blood-derived macrophages results in a 2.73 fold induction of benzopyrene hydroxylase as compared to untreated macrophage cultures. Experiment No. 2, Table III,

shows a 2.88 fold induction after treatment with benzanthracene. These results demonstrate the presence of benzopyrene hydroxylase activity in human macrophage cultures and provide a system for studying aryl hydrocarbon hydroxylase induction in these cells.

Summary. Macrophages of human and mouse origins were shown to contain inducible aryl hydrocarbon hydroxylase activity. Mononuclear phagocytic cells derived from circulating blood monocytes of healthy human donors and peritoneal macrophages from untreated C57 B1/6 mice exhibited low levels of enzyme activity. For mice, aryl hydrocarbon hydroxylase specific activity of freshly explanted macrophages was 1/70th the enzyme activity found in liver. Aryl hydrocarbon hydroxylase activity for both human and mouse macrophages maintained *in vitro* in the presence of benzanthracene exceeded by 2–3 fold the enzyme specific activity of cells cultivated in the absence of inducer.

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