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I. Drug Sensitivity of Rat Adjuvant Arthritis, Induced with 'Adjuvants' Containing no Mineral Oil Components (38168)

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Present evaluation of antiinflammatory agents centers on ameliorating the arthritis in rats induced with a Freunds adjuvant (FA) constituted with *Mycobact. tuberculosis* or *M. butyricum* in mineral oil (1–4).

We have found that by substituting certain metabolizable lipids for the mineral oil component of FA, powerful arthritogenic adjuvants are still obtained (5). However, the arthritis engendered by these alternative 'adjuvants' is more susceptible to treatment with some nonsteroid antiinflammatory drugs, which are active in man but do not assay too well against the FA-induced arthritis in rats.

By using such modified "Freund's adjuvants" to induce arthritis in rats, it may be feasible to detect both weaker novel drugs and less toxic doses of the powerful drugs with the adjuvant arthritis assay.

Methods. Outbred male HLA Wistar rats (180–220 g) from Hilltop Labs, Chatsworth, California, were injected on day 0 in the plantar region of one rear paw with 0.05 ml of a finely ground dispersion of delipidated, heat-killed *Mycobact. tuberculosis* (10 mg/ml) in the appropriate oily vehicle (5). The *M. tuberculosis* (human) was a mixture of strains, obtained from the Central Veterinary Lab., Weybridge, England, extracted twice with ethanol–ether (50:50) to remove lipids. Sources of the oils were: mineral oil (Protol, Witco); hexadecane (Aldrich); squalene, squalane and butyl stearate (Eastman Kodak); butyl palmitate (Calbiochem). Squalene was freshly purified (6) just before preparing this particular adjuvant. Mineral oil was sterilized by filtration, other oils by distillation.

Animals were housed in groups of 4 on sawdust and allowed free access to food and water. Body weights and rear paw measurements were determined periodically. Drugs suspended in 1% gum acacia were given orally from day −1 to day 13. Animals were sacrificed on day 14 and blood plasma was assayed for inflammation units (P.I.U.), albumin (7) and total thiol content. Thiols were determined colorimetrically using DTNB (8).

Results. Comparison of the data in Table IA with that in the footnote (for normal animals) shows that the arthritis induced in Wistar rats on varying the oil component of Freund's adjuvant may be as severe (or more so) than the disease induced with a conventional Freund's adjuvant. The weight loss, increased paw size, elevated plasma inflammation units (P.I.U.), and decreased albumin and thiol content of the blood were similar, regardless of the composition of these *arthritogenic* adjuvants. Each of the adjuvants induced a truly systemic disease with the tails, forepaws, ears and noses being also affected. The capacity of the liver to metabolize drugs (5, 7) was abnormally

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TABLE I. Effect of 3 Acidic Antiinflammatory Drugs on the Forms of Adjuvant Arthritis Produced in Rats by Freund's Adjuvant (FA) and Five Other Arthritogenic Adjuvants.

All values are $M \pm$ SEM

Arthritogenic vehicle*	Drug given	Av wt gain** days 1–14 (g)	Wt gain (% control)	Rear foot thickness increase (mm) **	Plasma levels** P.I.U.	Alb.	Thiol	A score***
A. Acetylsalicyclic acid 200 mg/kg/day × 14								
Mineral oil (FA)	–	51 ± 8	44	3.89 ± 0.38	510 ± 61	19 ± 1	10 ± 1	15
	+	58 ± 7	50	3.30 ± 0.53	370 ± 65	22 ± 3	11 ± 1	14
Hexadecane	–	28 ± 7	24	4.06 ± 0.47	358 ± 43	15 ± 1	12 ± 2	18
	+	18 ± 4	15	3.79 ± 0.51	329 ± 42	14 ± 1	12 ± 2	18
Squalene, $C_{30}H_{50}$	–	13 ± 5	12	3.44 ± 0.40	344 ± 67	20 ± 2	22 ± 3	15
	+	27 ± 28	23	3.73 ± 0.78	300 ± 185	19 ± 7	19 ± 9	13
Squalane, $C_{30}H_{62}$	–	16 ± 5	14	4.82 ± 0.41	391 ± 65	14 ± 1	9 ± 1	19
	+	51 ± 12	44	3.08 ± 0.50	380 ± 29	23 ± 4	16 ± 1	13
Butyl Stearate	–	32 ± 8	28	4.36 ± 0.47	462 ± 29	16 ± 1	8 ± 1	18
	+	54 ± 3	47	2.78 ± 0.56	318 ± 153	27 ± 2	12 ± 1	11
Butyl Palmitate	–	19 ± 6	16	5.02 ± 0.72	337 ± 44	14 ± 1	15 ± 3	18
	+	41 ± 7	35	2.53 ± 0.60	198 ± 84	23 ± 3	22 ± 8	9
B. Phenylbutazone 80 mg/kg/day × 14								
Mineral Oil	–	51 ± 8	44	3.89 ± 0.38	510 ± 61	19 ± 1	10 ± 1	15
	+	59 ± 13	51	2.73 ± 0.25	534 ± 94	24 ± 3	12 ± 1	13
Hexadecane	–	28 ± 7	24	4.06 ± 0.47	358 ± 48	15 ± 1	12 ± 2	18
	+	31 ± 6	27	2.13 ± 0.38	455 ± 46	18 ± 1	8 ± 1	15

