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Preliminary Report

Influence of Extraction of 'Bound Wax D' of BCG with Trichloroacetic Acid on Its Adjuvant Activity on Development of a Delayed Type of Hypersensitivity

In studies on the adjuvant activity of wax D fractions from various strains of *Mycobacterium*, White and cowerkers demonstrated that there was a striking difference between the strong activity of the wax D of human strains and inactivity of wax D from bovine, avian and saprophytic strains in both the production of circulating antibody and the development of a delayed type of hypersensitivity towards egg white albumin. It was suggested that the observed difference was due to the presence of peptides consisting of alanine, glutamic acid and α, ε-diaminopimelic acid in the former and their absence in the latter (White *et al.*, 1958). A recent study in this laboratory (Kotani *et al.*, 1963) offered further evidence in support of this suggestion, by showing that the 'bound wax D' isolated from 'delipidated' BCG cell walls digested with egg white lysozyme and the *Flavobacterium* L_{11} enzyme (Kato *et al.*, 1962), unlike the wax D separated from chloroform extracts of BCG whole cells by the method of Asselineau (1951), exhibits a marked adjuvant activity in sensitization of guinea pigs with egg white albumin and egg white lysozyme and contains peptides consisting of cell wall amino acids as one of major components. These findings seem to justify the conclusion that these peptides are an essential component of the wax D for manifestation of the adjuvant effect.

So far as the authors know, there has been no investigation as to whether another component of the wax D, namely carbonhydrates consisting of hexose, pentose and hexosamine, takes a part in the manifestation of the adjuvant activity. Experiments described here were undertaken to study this point, on the basis of the observation that a considerable part of the hexose and pentose present in 'delipidated' BCG cell walls was removed by repeated extraction with cold 5 per cent trichloroacetic acid (unpublished data).

A 100 mg specimen of a 'bound wax D' fraction isolated from 'delipidated' BCG cell walls digested with lysozyme and the L_{11} enzyme (specimen No. 2 listed in Table 3 in the previous paper, Kotani *et al*, 1963) was extracted six times with 10 ml volumes of 5 per cent trichloroacetic acid in the cold for 24 hours. A third of the extracted specimen was washed thoroughly with distilled water and then lyophilized (cold TCA-extracted 'bound wax D'). The remaining two thirds was further extracted with 6.6 ml of 10 per cent trichloroacetic acid at 60°C for 12 hours. After thorough washing with distilled water, the 'bound wax D' fraction extracted with hot trichloroacetic acid was lyophilized.

Table 1 shows the chemical analyses of the 'bound wax D' fractions before and after trichloroacetic acid treatment. Hexoses, pentoses, hexosamines and ninhydrin-positive substances were determined by the anthrone method (Aschwell,

Table 1. Changes in Some Chemical Properties of the 'Bound Wax D' of BCG by Extraction with Cold and Hot Trichloroacetic Acid

Test specimen	Hexose (as galactose) %	Pentose (as arabinose) %	Hexosamine (as glucosamine-HCl) %	Ninhydrin-positive substances (as leucine) %
Non-treated 'bound wax D'	20.0	16.0	2.6	21.3
Cold TCA-extracted 'bound wax D'	9.7	15.1	2.9	16.0
Hot TCA-extracted 'bound wax D'	3.6	5.7	1.7	13.9

Hexosamines and ninhydrin-positive substances were determined on specimens hydrolyzed with 4 N HCl for 8 hours and with 6 N HCl for 16 hours at 100°C, respectively. Hexose and pentose determinations were performed on specimens hydrolyzed with 2 N H_2SO_4 and 2 N HCl at 100°C for various periods and the maximum values (on the specimens hydrolyzed for 8 and 2 hours, respectively) are listed in the table.

1957), the orcin-HCl method of Bial, as modified by Dische (Aschwell, 1957), the method of Neuhaus and Letzring (1957) and the method of Moore and Steine (1948), respectively. As will be seen from this table, the contents of hexose and pentose in the 'bound wax D' fraction were reduced by cold and hot trichloroacetic acid treatments to nearly one sixth and one third, respectively, of those of the starting material. The decrease in the contents of hexosamine and ninhydrin-positive substances was not so marked.

The adjuvant activity of the cold and hot trichloroacetic acid-treated 'bound wax D' fractions on the development of a delayed type of hypersensitivity in guinea pigs was compared with that of the non-treated 'bound wax D' by corneal and intracutaneous tests, using crystalline egg white albumin (twice crystallized, Sigma Chemical Co., U.S.A.) as test antigen. The methods of sensitization and procedures used for corneal and intracutaneous tests were as described in the previous report (Kotani *et al.*, 1963). Table 2, summarizing the results, clearly shows that whereas guinea pigs sensitized with egg white albumin alone (group 1) exhibit no positvie corneal and intracutaneous reactions, the animals injected with the antigen plus the 'bound wax D' fractions, non-treated and extracted with trichloroacetic acid (groups 2, 3 and 4), respond with a definite positive reaction in both corneal and intracutaneous tests. No appreciable difference was recognized between the intensities of the reactions exhibited by the guinea pigs receiving the antigens mixed with the non-treated, cold TCA-extracted and hot TCA-extracted 'bound wax D' fractions.

The results indicate that the greater part of the hexose and pentose in the 'bound wax D' of BCG can be removed without a significant loss of adjuvant activity under the present experimental conditions, suggesting that the carbohydrate moiety extractable by cold and hot trichloroacetic acid may not be directly

Table 2. Influence of Trichloroacetic Acid Extraction on the Adjuvant Activity of the 'Bound Wax D' of BCG on the Development of a Delayed Type of Hypersensitivity

No. of animal groups	Sensitizing antigen (mg/animal)		Corneal reaction	Intracutaneous reaction *
1	Crystalline egg white albumin (5 mg) plus	None	0	— / 4×3
			0	
			0	
2		Non-treated 'bound wax D' (0.4 mg)	3C**	++ / 17×13
			3C	++ / 15×15 (19×23)
			3C	++ / 18×17
			3C	
3		Cold TCA-extracted 'bound wax D' (0.4 mg)	3C	
			3C	++ / 12×9 (24×17)
			3C	++ / 18×15
			3C	
4		Hot TCA-extracted 'bound wax D' (0.4 mg)	3C	++ / 19×17
			3C	++ / 14×13
			3C	

Corneal and intracutaneous tests were performed two and three weeks, respectively after the sensitizing injection. A 20 mg/ml solution of egg white albumin was injected into the cornea instilled with a drop of Xylocaine solution (Fujisawa Pharmaceutical Co., Osaka, containing lidocaine hydrochloride at a concentration of 40 mg/ml) to give a disc of opacity of about 2 mm in diameter. In the intracutaneous test, one tenth ml of 5 mg/ml egg white albumin solution was used.

*Readings after 48 hours are listed in the table

$$\frac{\text{Intensity of induration}}{\text{Size of redness (size of weak redness) mm} \times \text{mm}}$$

**C: chemosis

involved in the manifestation of the adjuvant activity of the 'bound wax D' fraction. However, the role of the residual carbohydrate components in the adjuvant activity is still being investigated.

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