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SHORT COMMUNICATIONS

Effect of benzo(a)pyrene induction of liver and lung metabolism in adjuvant-diseased rats

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Adjuvant-diseased rats have been employed as a model for chronic inflammations such as: rheumatoid arthritis, osteoarthritis and disseminated lupus erythematosis. These models parallel the human diseases in various aspects, even to the reversal with nonsteroidal drugs [1, 2]. In the case of adjuvant disease in rats, there are many parameters that change during the development of the disease, namely, serum proteins, paw edema, erythrocyte sedimentation rates and body weight. The interrelationship of these changes has been the subject of many studies [3–5]. The general subject of this paper is the microsomal drug-metabolizing system (MDMS), which is soon impaired after adjuvant injections in rats. Damage to the mixed-function oxidases or cytochrome P-450 can inhibit microsomal drug metabolism by 80 per cent on day 14 [3, 4]. Reversal by nonsteroidal drugs of the impairment of both the MDMS and inflammatory response (paw edema) has been reported [4]. Other studies [6, 7] investigating the MDMS relationship to inflammation have shown that a hepatic MDMS inducer, phenobarbital, reverses only the liver MDMS inhibition and can do so also when administered post adjuvant. This communication further explores this possible relationship by investigating the effect of adjuvant disease on the MDMS of a nonhepatic tissue, the lung, compared to liver. The possible reversal of this effect and/or paw edema by using benzo(a)pyrene, an inducer of both hepatic and nonhepatic microsomal mixed-function oxidases, was investigated by measuring the specific MDMS enzyme, aryl hydrocarbon hydroxylase (AHH).

Sprague–Dawley rats, males weighing 155–185 g, were housed three to a cage in a room with 12-hr light cycle and fed Purina Laboratory Chow *ad lib.* Two days before adjuvant, benzo(a)pyrene (Sigma) in suspension was injected at 25 mg/kg i.p. The injectable benzo(a)pyrene was prepared by dissolving it in undiluted Tween 80 with heat, and then sterile 0·9% saline was added to make the suspension, 4 mg/ml, in 5% Tween 90. *M. butyricum* in light mineral oil at 5 mg/ml (Difco Labs Co.) was administered on day 0 subcutaneously in the distal third of the tail at a dose of 0·1 ml. Control rats received only mineral oil. On days 0, 9 and 14, paw circumferences were measured at the lateral malleolus. The livers and lungs were removed from the various groups of rats on days 0, 7 and 14 and assayed for AHH activity by modifications of methods of Kuntzman *et al.* [8] and of Wattenberg *et al.* [9].

Preparation of liver and lung supernatants. Rats were decapitated using a guillotine and exsanguinated. Livers and lungs were immediately dissected out and washed in cold 0·9% saline to remove excess blood. All procedures, except the enzyme assay, were performed in the cold. The liver was cut into small pieces and weighed in a 50-ml beaker. The lung was scraped with a single-edged razor blade to remove lung tissue from excess connective tissue, bronchioles and blood vessels. Enough 0·25 M sucrose was added to the liver to make a 10% homogenate and 0·1 M phosphate buffer, pH 7·4, was added to the lung to make a 10% homogenate. The homogenates were prepared with a Thomas glass homogenizer (type B) with a Teflon pestle. The homogenate was placed into a plastic centrifuge tube and centrifuged at 4° for 20 min at 12,000 *g*. The liver supernatant was then diluted to 1% using cold 0·25 M sucrose.

Enzyme assay. All reagents were prepared in 0·1 M K_2HPO_4–KH_2PO_4 buffer at pH 7·4, except for the $MgCl_2$ solution where demineralized water was used.

Into 25-ml Erlenmeyer flasks the following solutions were pipetted: (A) a 1·4-ml reaction mixture containing 0·1 ml of 2 M KCl, 0·1 ml of 0·1 M $MgCl_2$ and 1·2 ml buffer; (B) cofactor mixture, 1·1 ml, containing 0·2 ml of 0·03 M glucose 6-phosphate, 0·1 ml of 4 mg/ml NADP, 0·1 ml of 4 mg/ml NAD and 0·7 ml buffer; the cofactors were obtained from Sigma; (C) 0·5 ml of 1% liver supernatant or 0·5 ml of 10% lung supernatnat. The Erlenmeyer flasks with the reaction medium and microsomal supernatant were placed into a Dubnoff metabolic shaking incubator for 2 min at 96 rev/min and 37° to equilibrate.

The following steps were carried out in dim light. The substrate, 50 μg benzo(a)pyrene in 0·1 ml acetone, was added to start the reaction. The reaction was allowed to continue for exactly 12 min for liver and 10 min for lung, when the reaction was stopped by adding 3 ml of cold acetone. Then 10 ml of ligroine (60–90° b.p.) was added to each flask and chilled at 4° for 5 min. The Erlenmeyer flasks were placed into an Eberbach shaker (180 rev/min) for 5 min to extract the metabolites of benzo(a)pyrene into the ligroine. After being shaken, the flasks were put on ice for 30 min to increase the fluorescence of the metabolites. Then 2 ml ligroine was removed from each flask and propipetted into a 50-ml culture tank containing 10 ml of 1 N NaOH. The culture tubes were vigorously shaken in an Eberbach shaker (180 rev/min) for 9 min. The fluorescence of the NaOH layer was measured at 10 min ± 30 sec, as the fluorescence disappears with time.

