SHORT COMMUNICATIONS

Fatty Acid Nature and Adjuvant Activity of Wax D from *Mycobacterium tuberculosis*

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Wax D, a chloroform-soluble and boiling-acetone-insoluble fraction extracted from various human strains of mycobacteria, showed characteristic biological activities such as Freund-type adjuvant activity (Raffel, 1950; White, Jollès, Samour & Lederer, 1964) and the induction of adjuvant arthritis in rats (Pearson, 1959; Pearson, Wood, McDaniel & Daft, 1963). Wax D from human strains was found to be peptidoglycolipid consisting of unsaturated fatty acid (mycolic acid) esters of mucopolysaccharide containing a peptide moiety of alanine, glutamic acid and *meso*- α - α' -diaminopimelic acid (Asselineau, 1951; Asselineau, Buc, Jollès & Lederer, 1958). Previous work from this laboratory has shown the importance of fatty acid content for the adjuvant activity of the wax D of a human strain, Peurois (Hiu, Amiel & Jollès, 1969). This communication reports an increase in adjuvant activity after hydrogenation of the fatty acids forming the lipid portion of the wax D from the human strains Peurois and Canetti.

METHODS

The wax D used in this investigation was the purified wax D, wax D_{p35}(C) and wax D_{p35}(P) (Jollès, Samour & Lederer, 1962), isolated from the human mycobacterial strains Peurois and Canetti originating from the Pasteur Institute, Paris. Wax D_{p35}(P) and wax D_{p35}(C) were prepared by a technique described earlier (Jollès *et al.* 1962). Saturation of the fatty acids by catalytic hydrogenation was achieved in an apparatus like that described by Brian & Gardner (1968). Samples of 200 mg. wax D_{p35} were taken up in 20 ml. anhydrous ether into 100 ml. Quick-Fit flasks and 5 mg. palladium catalyst (10% palladium oxide on charcoal) added. The flask was connected to a hydrogen source and the contents kept stirred with a magnetic stirrer, after flushing with hydrogen. When hydrogen uptake ceased (6h.) the catalyst was filtered off and the hydrogenated sample dried *in vacuo*. The hydrogenated sample was then ultracentrifuged in ether at 40,000 *g* (M.S.E. rotor 10 × 10) for 5 min. and the supernatant chromatographed on a silicic acid column eluted with chloroform. The substance was obtained in pure form as determined by thin-layer chromatography on silica gel GF-254 with chloroform-methanol (95:5, v/v) as solvent. Iodine values (Vilkas-Tenenbaum, 1960) measured for the hydrogenated wax D_{p35} derivatives, HD_{p35}(P) and HD_{p35}(C), showed that saturation was complete.

Female albino guinea pigs of 400 to 500 g. were sensitized by injection into each hind foot-pad of 0.1 ml. of a water-in-oil emulsion consisting of one part of paraffin oil containing the wax adjuvant, two parts of Tween 80 and seven parts of saline containing the protein antigen, crystalline egg white albumin (CEWA). On day 21 after sensitization the animals were skin-tested by intradermal injection of various amounts of CEWA in 0.1 ml. of saline into the clipped flank of the guinea pigs; the reactions were read 24 and 48 h. later.

RESULTS

When 0.1 mg. of antigen was used in skin tests positive reactions were observed in all animals sensitized with antigen emulsion whether containing wax D_{p35}(P) or wax D_{p35}(C) or their hydrogenated derivatives, HD_{p35}(P) or HD_{p35}(C) (Table 1). With test antigen doses of

Table 1. *Mean diameter of redness in guinea-pig skin at sites of injection of various amounts of test antigen CEWA in saline*

No. of animals in group	Sensitization		Delayed skin reactions		
	Sensitizing antigen (1 mg./animal)	Adjuvant incorporated in antigen emulsion (0.5 mg./animal)	Test antigen (mg./0.1 ml.)		
			0.1 mg. (mm.)	3 µg. (mm.)	0.2 µg. (mm.)
6	CEWA*	D _{p35} (C)	14 (N)†	10	0
			16 (N)	4	0
			7	0	0
			12	12	0
			12	8	0
			10	4	0
6	CEWA	HD _{p35} (C)	20 (N)	14	4
			20 (N)	16	9
			20 (N)	12	14
			20 (N)	14	10
			20 (N)	12	10
			20 (N)	12	6
6	CEWA	D _{p35} (P)	14	0	0
			15 (N)	4	0
			6	11	0
			12	5	0
			16	7	0
			14	0	0
6	CEWA	HD _{p35} (P)	20	10	10
			20 (N)	9	8
			20 (N)	11	7
			20 (N)	8	4
			20 (N)	10	4
			20 (N)	12	10
6	CEWA	None	0	0	0
			0	0	0
			0	0	0
			0	0	0
			0	0	0
			0	0	0

* Crystalline egg white albumin.

† Diameter of redness (mm.) at 48 h.; (N) = central necrosis.

3 µg., skin reactions were moderately positive in five out of six and four out of six animals in the groups which had received wax D_{p35}(C) and wax D_{p35}(P) respectively as adjuvant, and showed necrosis only in three cases out of 12. However, all 12 animals in the groups receiving the hydrogenated derivatives produced strong reactions – 11 out of 12 with central necrosis at the injection site. When the dose of test antigen was reduced to 0.2 µg. skin reactions were observed only in the groups which had received the hydrogenated wax derivatives.

DISCUSSION

The catalytic hydrogenation of the wax D_{p35} resulted in a substance in which all fatty acids forming the lipid moiety were fully saturated and which possessed significantly increased adjuvant activity. Since the principal chemical difference between wax D_{p35} and its hydrogenated derivative lies only in the presence of unsaturated fatty acids in the wax D_{p35}, it is suggested that the increase in adjuvant activity results from fatty acid saturation possibly by giving a more stable compound with a longer lasting immunostimulant activity.

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