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INHIBITION OF HEPATIC DRUG METABOLISM IN THE RAT AFTER *CORYNEBACTERIUM PARVUM* TREATMENT*

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Abstract—Drug-metabolizing enzyme activities, cytochrome concentration, and protein content of hepatic microsomal preparations from adult, female Sprague–Dawley rats were examined at 1-, 3-, 6-, 10-, 14- and 17-day intervals after administration of a single intravenous injection of *Corynebacterium parvum* (*C. parvum*) at a dose of 10 mg/m^2. Aniline hydroxylase (AH) activity, aminopyrine demethylase (APD) activity, and cytochrome P-450 concentration were reduced 20–50% on days 3–6 and, thereafter, gradually recovered to control levels by day 17. Cytochrome *c* reductase activity and cytochrome b_5 concentration were reduced significantly (24%) only on day 10. Microsomal protein concentration was unchanged. *C. parvum* added *in vitro* had no effect on AH or APD activity. Although livers of treated rats were only slightly (<20%) enlarged, gross splenomegaly was apparent, reaching a maximum on day 6. A marked inverse correlation existed between the temporal variation in the size of the spleen and APD activity. In rats killed 6 days after administration of *C. parvum* at 0.67 to 10.00 mg/m^2, a direct relationship was apparent between the adjuvant dose and the magnitude of reduction of APD activity. A similar relationship was apparent between splenomegaly and APD activity. Histopathologic examination of liver sections from treated rats revealed numerous granulomas throughout the parenchyma. The magnitude of enzyme inhibition generally paralleled the severity of the hepatic lesions.

Corynebacterium parvum (*C. parvum*), a potent, nonspecific stimulant of the reticuloendothelial system [1], is effective in the immunoprophylaxis and immunotherapy of a variety of transplantable animal tumors [2]. Added to chemotherapy, *C. parvum* increases the duration of drug-induced remissions and extends the survival of patients with malignant melanoma [3, 4], soft-tissue sarcoma [5], and carcinomas of the breast [5, 6] and lung [5]. We have reported previously [7] that bacillus Calmette–Guerin (BCG), another immunoadjuvant, administered intravenously to Sprague–Dawley rats at dosages equivalent to those used clinically, causes impairment of the hepatic oxidative drug-metabolizing enzyme system located in the endoplasmic reticulum. In a preliminary communication [8], we reported analogous findings after the administration of *C. parvum*. Our observations with the latter adjuvant in the rat have been confirmed [9], and similar results have been reported in the mouse [10]. We have also reported altered antipyrine plasma half-lives in patients receiving *C. parvum* by daily intravenous injection for 10 days [11]. Because of the current widespread clinical investigations of biological-response modifiers and the continuing interest in drug–adjuvant interactions [12–19], we wish to fully document our studies with *C. parvum*.

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MATERIALS AND METHODS

Aminopyrine and aniline were used as model substrates for drug metabolism *in vitro*. These compounds produce type I and type II binding spectra with microsomes respectively. The former compound undergoes *N*-demethylation to yield formaldehyde and 4-aminoantipyrine [20], while the latter compound undergoes ring hydroxylation to give *p*-aminophenol [21]. Cytochrome P-450 (cyt P-450), cytochrome b_5 (cyt b_5), and NADPH-dependent cytochrome *c* reductase (cyt *c* reductase) were also monitored, because these are important components of the electron transport chain responsible for drug metabolism.

Chemicals. The following drugs and reagent-grade chemicals were used: NADP sodium salt (Sigma), glucose-6-phosphate disodium salt (Sigma), glucose-6-phosphate dehydrogenase (Sigma), aniline (Eastman), aminopyrine (Aldrich), semicarbazide hydrochloride (Fisher), sodium dithionite (Fisher), sodium merthiolate (Schwartz-Mann), EDTA (Mallinckrodt), and pentobarbital sodium (Baker).

Animals. Adult, female Sprague–Dawley rats (Sprague–Dawley Laboratories, Madison, WI), weighing 200–250 g at the start of the experiments, were used. They were housed in metal cages in groups of four over hardwood bedding, with alternate periods of 12 hr light and 12 hr dark. Purina Lab Chow and water were provided *ad lib*. Both control and treated animals were weighed at the time of injection, and daily thereafter. Female animals were chosen for this study because of our previous observation [7] that this gender was more susceptible

than males to BCG-induced inhibition of the hepatic microsomal drug-metabolizing enzyme system.

