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EFFECT OF ADJUVANT DISEASE IN RATS ON CYCLOPHOSPHAMIDE AND ISOPHOSPHAMIDE METABOLISM

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Abstract—After the injection of a variety of arthritogenic adjuvants into male Wistar rats, hepatic activation of cyclophosphamide and isophosphamide is rapidly and profoundly depressed. This selective injury is largely reversible with phenobarbital and principally restricted to the liver microsomal protein fraction, which demethylates aminopyrine and *N,N*-dimethylaniline and generates "alkylating metabolites" from cyclophosphamide *in vitro*. Evidence is presented, based upon both metabolite excretion studies and the duration of hexabarbital-induced hypnosis, that this phenomenon is not an artifact *in vitro* and must be seriously considered in evaluating both the efficacy of potential anti-arthritic drugs against the rat adjuvant arthritis and their toxicity in these arthritic animals. A quantitative separation of two pathological responses to the same adjuvant may be obtained: (1) in Buffalo rats, whose liver metabolism may be profoundly impaired while they suffer minimal (or no) arthritis after being inoculated with adjuvants which are truly arthritogenic in other rat strains; (2) with *Mycobacterium tuberculosis* dispersed in methyl oleate, which induces minimal arthritis in Wistar rats, but nevertheless impairs their liver metabolism over a prolonged period (14 days or more). Drug metabolism appeared to be normal in rats with two other immunologically mediated "inflammatory diseases" (graft vs host disease and allergic encephalomyelitis) and in other rodents examined after adjuvant inoculations. A novel bioassay for cyclophosphamide and isophosphamide metabolites is described which utilizes their ability to prevent grafted rat lymphocytes from initiating a graft vs host reaction in tolerant recipient rats. At least four alkylating metabolites of cyclophosphamide were found in rat bile and tentatively identified by thin-layer chromatography. The possible error in relying on changes in urinary excretion (rather than biliary excretion) of drug metabolites as a guide to changes in hepatic xenobiotic metabolism is discussed.

ADJUVANT disease is a form of chronic arthritis in rats induced by the subdermal (or intranodal) injection of certain heat-killed Mycobacteria dispersed in an oily medium.[1] It is used extensively to assay both anti-inflammatory and immunosuppressant drugs,[2,3] including cyclophosphamide (CPA).[3,4,*] It is generally agreed that CPA has little or no effect on the signs of arthritis, once established. This is in strange

* Abbreviations used: CPA = cyclophosphamide (Cytoxan; 2-bis-β-chloroethyl-amino-tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide); IPA = isophosphamide (Ifosfamide; 3-β-chlorethyl-2-β-chlorethylamino-tetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide); EAE = experimental allergic encephalomyelitis; TCE = 2,2,2-trichloroethanol; NBP = 4-*p*-nitrobenzyl-pyridine; DMA = *N,N*-dimethylaniline; GvHR = graft vs host response; F_1 = first generation hybrid, tolerant to a lymphocyte graft from either parent; BCG = Bacillus Calmette-Guerin (*Mycobacterium bovis*); PB = phenobarbital; P.I.U. = plasma "inflammation units".

contrast to the effect of CPA on a related hypersensitivity disease in the rat, allergic encephalomyelitis (EAE).

CPA not only prevents (or at least considerably delays) the signs of EAE (weight loss, paralysis) when given prophylactically,[5,6] but also ameliorates the established disease,[7] i.e. CPA has a therapeutic action in this instance. Since CPA is probably controlling similar populations of lymphocytes which initiate and sustain the adjuvant arthritis and the EAE,[8,9] there must be some reason why CPA is so much less effective against established arthritis that established EAE. One explanation may be the effect of adjuvant disease on the bio-activation of cyclophosphamide.

In order to manifest its anti-tumor activity, CPA must be activated by metabolism *in vivo*, principally oxygenation by liver microsomes.[10,11] This report describes the impaired metabolism of CPA in rats with adjuvant disease.

In these experiments cyclophosphamide metabolism was determined by the production both *in vitro* and *in vivo* of alkylating metabolites, measured (1) colorimetrically with nitrobenzylpyridine, and (2) bioassayed by their effect in deactivating lymphocytes used to initiate a local graft vs host response in rats.

EXPERIMENTAL

Animals and materials. Inbred white mice were obtained from the following sources: Ajax (Jackson Memorial Lab, Bar Harbor, Me.); CD (Charles River Labs, Wilmington, Mass.). Buffalo strain white rats were obtained from Simonsen Labs, Gilroy, Calif. An outbred mouse strain (Swiss Webster) and other strains of rats were supplied by Hilltop Lab Animals, Chatsworth, Calif.; golden hamsters by Con Olsen, Inc., Madison, Wisc.; Syrian hamsters by Whitney Animal Labs, Horsehead, N.Y.; Chinese hamsters by Chick Line Co., St. Louis, Mo. *Mycobacterium tuberculosis* (human) was obtained from the Central Veterinary Laboratory, Weybridge, England; *Mycobacterium phlei* from General Biochemicals, Chagrin Falls, Ohio; *Corynebacterium pseudodiphthericum* (Hoffmanii) from Microbiological Labs, Inc., Maple Heights, Ohio. *Corynebacetium rubrum* and *Nocardia asteroides* were donated by Dr. R. Perrin, Calbiochem, San Diego, Calif.; *Corynebacterium lymphophilium* by Dr. C. Adlam, Wellcome Research Labs, Beckenham, England; cell walls from *Mycobacterium bovis* (BCG) by Dr. G. B. Mackaness, Trudeau Institute, Saranac Lake, N.Y. Whole bacterial preparations were dehydrated and delipidated by three extractions with ethanol–ether (1:1, v/v) at room temperature. Mineral oil (Protol) was a sterilized preparation from the UCLA Hospital Pharmacy originating from Witco Chemical Co., Sonneborn Division, N.Y. Paraffins and olefines were obtained from Aldrich Chemical Co., Milwaukee, Wisc., or Eastman Kodak Co., Rochester, N.Y.

