CONTRIBUTION TO THE STUDY OF THE STRUCTURE OF ADJUVANT ACTIVE WAXES D FROM MYCOBACTERIA: ISOLATION OF A PEPTIDOGLYCAN

D.MIGLIORE and P.JOLLES

Laboratory of Biochemistry, Faculty of Sciences, Paris, France

Received 28 October 1968

All mycobacteria contain a chloroform extractable, ether soluble, acetone insoluble glycolipid fraction referred to in previous communications as wax D [1,2]. Wax D fractions of human strains of *Mycobacterium tuberculosis* have important biological properties [3,4]: Freund's adjuvant effect [5]; induction of adjuvant arthritis [6], etc. Wax D fractions from several bovine strains of *M. tuberculosis* were found inactive. In an effort to purify this material, Jollès et al. [2] carried out a fractional ultracentrifugation in ether, yielding a non-sedimentable, "light" fraction, which was biologically inactive (wax D_S), and several "heavy" active fractions, sedimentable after different time intervals (waxes D_p). The principal chemical difference noted between active and inactive wax D fractions was the presence of a peptide moiety in the former and its absence in the latter. This peptide contains *meso*-α,α′-diaminopimelic acid (DAP), D-glutamic acid (Glu) and D- and L-alanine (Ala), in approximate molar ratios 2:2:3 (1D- and 2L-Ala). Jollès et al. [7] established later that only the "active" fractions contained amino sugars, mainly glucosamine and muramic acid. The same authors submitted the fractions obtained after ultracentrifugation to an acetylation followed by further purifications [8]. The arthritis-inducing ability of an "active" wax D was lost after acetylation. Furthermore, rats injected with an acetylated wax D were protected against the arthritis-inducing ability of unaltered wax D [9].

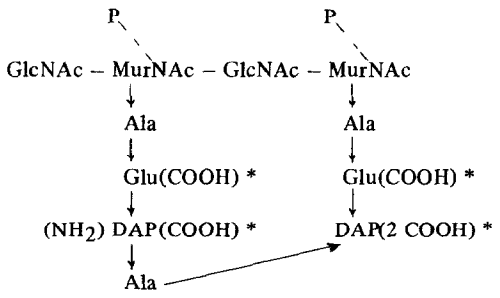
The acetylation of wax D was achieved in view to purify wax D as suggested by Tanaka [10]. However, we observed a deep modification of wax D during the treatment, when we worked with the material obtained from different human strains of *M. tuberculosis* (Brévannes, Canetti, $H_{37}RvSr$), as waxes Dp_{15} and $Dp_{35}v$. A part of the important "nitrogen containing moiety" was split off [8]. This latter substance, soluble in alcohol and in water, and which did not dialyse, was analyzed: it contained DAP, Glu, Ala, N-acetylglucosamine, N-acetylmuramic acid (molar ratios, 2:2:3:2:2) and only traces of non amino sugars. Almost all the phosphorus of the purified waxes D obtained by ultracentrifugation was found in this "nitrogen-containing moiety". In conclusion, acetylation was able to split off a *peptidoglycan* from an active wax D.

It is important to point out that the chemical analyses suggest a close relationship between the peptidoglycan obtained after acetylation of an active wax D and the material which constitutes the backbone of mycobacterial as well as other cell walls. As a peptidoglycan with a similar composition was also obtained during the acetylation of the mycobacterial cell walls [11] and as "bound wax D" was characterized by Kotani et al. [12] as a component of BCG cell walls which possessed the adjuvant activity, we suggest to attribute to the peptidoglycan, split off from waxes D of humain strains of *M. tuberculosis* during the acetylation, the formula indicated in table 1.

At the present state, this formula has not been entirely verified, but the following experiments are in its favour: a) the elemental analysis of the entirely acetylated peptidoglycan (with 1–2P residues) seemed in accordance with formula 1 (C: calc. 45.4%; found 46%; H: calc. 5.8%; found 6%; N: calc. 8.7%;

* 12th communication on waxes D of Mycobacteria; 11th communication : [9].