Corynebacterium parvum. A formalin-killed suspension of *C. parvum* (7 mg dry wt/ml; strain CN 6134, lot 196-M), obtained from the Burroughs Wellcome Co., Research Triangle Park, NC, was diluted with sterile physiologic saline and administered to each experimental rat through the tail vein at a dose of 10 mg/m^2 (1.53 mg/kg; about 0.35 mg/rat) in a volume of 0.2 ml. On the basis of body surface area [22], the dose is equivalent to the upper doses used in clinical studies [23]. Control rats were injected with a solution of 0.0065% sodium merthiolate in physiologic saline, corresponding to the amount of methiolate contained in the *C. parvum*.

Preparation of microsomes. At 1-, 3-, 6-, 10-, 14- and 17-day intervals after *C. parvum* treatment, groups of four experimental rats together with their respective controls were decapitated and exsanguinated for 10 sec. Spleens and livers were rapidly excised, rinsed in ice-cold 1.15% KCl solution, blotted, and weighed. Livers were homogenized with 3 vol. of 0.1 M Tris buffer, pH 7.7, at 0–4°, and the homogenates were centrifuged at 10,000 *g* for 20 min at 4°. The microsomal pellet was washed by resuspension in ice-cold 1.15% KCl–10 mM EDTA, followed by resedimentation at 105,000 *g* for 30 min. The final pellet was reconstituted in 0.1 M Tris buffer, pH 7.7 (pH 7.4 at 37°), so that each milliliter of suspension contained microsomes from 250 mg wet weight of liver (approximately 5–10 mg of microsomal protein/ml). Microsomal protein content was determined by the method of Lowry *et al.* [24]; crystalline bovine serum albumin was used as the reference standard.

Assays. Aniline hydroxylase (AH) activity was measured by determining the amount of *p*-aminophenol formed according to the method of Kato and Gillette [25]. Aminopyrine-*N*-demethylase (APD) was measured by determining the amount of formaldehyde formed, according to the procedure of Nash [26] as modified by Cochin and Axelrod [27]. NADPH–cyt *c* reductase was assayed as described by Mazel [28]. The cyt P-450 and the cyt b_5 content of microsomes were measured by the method of Omura and Sato [29]. The effect of *C. parvum* on enzyme activity *in vitro* was determined by adding 0.35 g of the adjuvant in saline (0.2 ml), either directly to whole liver slices prior to homogenization or to microsomal preparations derived from whole liver. All experiments were repeated twice to document the reliability and reproducibility of the data. Differences between control and experimental groups were evaluated by the two-tailed Student's *t*-test. Pearson's correlation coefficient was calculated by the standard formulae.

Sleeping times. Sodium pentobarbital, 0.2% in physiologic saline, was administered intraperitoneally to rats at individual doses of 20 mg/kg. Sleeping time was defined as the time between the loss and the recovery of the righting reflex in these animals.

Histology. Freshly excised livers were sliced and immediately fixed in a 10% formalin solution and embedded in paraffin-blocks. Sections were cut from these blocks and stained with hematoxylin-eosin and methyl green pyronin. Tissue sections were examined under the light microscope.

RESULTS

The temporal variation in enzyme activities and cyt P-450 content of microsomes after a single intravenous injection of *C. parvum* at 10 mg/m^2 is shown in Table 1. APD activity was reduced significantly for up to 14 days. Inhibition was apparent as early as 24 hr after adjuvant administration but was most pronounced (about 50% of the control) on days 3 and 6; by day 17, activity had recovered to near control levels. AH levels were likewise reduced; except on day 6, the temporal variation in activity closely paralleled that of APD. Qualitatively similar results were observed with the cyt P-450 concentration of microsomes. By contrast, levels of NADPH cyt *c* reductase and cyt b_5 were reduced significantly only on day 10 after *C. parvum* administration.

Direct addition of *C. parvum* (0.35 g) to microsomal preparations or to whole liver slices prior to homogenization caused no change in any of the above parameters.

