HYDROSOLUBLE ADJUVANT-ACTIVE MYCOBACTERIAL FRACTIONS OF LOW MOLECULAR WEIGHT

Danièle MIGLIORE-SAMOUR and Pierre JOLLÈS
Laboratory of Biochemistry, University of Paris VI, 96 Bd Raspail, F-75272 Paris 6e, France

Received 25 June 1973

1. Introduction

Mycobacteria [1], cell walls from human and non human mycobacterial strains [2, 3] as well as mycobacterial waxes D with a nitrogen-containing moiety [4, 5] possess a 'Freund's adjuvant effect' [1]; all these substances lead also to the production of delayed hypersensitivity and of certain disease states such as allergic encephalomyelitis or adjuvant arthritis [5, 6]. These different insoluble active fractions of high molecular weight contain a polysaccharide (Poly, mainly an arabinogalactan [7–9]) linked to a peptidoglycan (PA) [10–12]. Poly–PA constitutes the hydrosoluble moiety which is joined by ester linkages to the lipidic part (Lip, mycolic acids as in wax D). Previously we described [13] the preparation by a mild extraction technique of a hydrosoluble adjuvant-active substance (Poly–PA) from the delipidated cells of the human mycobacterial strain Peurois which did no more elicit the arthritis inducing effect. These studies were achieved quite independently from those reported at the same time by others [14, 15]; their hydrosoluble polysaccharide fractions obtained by different procedures required either the preparation of purified cell walls which were further submitted to an enzymic digestion or a chemical treatment (catalytic hydrogenation) of delipidated BCG cells.

All these adjuvant-active hydrosoluble fractions presented a high molecular weight. This note deals with the preparation of adjuvant-active fractions of low molecular weight. Two types of compounds were characterized: a first one where the sugars remained unchanged during the preparation steps ('native' type) and a second one where the sugars were acetylated.

2. Methods

2.1. *Mycobacterial residues*

The mycobacterial residues were prepared and their lipids eliminated according to Aebi et al. [16].

2.2. *Preparation of crude hydrosoluble substances*

2.2.1. 'Native' type

A first method was previously described [13] including homogenization in water and ammonium sulfate treatment until 70% saturation: the active substance remained in the supernatant (S_{70}). A second procedure included a pyridine extraction at 28°C during 24 or 36 hr followed by an addition of alcohol. The supernatant was dried and hydrosoluble substances were extracted by water.

2.2.2. 'Acetylated' type

The acetylation of delipidated cells was performed as previously reported [10, 17, 18] for waxes D. The fraction which remained alcohol-soluble was dried and submitted to extraction by water. The compounds which were soluble in alcohol and water were submitted for further purifications.

* 18th Communication on mycobacterial adjuvant-active substances.

Table 1

Yield of hydrosoluble extracts obtained from delipidated cells of mycobacterial strains by water extraction (W.E.) and acetylation (Ac.). The presence of the peptidoglycan is indicated by the DAP content (%).

Strain	Extraction procedure	Temperature (°C)	Extraction time (hr)	Crude extract (% of)	DAP (%) 'Native' purified extract*		Acetylation
					High mol wt. substance	Low mol wt. substance	(crude extract)
M. tuberculosis var. hominis							
$H_{37}Ra$	W.E.	20	6	2.7	2.6	7.6	
		30	18				
	Ac.	28	3	1.7			2.25
			24	3.0			
			72	1.9			
Peurois	W.E.	20	6	0.9	0.8		
		40	76	5.0	0.5	2.4	
	Ac.	28	24	2.0			0.6
Test	W.E.	40	48	3.7	1.0	6.0	
	Ac.	28	36	4.2			1.2
M. tuberculosis var. bovis							
BCG	W.E.	20	5	1.2	0.06		
		25	18				
	Ac.	28	36	0.2			0.07
Marmorek	W.E.	40	18	1.1	0.03		
Behring**	W.E.	40	18	2.6	0.4		
M. kansasii							
no. 4	W.E.	40	24	7.1	1.5		
		40	24				

* High and low mol wt.: high and low molecular weight fractions eluted from DEAE-cellulose calculated on the basis of 3 residus of Ala/mole (see parts 3.1 and 3.2).