Animal methods. EAE was initiated in inbred Dunning–Fischer rats by Newbould's technique.[12] Adjuvant disease was established in outbred Wistar rats by injecting into either their tail or one rear paw 50 μl of a finely dispersed suspension (10 mg/ml) of dried *C. rubrum*, heat killed *N. asteroides* or delipidated heat-killed *M. tuberculosis* or BCG cell walls in mineral oil or normal paraffins ($\geqslant C_{12}$) or olive oil. Non-arthritogenic "dummy adjuvants" were similarly prepared either using these same oils and delipidated (not heat-killed) *M. phlei* and *C. Hoffmanii*, or using short chain paraffins or methyl oleate or oleyl alcohol as the dispersion medium with arthritogenic bacteria

(*M. tuberculosis*, *C. rubrum*, *N. asteroides*) or BCG cell walls. All animals were bedded in sawdust with the exception of some in which drug-induced sleeping times were determined; these animals were bedded on gravel.

A local graft vs host response (GvHR) was established in F_1 Wistar × Fischer hybrid (F344 × W) rats by injecting 30–50 × 10^6 viable splenic lymphocytes from the parental Wistar strain into each footpad;[13] these lymphocytes were washed once in 0·85% (w/v) ammonium chloride buffered with 0·1 vol. of 0·15 M Tris hydrochloride (pH 7·4) to remove red cells and once again in Hanks' medium before use. The hybrid (cell recipient) animals were sacrificed 7 days later; the combined weights of each of the collateral lumbar and popliteal nodes were then determined as a measure of the GvHR elicited by the lymphocytes injected into that particular footpad. Only cell preparations that were at least 80 per cent viable by trypan blue exclusion after exposure to drugs *in vitro* were inoculated into the recipient animals. Drugged lymphocytes were always injected into one paw, and undrugged, but similarly manipulated, cells were injected in equal numbers into the other paw. This strategy effectively allowed for the inter-individual variation in the responsiveness of individual F_1 hybrids to the grafted lymphocytes, each animal effectively acting as its own control.

Carbon clearance studies of reticulo-endothelial activity *in vivo* in rats were carried out by injecting a carbon suspension (Pelikan, C11/1431a; John Henschel and Co., Inc., Farmingdale, N.Y.), 160 mg/kg, through a cannula inserted into a femoral vein and withdrawing 25 μl blood at intervals from a nick in the tail, and reading the optical density at 650 nm after shaking each sample with 4 ml of 0·1 %(w/v) sodium carbonate to lyse all blood cells.

Sleeping times were measured after i.p. injections of 200 mg/kg of trichloroethanol (Aldrich Chemical Co., Milwaukee, Wisc.) or 150 mg/kg of hexobarbital sodium (K & K Labs) in saline solutions adjusted to 0·3 osmolar with NaCl, using two or more animals drawn from each group of animals inoculated with any given adjuvant.

Urine was collected from animals restrained in Bollman cages[14] and continuously infused via a leg or tail vein cannula with saline (6–8 ml/hr) to ensure regular voiding of urine.

Plastic cannulae were inserted into the bile ducts of normal and adjuvant-inoculated animals under ether anesthesia; animals were restrained in Bollman cages for at least 15 hr before administering CPA via a leg vein cannula and liberally hydrated by saline infusions to replace fluid lost in the bile.

Methods in vitro. Liver tissue (5 g) was homogenized in chilled 0·15 M KCl and centrifuged at 15,000 *g* for 20 min at 4°. The cell-free supernatant was either used as such (15,000 *g* supernatant) or further centrifuged at 88,000 *g* for 60 min at 4° to derive a microsomal fraction and the "particle-free' cytosol. This microsomal fraction was washed by homogenization in 0·15 M KCl and re-isolated by further centrifugation at 88,000 *g*. These liver preparations (20–60 mg protein) were incubated in Erlenmeyer flasks with continuous shaking for 15 min at 37° with either 7 mM dimethylaniline or 7 mM aminopyrine (K & K Labs) or 3·6 mM isophosphamide or 1·2 mM cyclophosphamide (Mead Johnson & Co.) and the following co-factors: NADPH (1 mg), monosodium D-glucose 6-phosphate (G-6-P, 5·4 mg), *Torula* G-6-P dehydrogenase (0·32 unit) in a final volume of 3 ml with the composition of 2 mM $MgCl_2$, 100 mM KCl and 20 mM potassium phosphate, pH 7·4.

Total protein was measured with a biuret method.[15] Formaldehyde production from

aminopyrine and *N,N*-dimethylaniline was measured colorimetrically with pentan-2,4-dione.[16] Alkylating metabolites of cyclophosphamides were determined colorimetrically with 4-(*p*-nitrobenzyl)-pyridine (NBP),[17] as modified by Morita *et al.*[18] using mechlorethamine hydrochloride (HN2) as a standard. Acrolein was determined fluorimetrically using *m*-aminophenol.[19] As criteria of adjuvant disease, the following parameters were determined: serum albumin,[20] fibrinogen[21] and "plasma inflammation units" (P.I.U.).[22] Foot thickness was measured with a micrometer.

Thin-layer chromatography of cyclophosphamide and isophosphamide metabolites was carried out using 0·25 mm Silica plates (Silplates, rapid; Brinckmann Instruments, Westbury, N.Y.) irrigated with the following solvent systems: (1) methanol–chloroform (1:9, v/v); (2) *n*-butanol–glacial acetic acid–water (3:1:1, v/v); (3) methanol–chloroform (1:3, v/v); (4) *n*-butanol–dichlormethane (1:9, v/v).

Provisional identification of the various CPA metabolites separated by systems 1 and 2 has been described by Sladek.[23] Systems 3 and 4 are more suitable for characterizing hydroxylated derivatives of CPA and IFA.*

Both CPA and its alkylating metabolites were detected by spraying the plates with 5% (w/v) NBP in acetone, admixed with 0·2 vol. of 0·2 M sodium acetate, pH 4·4, followed by oven drying at 140° for 15 min and final spraying with 1 N NaOH to visualize these compounds as purple zones.