Table 1. Effect of an intravenous injection of *C. parvum* (10 mg/m^2) on various components of the hepatic drug-metabolizing enzyme system of female Sprague–Dawley rats

Days after *C. parvum*	APD	AH	Cyt P-450	Cyt b_5	Cyt *c* reductase
	Percentage of the control*				
1	78 ± 3†	74 ± 3†	79 ± 6	82 ± 4	102 ± 6
3	52 ± 5†	49 ± 5†	67 ± 5†	85 ± 5	105 ± 7
6	49 ± 5†	79 ± 5†	88 ± 5	96 ± 5	95 ± 6
10	71 ± 6†	77 ± 4†	79 ± 3†	76 ± 2†	76 ± 4†
14	63 ± 6†	73 ± 4†	73 ± 3†	83 ± 4	90 ± 5
17	87 ± 7	100 ± 5	105 ± 5	108 ± 4	81 ± 6

* Each value is the mean ± S.E. of observations from four rats. Average control values were: APD, 1.25 ± 0.06 nmoles formaldehyde formed per mg protein per min; AH, 0.84 ± 0.03 nmole *p*-aminophenol formed per mg protein per min; cyt P-450 0.69 ± 0.03 nmole cyt P-450/mg protein; cyt b_5, 0.37 ± 0.02 nmole cyt b_5/mg protein; cyt *c* reductase, 72.4 ± 4.1 nmoles cyt *c* reduced per mg protein per min. Abbreviations: APD, aminopyrine demethylase; and AH, aniline hydroxylase.

† Significantly different from the control: $P < 0.01$.

Table 2. Liver weight, spleen weight, and protein content of hepatic microsomes of female Sprague–Dawley rats after intravenous injection of *C. parvum* (10 mg/m²)*

Days after *C. parvum*		Liver wt (g/100 g body wt)†	Spleen wt (mg/100 g body wt)†	Hepatic microsomal protein (mg/g liver)
1	Control	3.20 ± 0.09	176 ± 13	27.4 ± 1.6
	Treated	3.58 ± 0.19 (112)‡	250 ± 12§ (142)	25.0 ± 1.3 (91)
3	Control	3.11 ± 0.04	194 ± 4	29.3 ± 0.5
	Treated	3.49 ± 0.10 (112)	401 ± 34‖ (207)	28.1 ± 1.3 (96)
6	Control	3.20 ± 0.10	189 ± 10	25.5 ± 2.0
	Treated	3.84 ± 0.21 (120)	478 ± 55‖ (253)	24.9 ± 1.6 (98)
10	Control	3.26 ± 0.09	195 ± 7	29.1 ± 0.7
	Treated	3.75 ± 0.13 (115)	366 ± 10‖ (188)	32.0 ± 1.6 (110)
14	Control	3.07 ± 0.10	216 ± 7	26.2 ± 1.6
	Treated	3.52 ± 0.16 (115)	409 ± 93§ (189)	28.6 ± 0.9 (109)
17	Control	3.17 ± 0.12	206 ± 7	27.2 ± 1.5
	Treated	3.22 ± 0.05 (101)	263 ± 14 (128)	29.1 ± 1.6 (107)

* Each value is the mean ± S.E. of observations from four rats.
† Average body weights of the control and the treated rats on day 0 were 246 ± 6 g and 248 ± 7 g respectively.
‡ Numbers in parentheses represent percentage of the control values.
§ Significantly different from the control, $P < 0.01$.
‖ Significantly different from the control, $P < 0.005$.

C. parvum-induced impairment of the hepatic drug-metabolizing enzyme system in the rat was also observed *in vivo*. Thus, pentobarbital-induced sleeping times in treated rats on day 6 (71 ± 9 min) were twice as great ($P < 0.01$) as that of controls (34 ± 6 min).

The rates of body weight gain of the treated and the control animals were similar over the 17-day observation period. Livers of treated rats were slightly enlarged compared to those of the control (<20%), but these differences were not statistically significant (Table 2). Levels of hepatic microsomal protein were similar for both groups of animals. By contrast, relative spleen weights increased markedly after *C. parvum* treatment. Spleen enlargement was evident as early as day 1. The average spleen weight of treated animals reached a maximum of 253% of that of the control on day 6; by day 17, spleen weights had returned to near normal values.

Correlation coefficients for the temporal relationships between the above variables are shown in Table 3. An excellent correlation exists between AH activity and cyt P-450 concentration. APD activity, on the other hand, is not strongly correlated with cyt P-450 levels. Neither AH nor APD activity shows a well-defined relationship with levels of cyt b_5 or cyt *c* reductase. A marked inverse correlation is apparent between spleen weight and APD activity; however, this relationship is not evident with the other components of the hepatic drug-metabolizing enzyme system.