** Bovine strain containing waxes D with a nitrogen-containing moiety [24].

2.3. *Purification of low molecular weight hydrosoluble adjuvants*

2.3.1. 'Native' hydrosoluble substances

The substances soluble in alcohol and in water obtained after the pyridine treatment were filtered on Biogel P 100 columns (250 × 1.2 cm) with 0.01 N acetic acid as eluent. The fractions were monitored at 220 and 280 nm. The delipidated residues were submitted for homogenization and ammonium sulfate treatment [13]. The 'native' hydrosoluble substances contained in S_{70} fractions were dialyzed on Diaflo-membranes UM2 (Amicon) with a view to eliminate all the compounds with a molecular weight lower than 1000. They were further chromatographed on DEAE-cellulose (Whatman DE 32) equilibrated with a 0.05 M phosphate buffer pH 7 and with a 0.05 M sodium citrate buffer pH 3 as eluent.

2.3.2. Acetylated hydrosoluble substances

They were also dialyzed on Diaflo-membranes and further purified on Biogel P 10 columns (250 × 2 cm) with 0.01 N acetic acid as eluent.

2.4. Characterization of the various constituents

The amino acid and amino sugar compositions were established with an Autoanalyzer after total hydrolysis (6 N HCl; 110°C; under vacuum) of 18 hr and 6 hr, respectively. The reducing non amino sugars were

Table 2

Amino acid and amino sugar compositions (in molar ratio, m.r. and per cent, %) of different 'native' purified hydrosoluble fractions from delipidated mycobacterial cells, strain Test. Molecular weights (mol. wt.) are calculated on the basis of 3 residues of Ala/mole.

Extraction procedure	Method of purification	Fractions		Amino acid composition					Amino sugar composition					M.W.
				Ala		Glu*		DAP	GlcN*		Mur*		GalN*	
				m.r.	%	m.r.	%	m.r.	%		m.r.	%	m.r.	%	
Pyridine-alcohol	Filtration on Biogel P 100	1		3.0	4.3	2.0	4.7	1.9	5.7		1.4	3.9	1.0	3.9	0	6,200
		2		3.0	3.3	1.8	3.8	1.5	4.1							6,500
		3		3.0	2.6	2.4	3.4	2.0	3.7							**
		4		3.0	1.0	6.2	3.3	0.6	0.4							**
Water [13] on cell residues after pyridine treatment	Chromatography on DEAE-cellulose	III + IV	[13]	3.0	1.0	2.2	1.2	1.4	1.0		3.0	2.0	1.6	1.5	1.2 0.8	26,700
		V		3.0	2.1	1.9	2.2	2.1	3.2		1.9	2.5	1.2	2.3	0.5 0.7	12,700
		VI		3.0	4.0	2.3	5.2	1.9	5.5		1.0	2.3	1.2	4.6		6,500
		VII		3.0	4.5	2.3	5.5	1.9	6.0		2.0	5.8	1.0	4.3		6,000

* Abbreviations: Glu, glutamic acid; GlcN, glucosamine; Mur, muramic acid; GalN, galactosamine. Structural studies showed that the figures concerning the N-substituted amino sugars are too low; other analytical procedures must thus be employed.

** Fractions not purified enough for a mol wt. determination.

determined according to Dische [19] and the phosphorous according to Fiske and Subbarow [20]. The glycolyl groups were characterized following the procedures of Calkins [21] and Eegriwe [22].

2.5. *Biological activities*

Adjuvant activity was determined according to White et al. [4, 5] and the arthrogenicity following Bonhomme et al. [23].