Reference compounds i.e. known CPA metabolites were generously provided by Drs. L. B. Mellet and R. F. Struck, Southern Research Institute, Birmingham, Ala.

RESULTS

Relationship of adjuvant composition to its arthritogenicity. Adjuvant arthritis was consistently induced in both Wistar and Lewis strains of rats with various dispersions of Mycobacteria in certain oily vehicles including mineral oil (liquid petrolatum, Protol), this latter medium constituting a so-called "complete Freund's adjuvant". Other oily dispersions of bacteria were not arthritogenic in Wistar rats, although they caused prolonged acute inflammation at the site of injection. They will be referred to hereafter as "dummy adjuvants". Examples of such dummy adjuvants are: *M. tuberculosis* in hexane, nonane, undecane; and commercially available strains of *M. phlei*, *C. Hoffmanii* and *C. lymphophilium*, each in mineral oil. By contrast, the following arthritogenic "adjuvants "(*M. tuberculosis* in olive oil, triolein, decane, dodecane, tetradecane, hexadecane and octadecane; *N. asteroides* and *C. rubrum* in hexadecane, mineral oil or olive oil; and Freund's adjuvant itself) caused loss of body weight, reduction in serum albumin, elevation of plasma fibrinogen and "inflammation units", paw swelling and an appreciable reduction in the capacity of liver preparations to metabolize (activate) cyclophosphamide *in vitro* (see below and Table 1). Closely related "adjuvants" constituted with *M. tuberculosis* and the corresponding Δ^1-(C_{12} through C_{18}) olefines caused a milder arthritis in Wistar rats with less impairment of liver cyclophosphamide metabolism *in vitro* than did the adjuvants prepared with the corresponding (C_{12} through C_{18}) paraffins.

The Buffalo rat develops only a minimal arthritis after injection with Freund's adjuvant, in contrast to the Wistar rat and other strains.[24] We found that Buffalo

* R. F. Struck, personal communication.

TABLE 1. METABOLISM OF CYCLOPHOSPHAMIDE (CPA) AND AMINOPYRINE (AP) OR DIMETHYLANILINE (DMA) BY LIVER PREPARATIONS FROM ADJUVANT-INOCULATED WISTAR (W) OR BUFFALO (B) RATS*

Strain	Animals injected with	Day†	No.	Criteria of adjuvant arthritis‡				Metabolite formation from§	
				Arthritic score	P.I.U.	Albumin (mg/ml)	Fibrinogen plasma)	CPA	AP or DMA
W	Nothing (normal control)		24		13 ± 24	41 ± 9	4 ± 2	100	100
W	Mineral oil only	14	2	0	0	34 ± 18	6 ± 1	83 ± 4	85 ± 26
W	Mineral oil with *M. phlei*	14	1	0	0	41	ND	134	88
W	Mineral oil with *C. hoffmanii*	14	2	0	90 ± 113	36 ± 7	ND	113 ± 18	ND
W	Mineral oil with *M. tb*	1	3	ND		ND	ND	41 ± 10	75 ± 18
		2	8		532 ± 246	24 ± 11	9 ± 4	41 ± 22	53 ± 18
		7	2		170	41	10	39 ± 36	51 ± 4
		14+	10	17	516 ± 131	12 ± 5	10 ± 4	33 ± 29	26 ± 14
W	*M. tb* with hexane	2	2		60 ± 25	26 ± 5	6 ± 1	58 ± 6	41 ± 18
		14+	7	0	75 ± 91	38 ± 6	6 ± 2	83 ± 24	93 ± 26
W	*M. tb* with dodecane	13+	6	9	395 ± 328	20 ± 9	9 ± 4	62 ± 16	50 ± 36
W	*M. tb* with octadecane	13+	6	16	213 ± 126	36 ± 19	6 ± 1	36 ± 18	36 ± 24
W	*M. tb* with methyl oleate	2	4		302 ± 240	26 ± 4	7 ± 4	37 + 21	53 ± 20
		3	2		ND	ND	ND	14 ± 2	30 ± 9
		14	3	2	22 ± 33	37 ± 9	6 ± 3	57 ± 12	84 ± 11
W	*M. tb* with Triolein	14	5	14	271 ± 217	19 ± 9	7 ± 2	22 ± 24	40 ± 5
W	Mineral oil with *C. rubrum*	14	2	12	27 ± 25	23 ± 21	3 ± 1	57 ± 33	58 ± 18
W	Mineral oil with *N. asteroides*	14	1	15	56	23	8	24	44
B	Nothing (normal control)		3		8 ± 14	39 ± 3	7 ± 3	100	100
B	*M. tb* with mineral oil (node)	14	2	0	288 ± 109	33 ± 0	10 ± 3	71	62
	(foot)	14	2	6	432 ± 420	19 ± 2	14 ± 3	52	81
	(tail)	14	2	5	735 ± 247	26 ± 3	11 ± 5	26	34

* CPA metabolites measured by NBP-positive chromogens and calculated as mechlorethamine equivalents. AP and DMA metabolism measured by formaldehyde fomation. Rats were injected with various adjuvants composed of *Mycobacterium phlei* or *tuberculosis* (*tb*), *Corynebacterium rubrum* or *hoffmanii, and Nocardia asteroides*, finely dispersed in various oils. Wistar rats were injected in the tail. Buffalo rats were injected in inguinal nodes, or tail or a rear paw.

† Day following adjuvant administration in tail or foot, + indicates within 3 following days, i.e. 14+ = days 14–17.

‡ Arthritic score was computed by summing the lesions of each rear foot (scored 0–4), tail and front feet (scored 0–2), reduction in body weight gain (scored 0–4) and general debility (scored 0–2) for a maximum score of 20 for a very sick animal determined at day 13 or thereafter. Data in these columns are mean values from groups of four or more animals. Biochemical criteria of adjuvant disease, probably reflecting deviations in normal liver function = plasma inflammation units (see Experimental), increased plasma fibrinogen and reduced albumin levels. ND = not determined.