The effects of the *C. parvum* dose on AH and APD activities, and on the cyt P-450 content of microsomes from rats killed 6 days after adjuvant administration, are shown in Table 4. A stepwise dose increase from 0.67 to 10.00 mg/m² was associated with a progressive decrease in APD activity. Although a similar trend was evident for AH activity and for cyt P-450 concentration, depression of these variables was statistically significant at $P < 0.05$ only at the highest adjuvant dose. A progressive increase in relative spleen weight was also evident. However, liver size and microsomal protein levels were not altered significantly.

An excellent inverse correlation existed between the *C. parvum* dose and APD activity (correlation coefficient, $r = -0.84$). Spleen weight was similarly correlated ($r = -0.88$) with APD activity. A perfect correlation existed between the *C. parvum* dose and splenomegaly ($r = 1.0$).

Livers of treated rats showed no gross morphologic abnormalities. However, histopathologic examina-

Table 3. Correlation coefficients for the temporal variation in APD activity, AH activity, cyt P-450 concentration, cyt b_5 concentration, cyt *c* reductase activity, and spleen weight (SW) after an intravenous injection of *C. parvum* (10 mg/m²) to female Sprague–Dawley rats

	Correlation coefficient				
	APD	Cyt P-450	Cyt b_5	Cyt *c* reductase	SW
AH	0.68	0.93	0.63	−0.68	−0.46
APD		0.57	0.24	−0.49	−0.95
Cyt P-450			0.84	−0.52	−0.38
Cyt b_5				−0.14	−0.17
Cyt *c* reductase					−0.24

Table 4. Effect of *C. parvum* dose on APD activity, AH activity, cytochrome P-450 concentration, liver microsomal protein concentration, liver weight, and spleen weight, in female Sprague–Dawley rats 6 days after intravenous administration of adjuvant (0.67 to 10.00 mg/m²)*

C. parvum dose (mg/m²)	APD	AH	Cyt P-450	Hepatic microsomal protein (mg/g liver)	Liver wt (g/100 g body wt)†	Spleen wt (mg/100 g body wt)†
	Percentage of the control					
None	98 ± 5	97 ± 4	98 ± 4	27.5 ± 1.1	3.63 ± 0.19	206 ± 12
0.67	80 ± 4§	95 ± 6	105 ± 5	27.2 ± 1.3 (99)‡	3.82 ± 0.20 (105)	222 ± 15 (108)
1.33	61 ± 3§	89 ± 3	92 ± 3	25.0 ± 1.2 (91)	3.82 ± 0.08 (105)	254 ± 19 (123)
3.33	52 ± 4‖	69 ± 3§	74 ± 5§	25.9 ± 0.9 (94)	3.90 ± 0.05 (107)	345 ± 76§ (167)
10.00				25.6 ± 1.4 (93)	4.28 ± 0.16 (118)	560 ± 43‖ (272)

* Each value is the mean ± S.E. of four observations. Abbreviations: APD, aminopyrine demethylase; and AH, aniline hydroxylase.
† Average body weights of the control rats on day 6 were 243 ± 9 g.
‡ Numbers in parentheses represent percentages of the control values.
§ Significantly different from the control, $P < 0.05$.
‖ Significantly different from the control, $P < 0.01$.

tion of liver sections from rats killed 6 days after adjuvant injection revealed numerous granulomas scattered throughout the parenchyma, as well as pronounced infiltration of the portal tracts by lymphohistiocytes. The severity of these lesions was greatest in rats receiving the highest dose (10 mg/m²) of adjuvant. Rats treated with an amount of merthiolate equivalent to that contained in the *C. parvum* dose showed no liver abnormalities.

DISCUSSION

The data show that *C. parvum* administered intravenously to Sprague–Dawley rats at doses similar to those used clinically, caused a time-dependent, dose-related impairment of the hepatic microsomal drug-metabolizing enzyme system. The mechanism of this effect is not clear. It is apparently not due to the systemic toxicity of the adjuvant, since treated rats appeared healthy and gained weight at the same rate as controls. Although the livers of treated animals were slightly enlarged, levels of microsomal protein were not altered significantly, and, therefore, cannot account for the observed reduction in enzyme activities. It is unlikely that reduced metabolism is due to the binding of *C. parvum* or its degradation products to the endoplasmic reticulum, since direct addition of the adjuvant to microsomal preparations or to whole liver slices prior to homogenization caused no change in enzyme activities.