Table 1
Tentative structure of the peptidoglycan obtained by acetylation from an adjuvant active wax D of *Mycobacterium tuberculosis var. hominis.*



* 2 carboxylic groups are amidated; from time to time, a residue of Ala is linked to one of the tow Glu residues (see discussion).

found 8.7%); b) the amino acid and sugar compositions (molar ratios) were in agreement with the indicated formula; c) after reduction by $LiBH_4$ in 0.05 M sodium tetraborate [13] of the peptidoglycan, one half of the muramic acid disappeared, but the glucosamine content remained stable; thus, only one residue of amino sugar out of the four seemed to have a free reducing group; d) hen egg-white lysozyme (EC 3.2.1.17) could not digest the peptidoglycan, as it was acetylated; after deacetylation in an alkaline medium, the amino sugar part of the peptidoglycan seemed modified as indicated by analyses; this explained again the absence of action of lysozyme; e) during the chromatography of the amino sugars, obtained after acid hydrolysis, on Amberlite CG-120 columns, a small peak was eluted at the place usually occupied by phospho-muramic acid [14]; recently, it was suggested that this phospho-amino sugar may constitute the link between peptidoglycans and polysaccharides [15]; f) no N-terminal amino acid was characterized. By Sanger's method, only mono-DNP-DAP was obtained; g) by hydrazinolysis, no C-terminal amino acid was obtained in high yield; only 0.3 residue of Ala, out of the 3 residues, was characterized; k) the presence of two amide groups was characterized; i) the peptidoglycan was submitted to partial hydrolyses (HCl 4N; 1 hr and 3 hr; 110°). The partial hydrolysate was purified by a two dimensional paper chromatography (Whatman No. 1; solvent No. 1: isopropanol-acetic acid-H_2O, 75 : 10 : 15, v/v; solvent No. 2: methyl-2-pyridine-NH_4OH-H_2O, 70 : 2 : 28, v/v; [16]. The peptides were eluted and analysed with an Autoanalyzer; their structure was established by usual methods. The yields of the peptides were closely related to the hydrolysis conditions. The following peptides were characterized: DAP-Ala; Ala-DAP; Glu-Ala; Ala-Glu.

Previously [7] these same peptides, as well as Glu-(DAP, Ala), were obtained from wax D_{p35}, strain Test and from total wax D (before ultracentrifugation), strain Brévannes; in this latter case [17], an additional peptide Glu-Glu was characterized which was no more obtained in more recent studies.

All the peptides are in accordance with formula 1 except Glu-Ala; its yield is low and we suggest that a part of the Glu residues are linked to an alanine residue, as suggested also by the result obtained by hydrazinolysis. Finally, it is worth mentioning that the bi-dimensional paper chromatograms ("fingerprint") of partial hydrolysates from wax D (Brévannes D_{p35}) and from cell walls of *M. tuberculosis var. hominis,* strain Peurois, sprayed with ninhydrin, seem identical [11].

This note is based on a part of a thesis submitted by one of us (D.M.) in partial fulfillment of the requirements for the degree of "Docteur ès Sciences", Paris (A.O. 2442).

References

[1] J.Asselineau, H.Buc, P.Jollès and E.Lederer, Bull. Soc. Chim. Biol. 40 (1960) 1953.
[2] P.Jollès, D.Samour and E.Lederer, Arch. Biochem. Biophys., Suppl. 1 (1963) 283.
[3] R.G.White, L.Berustock, R.G.Johus and E.Lederer, Immunology 1 (1958) 54.
[4] R.G.White, P.Jollès, D.Samour and E.Lederer, Immunology 7 (1964) 158.
[5] J.Freund, Adv. Tuberc. Res. 7 (1956) 130.
[6] B.H.Waksmann, C.M.Pearson and T.J.Sharp, J. Immunol. 85 (1960) 403.
[7] P.Jollès, D.Samour-Migliore, H. de Wijs and E.Lederer, Biochim. Biophys. Acta 83 (1964) 361.
[8] P.Jollès, D.Migliore and F.Bonhomme, Immunology 14 (1968) 159.
[9] F.Bonhomme, C.Boucheron, D.Migliore and P.Jollès, Experientia 24 (1968) 716.
[10] A.Tanaka and M.Kitagawa, Biochim. Biophys. Acta 98 (1965) 182.
[11] J.Hiu and P.Jollès, unpublished results (1968).

[12] S.Kotani, S.Hashimoto, T.Matsubara, K.Kato, K.Harada, J.Kogami, T.Kitaura and A.Tanaka, Biken. J. 6 (1963) 181.
[13] D.Mirelman and W.Sharon, J. Biol. Chem. 242 (1967) 3414.
[14] T.Y.Liu and E.C.Gotschlich, J. Biol. Chem. 238 (1963) 1928.
[15] W.D.Grant and A.J.Wicken, Biochem. Biophys. Res. Commun. 32 (1968) 122.
[16] K.H.Schleifer and O.Kandler, Arch. Microbiol. 57 (1967) 335.
[17] P.Jollès, H.Nguyen-Trung-Cros and E.Lederer, Biochim. Biophys. Acta 43 (1960) 559.