§ As a percentage of concurrent control, run simultaneously (±S.D.). Average values in these controls were 5·3 ± 3·5 nmoles of alkylating metabolites (calculated as equivalents of mechlorethamine)/15 min/mg protein (n = 43) from CPA, and 80 ± 74 nmoles formaldehyde/15 min/mg protein (n = 28) from AP or DMA.

rats injected in the inguinal nodes or tail or foot with Freund's adjuvant had impaired CPA metabolism *in vitro* (Table 1) with little or no evident arthritis, but did suffer changes in the level of plasma albumin, fibrinogen and inflammation units (P.I.U.) such as are associated with the development of arthritis in susceptible rat strains.

It was not necessary to inject the whole dried (arthritogenic) bacteria to observe this dysfunction in cyclophosphamide metabolism. As little as 40 μg of a purified BCG cell wall preparation, administered as a dispersion in mineral oil or hexadecane, induced both a chronic arthritis and loss of drug-metabolizing capacity in rats. When the same BCG cell wall dispersions were inoculated into the footpads of three strains of mice, they induced a mild systemic (but transient) arthritis 12–14 days later without causing any evident impairment of hexobarbital or cyclophosphamide metabolism.

Studies of drug metabolism in vitro. For these studies liver homogenates or liver microsomal fractions were always prepared simultaneously from adjuvant-inoculated animals and either from their untreated littermates or from weight-matched normal animals. When compared with the respective drug-metabolizing activities of homogenates of livers taken from the control rats, the demethylation of *N,N*-dimethylaniline or aminopyrine by liver homogenates prepared from adjuvant-treated animals was always impaired when CPA metabolism was found to be subnormal in these same homogenates (Table 1). Impaired drug metabolism was apparent even after 1 day following tail injection of *M. tuberculosis* in mineral oil, and sometimes so gross that cyclophosphamide was not metabolized at all *in vitro* by liver tissue harvested on day 14 after an adjuvant injection (Table 1). Table 2 presents data indicating that this

TABLE 2. CYCLOPHOSPHAMIDE (CPA) METABOLISM BY LIVER MICROSOMES FROM ARTHRITIC (A) AND NORMAL (N) RATS*

Microsomes from	CPA metabolites†	
	Expt I	Expt II
N only	4·7	8·2
N plus cytosol from N	4·9	11·8
N plus cytosol from A	3·2	8·1
A only	2·4	3·4
A plus cytosol from N	2·5	6·4
A plus cytosol from A	1·8	4·7

* Microsomes were washed once with 0·15 M KCl.
† Calculated as nanomoles of mechlorethamine (HN2) equivalents formed/mg microsomal protein/15 min.

apparent loss of enzymic activity was primarily located in the microsomal fraction, and was not solely due to the presence of an inhibitor in the liver cytosol of the arthritic animal. The occasional stimulation of CPA metabolism by liver microsomes from arthritic rats on adding the cytosol from normal rat livers (experiment 2, Table 2) may possibly reflect some enhancement of microsomal activity by mitochondrial products[25] or even albumin, these being present in greater quantity in the extramitochondrial compartment of normal liver tissue than in the same compartment of arthritic rat livers. Addition of rat fibrinogen (from arthritic animals) did not impair CPA metabolism by microsomes from normal animals.

Injection of non-arthritogenic "dummy adjuvants" (e.g. *M. tuberculosis* in hexane) not only impaired the liver microsomal metabolism of CPA and aminopyrine measured 2 or 3 days after the "adjuvant" inoculation (Table 1), but also caused an acute local inflammation (e.g. foot swelling) accompanied by increased levels of plasma fibrinogen. The liver metabolism was subsequently restored toward normal as these animals recovered from the acute inflammation, so that by day 14 after inoculating these dummy adjuvants, the animals showed no signs of chronic inflammation and their livers metabolized aminopyrine and cyclophosphamide *in vitro* as efficiently as those of the untreated controls (see data for *M. tuberculosis* in hexane at day 14, Table 1).

One very marginally arthritogenic adjuvant (*M. tuberculosis* in methyl oleate), however, consistently depressed CPA metabolism even as late as 14 days after its inoculation, although it only elicited an arthritis in less than 10 per cent of the animals in which it was inoculated.

Injection of 0·1 ml Freund's adjuvant (a potent arthritogen) into the base of the tails of 80 g golden hamsters or 80 g Syrian white hamsters or 20 g Chinese hamsters caused no visible paw lesions, and appeared only to stimulate the aminopyrine-metabolizing activity by 28 per cent at most (four animals per group). Rats which were debilitated with EAE during the time period 10–17 days after inoculating the encephalitogen had smaller livers (commensurate with the overall weight loss of these animals) which, on a weight-for-weight basis, yielded subcellular fractions at least 80 per cent as active in metabolizing CPA or aminopyrine as similar liver fractions prepared from the normal healthy control animals. Likewise, F_1 hybrid rats suffering from the immunological onslaught of an active graft vs host reaction (sacrificed 7 days after grafting parental lymphocytes) yielded liver homogenates which metabolized CPA and aminopyrine at least 90 per cent as efficiently as those from the normal hybrid controls.

Bioassay for CPA metabolism in vitro. Further evidence that CPA metabolism was subnormal in liver preparations from adjuvant-diseased animals was obtained in the following bioassay. Parental splenic lymphocytes (150×10^6) were incubated for 15 min at 37° in the liver incubation media (to which CPA had been added 15 min before adding the lymphocytes) and thereby exposed to the lymphocyte-deactivating CPA metabolite(s). The ability of these drugged cells to elicit the GvHR was determined by injecting $30–50 \times 10^6$ cells (separated from the incubation medium) into the foot pads of at least two F_1 hybrids; the contralateral paws in the same animals received the same number of undrugged cells, i.e. cells incubated under identical conditions with the same liver preparation but without any CPA present. Table 3 summarizes the results of these experiments.