Squalene	–	13 ± 5	12	3.44 ± 0.40	344 ± 67	20 ± 2	22 ± 3	15
	+	22 ± 8	19	1.34 ± 0.28	211 ± 95	20 ± 2	19 ± 3	11
Squalane	–	16 ± 5	14	4.82 ± 0.41	391 ± 65	14 ± 1	9 ± 1	19
	+	61 ± 10	53	1.69 ± 0.46	196 ± 42	25 ± 2	24 ± 5	7
Butyl Stearate	–	32 ± 8	28	4.36 ± 0.47	462 ± 29	16 ± 1	8 ± 1	18
	+	66 ± 8	57	2.05 ± 0.67	268 ± 28	23 ± 3	10 ± 1	10
Butyl Palmitate	–	19 ± 6	16	5.02 ± 0.72	337 ± 44	14 ± 1	15 ± 3	18
	+	45 ± 4	39	1.70 ± 0.46	81 ± 38	22 ± 2	15 ± 2	6
C. Flufenamic acid 20 mg/kg/day 114								
Mineral Oil	–	51 ± 8	44	3.89 ± 0.38	510 ± 61	19 ± 1	10 ± 1	15
	+	26 ± 14	22	2.14 ± 0.16	500 ± 67	23 ± 4	15 ± 2	13
Hexadecane	–	28 ± 7	24	4.06 ± 0.47	358 ± 48	15 ± 1	12 ± 2	18
	+	48 ± 13	42	2.06 ± 0.29	352 ± 21	18 ± 2	8 ± 1	14
Squalene	–	13 ± 5	12	3.44 ± 0.40	344 ± 67	20 ± 2	22 ± 3	15
	+	40 ± 15	34	1.67 ± 0.38	241 ± 94	27 ± 3	16 ± 5	9
Squalane	–	16 ± 5	14	4.82 ± 0.41	391 ± 65	14 ± 1	9 ± 1	19
	+	62 ± 8	53	1.80 ± 0.41	322 ± 9	22 ± 2	11 ± 2	11
Butyl Stearate	–	32 ± 8	28	4.36 ± 0.47	462 ± 29	16 ± 1	8 ± 1	18
	+	77 ± 12	66	2.06 ± 0.49	302 ± 60	26 ± 5	11 ± 1	11
Butyl Palmitate	–	19 ± 6	16	5.02 ± 0.72	337 ± 44	14 ± 1	15 ± 3	18
	+	56 ± 15	48	2.43 ± 0.52	306 ± 28	22 ± 2	11 ± 1	13

* Admixed with *M. tuberculosis* (human) to initiate adjuvant disease.

** Normal values for rats ($N = 21$) without adjuvant were: wt gain = 116 ± 5 g; increased paw thickness = 0.34 ± 0.06 mm: P.I.U. = 15 ± 8; Alb. = 36 ± 1 mg/ml; Thiols 27 ± 2 colorimeters units.

