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Adjuvant Activity in Development of a Delayed Type of Hypersensitivity of a Lipid Fraction Isolated from Enzymically Digested Cell Walls of *Corynebacterium diphtheriae*

There is much similarity in the chemical composition of the cell walls of *Corynebacterium diphtheriae* and of BCG. Both have the same component amino acids and sugars and are characterized by their high content of lipids, hexoses and pentoses (Cummins and Harris, 1958; Kotani *et al.*, 1959b; Mori *et al.*, 1960; Cummins, 1962).

A recent study carried out in this laboratory showed that an insoluble residue ('bound wax D'), isolated from 'delipidated' BCG cell walls by digestion with egg white lysozyme and *Flavobacterium* L_{11} enzyme, is virtually chemically identical with a wax D fraction separated from a human type tubercle bacilli and exhibits a marked adjuvant effect in the development of a delayed type of hypersensitivity and in the production of circulating antibody when injected into guinea pigs with egg white albumin or egg white lysozyme (Kotani *et al.*, 1963). It appeared of interest, in view of these findings, to see if a similar compound with adjuvant activity was present in the cell walls of *C. diphtheriae*.

A specimen of *C. diphtheriae* cell walls, isolated as previously described (Kotani *et al.*, 1959a) was incubated, with stirring at 37°C, with a *Streptomyces* L_3 enzyme (0.2 units/mg cell walls) in the presence of 0.01 M phosphate buffer, pH 8.0, containing a few drops of chloroform. Details of the method of preparation of the L_3 enzyme and its properties have been given by Mori *et al.* (1960, 1962). During a 96 hour incubation period, about 60 per cent of the cell wall materials became soluble, and there was about 65 per cent reduction in the optical density of the reaction mixture, a marked increase in free amino groups and a slight uncovering of reducing groups in the soluble products (unpublished observation). The insoluble residue was isolated as a sediment by centrifuging the reaction mixture at $12,000 \times g$ for 60 minutes and was washed with distilled water. The completeness of the digestion was shown by the fact that the washed sediment was not affected by further addition of the L_3 enzyme. The insoluble residue, after being freeze-dried, was exhaustively extracted with ethanol-ther (1:1) and then with chloroform-methanol-water (100:10:1, v/v). The latter extract was dried by evaporation and re-extracted successively with ether and the above chloroform-methanol-water mixture. Two fractions, an ether-soluble Fraction A and an ether-insoluble Fraction B, were thus obtained.

The adjuvant activity of each of these fractions was studied by sensitizing guinea pigs with crystalline egg white albumin (Twice crystallized, Sigma Chemical Co., U.S.A.), giving 5 mg per animal, alone or with either Fraction

A or B (0.4 mg each per animal). Development of a delayed type hypersensitivity against the albumin was examined by corneal and intracutaneous tests three weeks after the sensitization. The methods used for sensitization and corneal tests were essentially as described for the study for the adjuvant activity of the 'bound wax D' of BCG cell walls (Kotani *et al.*, 1963). As shown in the accompanying table, animals sensitized with the antigen containing Fraction A exhibited an intense reaction of the delayed type towards the albumin in both the corneal and intracutaneous tests, while the animals receiving albumin alone or albumin with Fraction B gave only negative or doubtful positive reactions.

Although no investigation has yet been made of the chemical nature of Fraction A except its solubility in the chloroform-methanol-water mixture and ether,

Table I. Adjuvant Activity of Fractions Isolated from the Insoluble Residue of the Enzymically Digested Cell Walls of *Corynebacterium diphtheriae*

Sensitizing antigen	Guinea pig No.	Corneal reaction*	Intracutaneous reaction**
Egg white albumin (5 mg) alone	1	0	$\frac{-}{2\times2\ (16\times12)}$
	2	0	$\frac{-}{(11\times9)}$
Egg white albumin (5 mg) and Fraction B (0.4 mg)	1	0	$\frac{-}{12\times9}$
	2	0	$\frac{-}{2\times2}$
	3	1	$\frac{\pm}{19\times13}$
Egg white albumin (5 mg) and Fraction A (0.4 mg)	1	3C	$\frac{\#\ CN}{14\times12\ (41\times28)}$
	2	3C	$\frac{\#\ CN}{24\times20}$
	3	2C	$\frac{\#\ CN}{14\times11}$
	4	3C	$\frac{\#\ CN}{19\times14}$
	5	1C	$\frac{\pm}{14\times13}$

Sensitizing antigens were given to guinea pigs by intracutaneous injection into the foot pad of 0.2 ml of a mixture of Bayol F, Arlacel A and water (3 : 1 : 1, v/v) containing the indicated dose of the test specimen. The concentrations of egg white albumin solutions used for the corneal and intracutaneous tests were 20 and 5 mg/ml, respectively.

* Extent of the reaction (arbitrarily graded). C: chemosis.

** $\frac{\text{Intensity of induration}}{\text{Size of redness (size of weak redness) mm}\times\text{mm}}$ CN: central necrosis.

since the yield of the fraction was only one to two per cent of the original cell wall preparations, a comparative study of the 'bound wax D' of BCG cell walls and the Fraction A isolated from the enzymically digested *C. diphtheriae* cell walls would provide a useful clue for elucidation of the chemical structure responsible for manifestation of the adjuvant activity.

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Shozo Kotani
Toshiro Matsubara
Toshiyuki Kitaura
Yuji Mori
Masakazu Chimori
Hideo Kishida

Department of Bacteriology
Nara Medical College
Kashiwara, Nara

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