In these bioassays the depression of the GvHR is a measure of active alkylating metabolites (nitrogen mustards) but not of CPA itself. Since CPA is only a latent drug, it has no effect on the GvHR activity of these lymphocytes in the absence of liver microsomes. By contrast, incubating these same lymphocytes in Hanks' medium buffered with 0·2 vol. of sodium phosphate, pH 7·4, together with active mustards such as chlorambucil (20 μM) and mechlorethamine (2 μM) inhibited the GvHR by approximately 50 per cent.

This bioassay independently confirms the data from the colorimetric assays for alkylating metabolites which indicated that the metabolism of CPA *in vitro* by liver preparations from animals with adjuvant disease was distinctly subnormal.

TABLE 3. BIOASSAY OF CYCLOPHOSPHAMIDE (CPA) METABOLITES BY A GvHR REACTION*

CPA incubated with	No.†	GvHR (%)	Inhibition by CPA metabolites (%)
KCl (0·15 M)	7	99 ± 17	1
Liver tissue from normal rats	42	25 ± 16	75
Liver tissue from arthritic rats‡	23	74 ± 30	26
Liver tissue from Co^{2+}-pretreated rats	6	65 ± 10	35
Liver tissue from arthritic controls§	6	45 ± 23	55

* See text for description of experiment. Magnitude of GvHR elicited in controls (i.e. by cells incubated with KCl alone or liver preparations without CPA = 100%.
† Number of recipient (F_1) animals.
‡ Rats inoculated with adjuvant 13 or more days previously.
§ Rats inoculated with non-arthritogenic (dummy) adjuvants.

Effect of cobalt chloride. The rat liver microsomal drug-metabolizing system is rapidly inactivated after administering cobaltous salts[26,27] by inhibition of ferrochelatase,* preventing cytochrome P_{450} synthesis *de novo*. Wistar rats were injected subcutaneously with 45 mg/kg of $CoCl_2 . 6\ H_2O$ in saline on each of 2 days preceding sacrifice. Cyclophosphamide metabolism was then studied *in vitro* using liver homogenates prepared from these Co^{2+}-dosed animals and from the saline-injected controls. The yield of alkylating metabolites, assayed with NBP, was only 62 per cent that of controls after this cobalt pretreatment. The CPA metabolites formed by liver homogenates prepared from Co^{2+} -treated animals inhibited the GvHR to a much lesser degree than did the CPA metabolites formed by liver homogenates prepared from the saline-injected controls (Table 3). Thus cobalt pretreatment, like the adjuvant arthritis, diminished the ability of rat hepatic microsomes to generate lymphocyte deactivating metabolites from CPA .The absolute magnitude of the GvHR appeared to be unaffected by any residual cobalt ions in the liver preparations, since in the absence of CPA these same liver extracts had no effect on the GvHR.

These findings confirm the impairment of CPA metabolism after pretreatment with $CoCl_2$[27] and show that the GvHR bioassay system, as used in these experiments, adequately "recognizes" the impaired activation of CPA *in vitro* when normal microsomal drug metabolism is significantly inhibited (such as after cobalt intoxication). This latter conclusion is rather important as it validates our interpretation of the previous experiments using a GvHR bioassay to "diagnose" impaired CPA activation by liver preparations from adjuvant-arthritic animals.

Studies with isophosphamide. Isophosphamide (IPA) is a structural isomer of CPA which is somewhat less potent as an immunosuppressant.[28,29] When IPA (3·6 mM) was incubated with all the cofactors, but without any liver enzymes, low levels of NBP-reactive products were spontaneously generated which, when incubated with splenic lymphocytes *in vitro*, did not inhibit the GvHR. When IPA was incubated with normal liver microsomes, the yield of NBP-reactive products formed *in vitro* was not always increased, but a product was formed which consistently inhibited the GvHR (by 61 ± 11 per cent, $n = 6$). However, when IPA was incubated with micro-

* T. R. Tephly, personal communication.

somes from adjuvant-arthritic rats, the GvHR was always inhibited to a lesser degree (39 ± 22 per cent, $n = 5$).

These preliminary studies show that adjuvant disease probably also influences isophosphamide activation by rat liver tissue. Unambiguous studies to verify this conclusion must await identification of the pathway of IPA metabolism by rat liver extracts *in vitro*.

Further characterization of the adjuvant-induced lesion in drug metabolism. Animals with established adjuvant disease had approximately normal carbon clearance times ($t_{1/2}$ = approximately 10 min), indicating that the arthritogenic adjuvants impaired liver function rather selectively, having little effect on the phagocytic activity of the liver RES cells.

In normal male Wistar and Buffalo rats, trichloroethanol (200 mg/kg) and sodium hexobarbital (150 mg/kg) injected i.p. gave mean sleeping times which were not profoundly altered by bedding the animals on gravel instead of sawdust (to avoid induction of drug-metabolizing enzymes by volatile sawdust constituents). However, all groups of arthritic Wistar rats (i.e. with established disease) studied had mean sleeping times approximately four times that of the normal controls. These animals were drawn from groups which received eight different types of arthritogenic adjuvant based on *M. tuberculosis*. Prolonged sleeping times were evident even 2 days after adjuvant administration to Wistar and Buffalo animals. By contrast, animals which had been inoculated with non-arthritogenic adjuvants or with mineral oil exhibited almost normal sleeping times 14 days after these inoculations (Table 4).

TABLE 4. SLEEPING TIMES AFTER ADMINISTRATION OF HEXOBARBITAL (150 mg/kg) OR TRICHLOROETHANOL (200 mg/kg)*

Group	P value†	Hexobarbital	Trichloroethanol
Control		28 ± 26 (49)	15 ± 23 (17)
Adjuvant arthritic, days 14+	0·001	112 ± 73 (80)	72 ± 42 (16)
Dummy adjuvant,‡ days 14+	0·75	35 ± 55 (13)	24 ± 27 (4)
Dummy adjuvant, day 2	0·001	99 ± 48 (8)	
Freund's adjuvant, day 2	0·001	72 ± 30 (7)	

* Numbers in parentheses = number of animals studied. Sleeping times (min) ± S.D.
† For hexobarbital sleeping times compared with controls.
‡ Non-arthritogenic (*M. hoffmanii* in mineral oil or *M. tuberculosis* in either methyl oleate or hexane).