*** Arthritic Score computed by averaging increase of rear paws (0–4) incidence of tail, nose, ear, forepaw involvement (0–3) reduction in body wt (0–4) elevated P.I.U. (0–4) depressed albumin level (0–4) and depressed thiol groups (0–3). Maximum score = 22 per animal.

low following administration of each type of adjuvant.

Relatively low doses of aspirin (A.S.A.), phenylbutazone and flufenamic acid ameliorated the symptoms of the arthritis induced with some of these adjuvants but not others. Thus, 200 mg/kg A.S.A. only marginally affected the arthritis induced by the hexadecane, mineral oil or squalene constituted adjuvants. Arthritis induced with a squalane adjuvant was more sensitive to A.S.A. but not as sensitive as the arthritis which develops after injecting either of the 2 butyl ester adjuvants, as can be seen in the reduction of total arthritic score. Arthritis induced with adjuvants containing squalane, or butyl stearate or butyl palmitate was quite sensitive to 80 mg/kg/day phenylbutazone (Table IB). Flufenamic acid 20 mg/kg/day ameliorated the arthritis induced with adjuvants containing any of these four metabolizable lipids, while hardly affecting the arthritis induced by hexadecane or mineral oil adjuvants (Table IC).

Discussion. In our laboratory, repeated assays over a period of 5 years failed to consistently disclose the antiarthritic activity in rats of flufenamic acid and aspirin, despite testing numerous samples of different origin, when conventional mineral oil adjuvants were employed to initiate arthritis in 3 outbred strains of Wistar rat. This is disturbing as these 2 drugs are undoubtedly effective against several forms of arthritis in man.

As Table I shows, simplifying the constitutions of the mineral oil adjuvant by using hexadecane alone as the paraffin component still yielded an aspirin and flufenamate-resistant adjuvant disease. A more drug-responsive form of inflammatory disease was noticed when certain other arthritogenic oily vehicles were used *in lieu* of mineral oil to constitute the adjuvant. These particular oily vehicles had the common property of being more biolabile than the bulk of the mineral oil components or hexadecane. They included certain highly branched aliphatic hydrocarbons (squalane), unsaturated materials (squalene, butyl oleate) or esters containing more than 16 C atoms (5). Though probably possessing shorter biological half lives than hexadecane, they nevertheless function as powerful co-arthritogens when admixed with the appropriate arthritogenic bacteria. The arthritis they induce is superficially just as severe as that induced by mineral oil (or n. alkane) adjuvants. For practical purposes, they offer the advantage that weaker drugs such as aspirin may be more confidently detected when administered prophylactically by their ameliorative action in diminishing the signs of *chronic* inflammation that develop after administering *Mycobacteria* dispersed in one of these 'metabolizable' oils.

Many of the drugs which efficiently prevent full development of the arthritis induced with mineral oil/hexadecane adjuvants may be too powerful to be very useful in man as antiinflammatory agents without the closest medical supervision. The repeated discovery of ulcerogens for man, by testing drugs against the arthritis induced with conventional adjuvants in rats, illustrates the moral of this argument.

For so long as there is still a need for safer aspirin-like drugs to treat arthritis in man, it would seem reasonable to (i) experiment further with alternate (metabolizable) adjuvants for arthritis induction in rats, and (ii) recheck some of those compounds already eliminated from consideration because they failed to drastically offset the rat arthritis induced with mineral oil adjuvants.

Addendum. Arthritogenic adjuvants based on squalane (perhydrosqualene) and butyl palmitate will soon be commercially available from Calbiochem, San Diego, Ca., under the designations, Perrigen (W series).

Summary. 1. Adjuvant arthritis induced with mineral oil adjuvants was resistant to acetylsalicylic acid and flufenamic acid when given prophylactically, and only moderately sensitive to phenylbutazone. 2. Adjuvant arthritis induced in the same rat strain with 4 metabolizable adjuvants (*M. tuberculosis* with squalene or squalane or butyl palmitate or butyl stearate) was considerably more sensitive to acetylsalicylic acid, phenylbutazone and flufenamic acid. 3. These alternative adjuvants offer promise for screening weaker, aspirin-like (antiarthritic)

drugs in rats, to derive less toxic (ulcerogenic, leukopenic) drugs for use in man.

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