3. Results

Only mycobacterial strains from which waxes D with a nitrogen containing moiety could be extracted, such as human, atypical or certain bovine strains [5, 24], gave rise to hydrosoluble substances either by homogenization in water or by acetylation (table 1) which contained meso-α,α'-diaminopimelic acid (DAP), one of the main constituents of PA.

3.1. *'Native' hydrosoluble substances (adjuvants)*

Some analytical data concerning the hydrosoluble substances isolated by the pyridine treatment are reported in table 2. Four substances (1–4) soluble in alcohol and containing DAP were characterized on Biogel P 100. The cell residues were homogenized by water [13]. The chromatography on DEAE-cellulose of S_{70} fractions isolated from strains Peurois, $H_{37}Ra$ or Test gave rise to 8 fractions (I–VIII); 6 of them (III–VIII) contained DAP. Peaks III and IV where previously characterized [13]; they corresponded to various Poly-PA with molecular weights ranging from 12 000–26 000 on the basis of 3 residues of Ala/mole. The substances contained in fractions VI and VII had lower mol. wt. (6000–7000). They behaved as adjuvants [25] as determined according to White et al. [4, 5] but were no more arthrogenic [23].

3.2. *Acetylated hydrosoluble substances*

Fig. 1 indicates the purification of the crude acetylated adjuvant-active [4, 25] hydrosoluble fraction from strain $H_{37}Ra$ (or Peurois) on Biogel P 10 and table 3 summarizes some analytical data. Four main peaks were characterized. Two of them (1 and 2) representing 10% of the crude extract, were more particularly studied. Their molecular weights were slightly below 4000, on the basis of 3 residues of

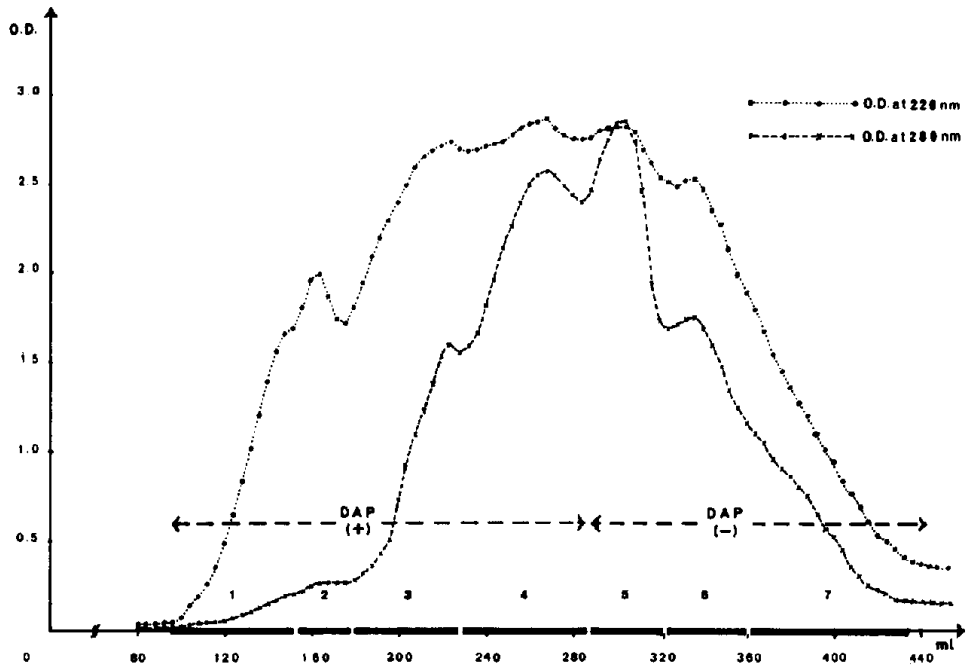

Fig. 1. Filtration on Biogel P 10 (250 × 2 cm) of the crude acetylated hydrosoluble extract of mycobacterial cells, strain $H_{37}Ra$; 0.01 N acetic acid as solvent.