The adjuvant-induced lesion was largely reversed by treatment with phenobarbital, which shortened the hexobarbital sleeping times in arthritic rats and increased the rate of cyclophosphamide and aminopyrine metabolism by their liver tissue *in vitro* (Table 5). By contrast, the signs of arthritis (data not given here) and the plasma albumin, fibrinogen and other inflammation marker protein (P.I.U.) levels in the arthritic rats were not modified by phenobarbital. This indicates that phenobarbital rather selectively stimulates microsomal drug metabolism, without significantly modifying either the lesion in hepatic albumin synthesis or the hyperproduction of fibrinogen and α_2-glycoproteins that are associated with adjuvant disease.

TABLE 5. EFFECT OF PHENOBARBITAL (PB) ON THE ADJUVANT-INDUCED LESION IN DRUG METABOLISM*

Group	Treatment	Sleeping times (min) Pretreatment	Sleeping times (min) Post-treatment	Metabolism *in vitro* CPA† (%)	Metabolism *in vitro* AP‡ (%)	Plasma levels Albumin (mg/ml)	Plasma levels P.I.U.§
Normal	None	13 ± 16	10 ± 12	100	100	49 ± 11	34 ± 39
Normal	PB	14 ± 20	7 ± 14	203 ± 2		41 ± 2	44 ± 61
Arthritic	None	183 ± 97	196 ± 84	38 ± 36	57 ± 13	24 ± 14	257 ± 247
Arthritic	PB	170 ± 102	41 ± 19	170 ± 55	104 ± 23	17 ± 7	312 ± 296

* Arthritic animals were inoculated with adjuvant 14 days before oral dosing with PB (50 mg/kg × 7 days) or with saline. Sleeping times in response to i.p. hexobarbital (150 mg/kg) are for groups of five or more animals. Metabolism and plasma determinations *in vitro* were conducted 1 day after the final PB dose (day 22) using four or more animals/group. All data ± S.D.

† Formation of alkylating metabolites from cyclophosphamide (CPA) by liver preparations (see Table 1): normal untreated control = 100%, being equivalent to 5·2 ± 2·1 nmoles mechlorethamine/mg protein/15 min.

‡ Formation of formaldehyde from aminopyrine (AP): normal untreated control = 100%, being equivalent to 60 ± 3 nmoles formaldehyde/mg protein/15 min.

§ Plasma inflammation units.

Studies with CPA in vivo. In the 3-hr period following a single i.v. dose of 7·5 mg (27 μmoles) cyclophosphamide, the urinary excretion of alkylating metabolites was uniformly depressed in the adjuvant-diseased animals (Table 6). The levels of these (NBP-reactive) metabolites in urine collected from arthritic animals varied from 35 to 75 per cent of that present in the urine of control animals collected over the same time period under forced diuresis with constant saline infusion. The greatest reduction in metabolite excretion was observed with animals which had established disease induced with an optimal arthritogenic adjuvant (i.e. *M. tuberculosis* in mineral oil or olive oil). Animals receiving the (weaker) olefine adjuvants excreted greater amounts of the urinary metabolites, but their total output of CPA metabolites was still less than that of the normal control animals.

TABLE 6. EFFECT OF PRIOR ADJUVANT ADMINISTRATION ON THE LEVELS OF ALKYLATING METABOLITES OF CYCLOPHOSPHAMIDE (CPA) EXCRETED INTO URINE AND BILE BY MALE WISTAR RATS*

Animals	Urine (3 hr) No.	Urine (3 hr) % of Control†	Bile (4 hr) No.	Bile (4 hr) % of Control‡
No treatment (controls)	11	100	9	100
Established arthritis	14	60 ± 15	6	49 ± 26
2–4 days after FA	6	104 ± 24	2	31 ± 3

* CPA was administered as a single i.v. dose of 7·5 mg/rat (200–250 g). Urine was collected for a 3-hr period and bile for 4 hr. FA = *M. tuberculosis* in mineral oil, inoculated in tail. Controls were littermates or weight-matched normal animals studied simultaneously (usually two per experiment). Data are composite from several experiments.

† 7·2 ± 3·75 μmoles HN2 equivalents excreted in urine of normal rats over a 3-hr period.

‡ 0·52 ± 0·375 μmole HN2 equivalents excreted in bile flow of normal rats over a 4-hr period.

The biliary excretion of NBP-reacting chromogens in the 4-hr period after i.v. administration of CPA (7·5 mg) to arthritic Wistar rats averaged 49 per cent ($n = 6$) of that excreted by weight-matched normal Wistar rats. The biliary excretion of alkylating metabolites by animals inoculated with Freund's adjuvant 2 and 4 days previously (i.e. 10 days before the onset of arthritis) was also depressed (Table 6). The average yield of the alkylating metabolites in the bile from all animals was only 7 per cent of the alkylating metabolites excreted in the urine at the same time. The rate of bile flow was not changed by CPA.

Groups of at least three diseased animals were dosed once daily p.o. with 20, 30 and 40 mg/kg of CPA until they died or for a maximum of 14 days. Doses of CPA which proved lethal to all normal animals (30 mg/kg for 11 days) were tolerated by animals with established adjuvant arthritis (3/3 survivors after 14 days of dosing). Sublethal doses of CPA (20 mg/kg for 14 days) caused an average weight loss of 10 g in normal rats but no weight loss in arthritic animals (average weight gain, +17 g). These findings show that CPA is certainly less toxic in adjuvant-diseased rats than in their undiseased littermates.