An Aminco–Bowman spectrophotofluorometer measured the fluorescence. The settings were as follows: excitation wavelength, 400 nm; emission wavelength, 522 nm; sensitivity, 30; Slit widths: excitation, 5·0 mm; emission, 1·5 mm and photomultiplier, 3·0 mm. The fluorescence spectra of the metabolites formed were identical to that of 3-OH-benzo(a)pyrene in 1 N NaOH. Although a mixture of hydroxylated products is formed during the reaction and therefore the fluorescence measured represents a mixture of hydroxylated products, one aryl hydrocarbon hydroxylase (AHH) unit was designated to equal 100 pg 3-OH-benzo(a)pyrene/mg wet weight of tissue/min.

A standard curve was made with 3-OH-benzo(a)pyrene* serially diluted to 100 pg/ml, and a quinine sulfate solution (0·6 μg/ml in 0·1 N H_2SO_4), which was stable for months, was used as a fluorescent standard for everyday adjustments of the spectrophotofluorometer.

The statistical procedures used in the analysis of the data were: (1) analysis of variance, (2) Duncan's multiple comparison, (3) rankit transformation and (4) the Student *t*-test [10].

Table 1 shows the effect of benzo(a)pyrene administration on paw circumference of normal and adjuvant-diseased rats during the first 14 days of adjuvant dis-

* Dr. Harry V. Gelboin of NIH kindly supplied the 3-OH-benzo(a)pyrene.

Table 1. Effect of benzo(a)pyrene on paw circumference of normal and adjuvant-diseased rats*

Day	Paw circumference (mm) Normal controls	Normal benzo(a)pyrene	Adjuvant controls	Adjuvant benzo(a)pyrene
0	22·2 ± 0·2† (7)	22·1 ± 0·5† (7)		
9	22·3 ± 0·1† (6)	22·6 ± 0·3† (7)	22·4 ± 0·6† (12)	21·7 ± 0·9† (9)
14	22·6 ± 0·4† (6)	22·8 ± 0·1† (7)	28·0 ± 1·3‡ (12)	28·5 ± 1·4‡ (9)

* Benzo(a)pyrene was given i.p. at 25 mg/kg on day −2, while adjuvant was administered on day 0 as described in Methods. Paw circumference was measured in mm at the lateral malleolus. Results are given as means ± S.E.M. The number of rats is reported in parentheses.

†,‡ Unlike superscripts indicate that two group averages are statistically different at $P < 0.05$ when compared on the same day.

ease development. The data indicate that paw edema is not evidenced until after the ninth day and that benzo(a)-pyrene appears to have no effect on the development of paw edema.

In order to contrast paw edema with depression of MDMS activity, the experiment was designed so that AHH activity was measured before adjuvant treatment (day 0), during a high disease state (day 14) and at a point (day 7) days before development of paw edema. Tables 2 and 3 exhibit the effect of benzo(a)pyrene treatment on the AHH level of two tissues of normal and adjuvant-diseased animals. Table 2 summarizes the liver AHH activity at days 0, 7 and 14 in four groups: untreated controls, benzo(a)pyrene-treated animals, adjuvant-treated animals, and animals treated with both benzo(a)pyrene and adjuvant. The data in this table indicate that the liver AHH activity was elevated 4-fold within 2 days by benzo(a)pyrene treatment (i.e. treatment on day −2 and comparison on day 0). However, 9 days after benzo(a)pyrene administration, the liver AHH lost its induced activity, as shown by comparison of the normal controls to normal benzo(a)pyrene-treated animals. AHH activity in adjuvant–benzo(a)pyrene-

Table 2. Effect of benzo(a)pyrene on aryl hydrocarbon hydroxylase (AHH) activity in liver of normal and adjuvant-diseased rats*

Day	Liver AHH units Normal controls	Normal benzo(a)pyrene	Adjuvant controls	Adjuvant benzo(a)pyrene
0	17·9 ± 1·7† (7)	64·4 ± 5·0‡ (7)		
7	25·5 ± 5·1† (6)	26·1 ± 4·8† (7)	6·8 ± 1·0‡ (11)	10·7 ± 1·6§ (11)
14	18·9 ± 3·3† (11)	25·0 ± 5·4† (7)	4·4 ± 0·7‡ (12)	4·5 ± 0·8‡ (9)

* Benzo(a)pyrene at 25 mg/kg was given i.p. on day −2, while adjuvant was administered on day 0 as described in Methods. One AHH unit = 100 pg 3-OH-benzo(a)pyrene formed/mg wet weight of tissue/min. Results are given as means ± S.E.M. The number of rats is reported in parentheses.