Inhibition of the hepatic drug-metabolizing enzyme system by *C. parvum* is, to some extent, due to the decrease in the cyt P-450 content of microsomes. Thus, an excellent correlation was observed between the temporal variation in AH activity and cyt P-450 concentration. A similar relationship was not observed with APD activity; this finding is rather anomalous, since changes in the rates of metabolism of type I monooxygenase substrates, such as aminopyrine, often parallel changes in cyt P-450 levels [30].

The inverse relationship between enzyme activity and splenomegaly in both temporal and dose–response studies strongly suggests that the depression of the hepatic drug-metabolizing enzyme system by *C. parvum* is mediated through the reticuloendothelial system (RES). Soyka *et al.* [18] recently reported similar conclusions following comprehensive studies in a mouse model. These investigators have also shown that the same effect can be abrogated by prior splenectomy. In previous studies with BCG, we suggested [7] that activated macrophages may play a role in the pathogenesis of adjuvant-induced inhibition of hepatic monooxygenases. In both the rat and the mouse, *C. parvum* is a potent RES stimulant, inducing hyperplasia of the spleen and the liver and markedly enhancing nonspecific phagocytosis [1, 2]. Activated macrophages contain elevated levels of hydrolytic enzymes [31, 32] and possess increased ability to generate oxidative species, such as hydrogen peroxide [33] and superoxide anion [34]. Release of these products from hepatic granulomas could damage the endoplasmic reticulum of proximate parenchymal cells and, therefore, impair the integrity of the associated drug-metabolizing enzymes. However, because of the

large number of immunologic and pathologic changes that are known to accompany the intravenous administration of *C. parvum* to rodents [35], many other mechanisms might cause the same effect.

C. parvum-induced inhibition of hepatic microsomal enzymes has important implications for experimental and clinical studies in which adjuvants of immunity are combined with drugs. In particular, many antitumor agents [36], carcinogens, [37] and hypnotics [38] are biotransformed by these enzymes to products with greatly altered biological activity. Prior or concurrent administration of *C. parvum* could alter significantly the rate of biotransformation and excretion of these drugs and, therefore, could change their toxicologic and therapeutic properties. Several recent studies suggest this possibility. Mosedale and Smith [39] have reported that mice treated intravenously with *C. parvum* become lethally sensitized to normally safe doses of pentobarbital and tribromoethanol. Fisher *et al.* [13] have demonstrated altered cyclophosphamide metabolism in mice injected intraperitoneally with *C. parvum*. In a clinical study, we found [11] that nine of thirteen patients administered *C. parvum* intravenously at 2 mg/m^2 per day for ten consecutive days showed increased antipyrine plasma half-lives on days 5–7, but not on day 10, of treatment. In another study [12], however, we observed no alteration in the plasma half-lives of dacarbazine (an antineoplastic agent that requires microsomal enzyme activation) or in the 24-hr cumulative urinary excretion of 4-aminoimidazole-5-carboxamide, its principal metabolite, in sixteen patients with melanomas who had previously received *C. parvum* intravenously at 0.25 to 2.0 mg/m^2 for 10–14 consecutive days. More recently, Lipton *et al.* [16] reported decreased aminopyrine metabolism in three of eleven patients after intradermal administration of BCG (3×10^7 organisms) and in six of seven patients after intravenous administration of *C. parvum* (5 mg/m^2) in combination with cyclophosphamide. In contrast, Wan *et al.* [17] found no alteration in the plasma clearance of diphenylhydantoin or in the urinary elimination of its major metabolite in three patients with malignant melanoma and in one patient with hypernephroma, 10 days after a single intravenous injection of *C. parvum* at 2 mg/m^2. More recently, Hamilton *et al.* [19] reported no change in the antipyrine plasma half-life in seven patients receiving *C. parvum* (5 mg/m^2) in combination with various drug regimens.

Since a common trend is not evident in these clinical studies, general conclusions cannot be drawn at the present time concerning the effects of *C. parvum* and BCG on the hepatic drug-metabolizing enzyme system in man. Several factors may account for the divergent clinical findings, including differences in the composition and dose of the adjuvant, in the route, schedule, and duration of adjuvant administration, and in the time interval between administration of the adjuvant and the drug. Because of the complexity and variability of most clinical chemoimmunotherapeutic regimens, it seems unlikely that drug–adjuvant interactions in man can be predicted with confidence. Rather, such interactions will have to be investigated, when appropriate, for each specific protocol. Certainly, our observations in experimental animals and in man clearly suggest that drug–adjuvant interaction should be an important consideration in the design and the evaluation of studies in which drugs are combined with adjuvants of immunity.

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