Adjuvant disease was allowed to develop for 21 days after injections of Nocardia adjuvants, and the severity of arthritis at this stage was determined by measuring the swollen rear paws of each animal with a micrometer screw gauge. These arthritic animals were then randomized into various groups for treatment with either CPA or saline, administered p.o. Animals given sublethal doses of CPA (20 or 30 mg/kg/day) for 14 days suffered the same further increase in paw thickness (average = 2·13 mm, $n = 12$) as did the saline-dosed groups (average = 2·14 mm, $n = 8$), confirming the inability of CPA to control established arthritis at sublethal doses.

Quantitative analysis of CPA metabolites. Acrolein may be formed by the breakdown of one CPA metabolite, namely aldophosphamide.[30] We found no evidence, using a fluorimetric assay,[19] that acrolein was present in homogenates of either normal rat livers or liver tissue from adjuvant-pretreated animals after incubation with CPA.

Thin-layer chromatography (TLC) of CPA metabolites formed in normal rat liver homogenates indicated the presence of 4-oxo-CPA, carboxyphosphamide, aldophosphamide and an almost immobile compound in systems 1, 3 and 4 (phosphoramide

TABLE 7. THIN-LAYER CHROMATOGRAPHY OF CPA METABOLITES IN BILE*

Relative intensity	R_f in system 1	R_f in system 2	Tentative identifications
±	0	0·14	
±	0	0·36	(Sladek's cpd N?)
±	0	0·50	(Sladek's cpd M?)
+	0·07	0·78	Aldophosphamide
+	0	0·88	
++	0·08	0·90	Carboxyphosphamide
+	0·75	0·93	4-oxo-CPA?

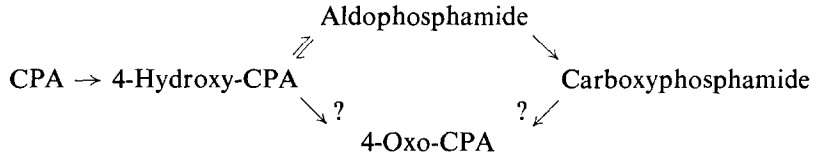
* Two-dimensional chromatography with Sladek's solvent system[23] for urinary metabolites (see Experimental).

mustard?) as the principal metabolites in addition to unchanged CPA, in general agreement with Sladek's report.[23] Incubating CPA with liver homogenates prepared from animals pretreated with adjuvants 2 or 14 days earlier gave the same qualitative spectrum of metabolites when analyzed by TLC, but with a relative increase in the intensity of the zone due to unmetabolized CPA (when compared with controls), and a decrease in intensity of the zones associated with the various metabolic products.

TLC of bile secreted in the first 4 hr after administering CPA i.v. indicated the presence of at least four NBP-reactive products (Table 7). None of these compounds was detected in normal bile, i.e. before the CPA administration. Adjuvant treatment (2 or 14 days previously) did not change the spectrum of bile metabolites qualitatively.

DISCUSSION

The hepatic metabolism of cyclophosphamide (CPA) is believed to be as follows:[10,11,23,31]



Excretion of cyclophosphamide metabolites. The presence of CPA metabolites in bile has been noted previously.[32,33] In these present experiments we observed no gross reduction in the urinary excretion of CPA metabolites when the bile flow was drained off. This suggests that the striking reduction in the blood levels of CPA metabolites in rats observed after occluding the bile duct[27] was not simply due to interrupting the following (theoretical) sequence for distribution of a hepatic metabolite: liver → bile → intestine → blood.

There was a rather considerable discrepancy between the consistent reduction in the biliary excretion of alkylating CPA metabolites and the relatively minor changes in the urinary excretion of alkylating CPA metabolites, associated with the early (i.e. pre-arthritic) hepatic response to arthritogenic adjuvants (Table 6). This points to a possible pitfall in using urinary excretion data as an index of liver metabolism of a drug, if the level of metabolites in the bile truly reflects the activity of the CPA-activating enzymes in the liver. If this is the case, then the apparently near normal level of CPA metabolites excreted in the urine by these same animals might reflect some considerable extrahepatic metabolism of CPA. Thus any experimentally induced reduction in hepatic drug metabolism (which certainly minimizes CPA toxicity *in vivo*) might be accompanied at first by little or no change in the extent of CPA metabolism or decomposition elsewhere in the animal.

The recovery of alkylating metabolites in the urine, computed as micromolar equivalents of mechlorethamine (HN2), rarely exceeded 30 per cent of the input of cyclophosphamide. Carboxyphosphamide, one of the principal urinary metabolites of CPA, gave a color yield with NBP approximately 70 per cent that of HN2. Another major urinary metabolite, 4-oxo-CPA, and CPA itself both gave color yields less than 5 per cent that of HN2. These values were also reduced by adding normal urine to these compounds before determining their respective color yields with NBP. Thus attempts to estimate recoveries of CPA metabolites excreted via the kidney, using the

nitrobenzylpyridine reagent, may be somewhat inadequate. Nevertheless, it is rather unlikely that the consistent reduction in the urinary alkylating metabolites with established adjuvant disease can be attributed to a more rapid excretion of unmetabolized CPA or increased formation of 4-oxo-CPA. (No chromatographic evidence was obtained to support the latter possibility.) Several other lines of evidence (sleeping times, metabolism *in vitro* of aminopyrine, toxicity studies, quantitation of biliary metabolites) confirm the effect of various adjuvants in depressing hepatic drug metabolism in rats.

Effect of adjuvants on drug metabolism. These studies considerably amplify earlier observations[34–36] that adjuvants used to induce chronic arthritis in rats may profoundly depress liver microsomal metabolism. Extended sleeping times were noted with three barbiturates administered 25–50 days after injection of *M. butyricum* in "paraffin oil".[34] Morton and Chatfield[35] showed that the capacity of liver microsomes to *N*-demethylate propoxyphene and form a glucuronide conjugate from 4-acetylaminophenol (paracetamol) decreased progressively from the fourth day after injecting a fine suspension of *M. tuberculosis* in liquid paraffin. These authors also reported that the toxicity of phenobarbital (300 mg/kg) was considerably potentiated 15 days after adjuvant inoculation, and the percentage of unconjugated paracetamol excreted in the urine increased from approximately 25 per cent to greater than 50 per cent.