†,‡,§ Unlike superscripts indicate that two group averages are statistically different at $P < 0.05$ when compared on the same day.

Table 3. Effect of benzo(a)pyrene of aryl hydrocarbon hydroxylase (AHH) activity in lung of normal and adjuvant-diseased rats*

Day	Lung AHH units Normal controls	Normal benzo(a)pyrene	Adjuvant controls	Adjuvant benzo(a)pyrene
0	0·18 ± 0·03† (7)	1·50 ± 0·20‡ (7)		
7	0·13 ± 0·02† (6)	1·22 ± 0·18§ (6)	0·08 ± 0·01‡ (11)	1·13 ± 0·23§ (8)
14	0·12 ± 0·02† (11)	0·76 ± 0·20§ (7)	0·02 ± 0·02‡ (12)	0·77 ± 0·10§ (9)

* Benzo(a)pyrene at 25 mg/kg was given i.p. on day −2, while adjuvant was administered on day 0 as decribed in Methods. One AHH unit = 100 pg 3-OH-benzo(a)pyrene formed/mg wet weight of tissue/min. Results are given as means ± S.E.M. The number of rats is reported in parentheses.

†,‡,§ Unlike superscripts indicate that two group averages are statistically different at $P < 0.05$ when compared on the same day.

treated rats was only slightly higher than that of adjuvant controls at day 7, but the difference was statistically significant, $P < 0.05$.

In contrast to the liver, the lung AHH responds quite differently to adjuvant disease after induction by benzo(a)-pyrene, as summarized in Table 3. Benzo(a)pyrene treatment raises the lung AHH levels approximately 9-fold and this activity remained elevated much longer in lung than in liver. After 14 days the activity was still 5- to 6-fold higher than in the normal controls. Benzo(a)pyrene injections also appeared to protect against the impairment of lung MDMS by adjuvant disease for 14 days (Table 3). The AHH level in adjuvant controls is only one-sixth that of normal controls, while the level of AHH in adjuvant rats pretreated with benzo(a)pyrene is equal to that of normal rats pretreated with benzo(a)pyrene at 7 as well as 14 days of the experiment.

Rats with adjuvant disease exhibited increasingly severe impairment of the liver MDMS before a significant amount of paw edema could be measured. Whether the much more sensitive MDMS impairment is associated with the inflammatory process or is merely another manifestation of a toxis substance that also causes the allergic reaction of adjuvant arthritis is a most important question. In order to try to answer this question, an attempt at stimulating the MDMS was made. A previous attempt had used phenobarbital as the MDMS-inducing agent and was not successful in ameliorating the disease [7]. As in nonhepatic tissues, however, phenobarbital is a poor inducer, therefore benzo(a)pyrene was used in order to determine if an increased extrahepatic MDMS activity might have an effect on the course of the disease state [8]. In our experiments, when benzo(a)pyrene was given 2 days before adjuvant, neither paw edema nor impairment of hepatic drug metabolism was prevented. Since benzo(a)pyrene is a good inducer extrahepatically, the lung was significantly induced by this treatment and remained induced over a long period of time. Our experiments showed that lung AHH activity was still induced 14 days after adjuvant treatment. Possibly, slower turnover of protein in the lung than in the liver accounts for this result.

From this study it appears that benzo(a)pyrene does not act as an inducer for detoxification mechanisms of adjuvant, even though it will prevent the deterioration of specific extrahepatic MDMS enzymes as represented by AHH. The relationship of auto-immunity and the impairment of the MDMS is not limited to adjuvant arthritis, since other animal auto-immune models also appear to produce this effect. In preliminary experiments in this laboratory it was found that rats with experimental allergic encephalomyelitis (EAE) also had impaired MDMS as measured by ketamine sleeping times and AHH levels. This is in contrast to the work Beck and Whitehouse [7], who found no impairment of metabolism of cyclophosphamide *in vitro*, indicating either a specific rather than a general effect on the MDMS or different methods of induction of EAE. An excellent discussion of the different methods of induction can be found in Whitehouse *et al.* [11]. However, an interesting preliminary report by Farquhar and Loo [12] indicates that the immunostimulant BCG can impair the drug-metabolizing enzyme activities of the rat liver. From the above, one should also ask: "Is the liver MDMS inhibited in other immune phenomena—those not using adjuvant?" An answer to this question could be of importance in patients with various immune diseases. In addition, since the immunostimulants BCG [12] and *C. parum* are being added to cancer chemotherapy combinations to determine whether treatment results can be improved, an investigation of this question may be of clinical importance [13]. In those cases where the liver MDMS is damaged, the usual doses of toxic drugs metabolized by the MDMS may be too high and hence toxic. A better understanding of the metabolism of the drug in question would, therefore, be helpful in planning rational therapy for these patients. Also of importance is the fact that AHH is considered a key enzyme in the conversion of many precarcinogens to proximate carcinogens [14]. Thus influences on AHH levels are of great interest in the field of carcinogenesis. Further work is necessary before the mechanisms of the impairment of the MDMS by immune diseases can be elucidated.

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