The first stage in the bioactivation of cyclophosphamide is believed to be the microsomal hydroxylation of the C-4 methylene group.[10,31] Termination of the hypnotic activity of hexobarbital in rats is primarily by microsomal hydroxylation,[37] that of trichloroethanol by *O*-glucuronidation.[38] In the experiments reported here, changes in liver microsomal activity were routinely monitored by studying the *N*-demethylation of aminopyrine or *N*,*N*-dimethylaniline or of both. From these and other studies,[35,36] it is evident that the adjuvant-induced injury to the drug-metabolizing system is: (1) a fairly general phenomenon, depressing the metabolism of at least ten drugs involving three or more pathways of metabolism; and (2) not an artifact *in vitro*, since drug responses *in vivo* to active drugs (hypnotics) are potentiated, but the drug response to and metabolism of two latent drugs, cyclophosphamide and isophosphamide, are consistently impaired (compared to the responses of and metabolism by the normal control animals). Furthermore, this is not a unique response by a very susceptible strain of rat; at least one adjuvant-resistant strain (Buffalo rat), which develops only minimal arthritis in response to several powerful arthritogenic adjuvants, still displayed fairly gross changes in hepatic drug-metabolizing capacity. Nor is it a unique response to a specific adjuvant, for the composition of the latter could be varied both with respect to the nature of the arthritogenic microorganism and the arthritogenic oily phase. So far we have examined over 30 types of adjuvant preparations but discovered only one non-arthritogenic adjuvant which consistently impairs hepatic drug metabolism to a significant degree (>30 per cent) after 14 days. Other non-arthritogenic adjuvants caused only a transient depression in hepatic drug metabolism. Yet other forms of immunologically induced stress and injury in rats, such as induction of a graft vs host response or allergic encephalomyelitis, had little or no effect on cyclophosphamide, hexobarbital and aminopyrine metabolism, indicating that this arthritogen-related hepatic dysfunction is probably not just a manifestation of some general, nonspecific effect of disease on metabolism.

The hepatic dysfunction following an adjuvant inoculation is evidently complex. One component, the loss of drug-metabolizing activity, was reversed in our animals by administering phenobarbital; this agrees with one report,[36] but not with another.[35] Another change in hepatic function, namely albumin synthesis, which is impaired in the arthritic animal,* was probably not affected by this phenobarbital treatment, since the depressed albumin levels in the arthritic animals were not altered by phenobarbital (confirming another report[36]).

These findings may have important implications for the experimental evaluation of potential anti-arthritic drugs using adjuvant-induced arthritis in rats. Certain drugs, especially when tested therapeutically against the established disease (when liver impairment is maximal), may be either prematurely discarded from the drug-screening program if they show toxicity at this stage or therapeutically overrated, by virtue of being "under-metabolized". Conversely, drugs such as cyclophosphamide, which need to be metabolized *in vivo* to generate pharmaco-active moieties, might be considerably undervalued if tested in adjuvant-diseased animals. We know of only one report which clearly ascribes anti-arthritic activity to cyclophosphamide (2·5 mg/kg/day) used therapeutically to treat established adjuvant arthritis, following injections of an undefined "Freund's adjuvant".[39]

Some uncertainties. These comments should be tempered by at least three observations relating to the properties of CPA *in vivo* in rats:

(1) The discrepancy between the low output of biliary metabolites of CPA by animals with pre-arthritic adjuvant disease (when compared with normal animals with equal rates of bile flow), and the near normal excretion of urinary metabolites by these same animals. This might indicate that considerable extrahepatic metabolism (or decomposition) of CPA occurs in the rats, which is independent of any hepatic dysfunction associated with adjuvants.

(2) Sladek's[27,40] findings that neither drugs which inhibit cyclophosphamide metabolism by liver microsomes nor the well known sex difference in the rat (male > female) concerning hepatic microsomal enzyme activity, affect the efficacy of cyclophosphamide as an anti-tumor agent when administered in a single dose. He argues that this was because, given a set of conditions which he carefully defines,[40] the probability that a given cell will be exposed to the lethal action of the bioeffective form of the drug remains unchanged.

(3) The somewhat parallel findings that the (anti-proliferative) effect of CPA *in vivo* in inhibiting the local graft vs host reaction in F_1 hybrid rats was not diminished in female rats or in adjuvant-treated males when compared with normal male rats;[41] again emphasizing the fact that factors (e.g. sex, adjuvants) altering rates of hepatic metabolism may still not alter appreciably the cytostatic activity of the hepatic metabolites (if these are really the bioeffective agents in the two assay systems used here[40,41]).

Some of these paradoxical findings can probably be fully interpreted only when we know the answers to some of the following questions: How extensively may extrahepatic transformation of CPA contribute to drug efficacy *in vivo*? What are the active immunosuppressant (anti-arthritic) metabolites of cyclophosphamide, which may not perhaps be those principally responsible for the anti-tumor activity or other forms of

* M. J. Billingham, personal communication.

immunosuppressant (e.g. anti-proliferative) activity? Do measurements of alkylating activity (i.e. NBP-reacting chromogens) truly reflect the formation and availability of immunoregulatory metabolites? How analogous are the sex-linked or chemically induced alterations in activity of liver microsomal enzymes, studied by Sladek[27,40] and others, to the hepatic dysfunction induced by arthritogenic adjuvants? Does this hepatic dysfunction actually reflect a more generalized liver response to a "toxohormone" (as suggested by Dr. Sladek in a private conversation), in this case formed either locally in draining lymph nodes or at the site of injection of the arthritogenic adjuvant?

Finally, in reviewing our own observations, we are not convinced that the lesions in drug-metabolizing capacity of the liver can account for the failure of cyclophosphamide to moderate the signs of arthritis in established adjuvant disease. Other mustards which do not require bioactivation (e.g. mechlorethamine, melphalan) seem to be equally ineffective in treating the signs of arthritis at subtoxic doses.*

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