Ala/mole. Table 3 indicates that these two compounds contain principally the peptidoglycan and only small amounts of reducing non amino sugars: 13 ± 2% for peak 1, and 11–12% for peak 2 devoid of pentose (arabinose). The presence of glycol groups was characterized but no phosphorous could be detected. The rest of the molecules were constituted by the acetyl groups.

4. Conclusion

This note describes the purification by various methods of hydrosoluble adjuvant-active low molecular weight substances from mycobacterial strains. The 'native' substances contain yet a polysaccharide part, whereas the acetylated compounds are mainly constituted by the peptidoglycan (PA); the latter remain

Table 3

Amino acid, amino sugar and reducing non amino sugar compositions (m.r. and %) of different acetylated purified hydrosoluble fractions characterized on Biogel P 10 from delipidated mycobacetrial cells, strain $H_{37}Ra$.

Fractions	Amino acids						Amino sugars				Reducing non-amino sugars
	Ala		Glu		DAP		GlcN		Mur		
	m.r.	%	m.r.	%	m.r.	%	m.r.	%	m.r.	%	%
1	3.0	8.0	2.1	9.2	1.5	8.6	1.8	11.4	1.5	13.1	13 ± 2
2	3.0	8.5	2.3	10.7	1.5	8.9	1.5	10.3	2.0	15.3	11–12
3	3.0	3.0	2.2	3.9	1.0	2.2	3.0	7.2	1.7	5.6	20.5
4	3.0	0.9	3.4	1.8	0.3	0.2	3.3	2.6	0.5	0.5	

however adjuvant-active [13, 25]. The biological properties of the different purified substances will be described in detail in a forthcoming paper [25].

Acknowledgements

This research was supported in part by the C.N.R.S. (ER 102) and the I.N.S.E.R.M. (groupe U 116). The authors wish to thank Mr. F. Bonhomme (Institut Prophylactique, Paris) for his help in the determination of the arthritis inducing ability of the various fractions.

References

[1] Freund, J. (1956) Advan. Tuberc. Res. 7, 130.
[2] Bonhomme, F., Boucheron, C., Migliore, D. and Jollès, P. (1969) Intern. Arch. Allergy Appl. Immunol. 36, 317.
[3] Misaki, A., Yukawa, S., Tsuchiya, K. and Yamasaki, T. (1966) J. Biochem. (Tokyo) 59, 388.
[4] White, R.G., Bernstock, L., Johns, R.G. and Lederer, E. (1958) Immunology 1, 54.
[5] White, R.G., Jollès, P., Samour, D. and Lederer, E. (1964) Immunology 7, 158.
[6] Waksmann, B.H., Pearson, C.M. and Sharp, T.J. (1960) J. Immuol. 85, 403.
[7] Vilkas, E., Amar, C., Markovits, J., Vliegenthart, J.F.G. and Kamerling, J.P. (1973) Biochim. Biophys. Acta 297, 423.
[8] Azuma, I., Yamamura, Y. and Misaki, A. (1969) J. Bacteriol. 98, 331.
[9] Misaki, A. and Yukawa, S. (1966) J. Biochem. (Tokyo) 59, 511.
[10] Migliore, D. and Jollès, P. (1968) FEBS Letters 2, 7.
[11] Migliore, D. and Jollès, P. (1969) Compt. Rend. Série D, 269, 2268.
[12] Wietzerbin-Falszpan, J., Das, B.C., Azuma, I., Adam, A., Petit, J.F. and Lederer, E. (1970) Biochem. Biophys. Res. Commun. 40, 57.
[13] Migliore-Samour, D. and Jollès, P. (1972) FEBS Letters 25, 301.
[14] Adam, A., Ciorbaru, R., Petit, J.F. and Lederer, E. (1972) Proc. Natl. Acad. Sci. U.S. 69, 851.
[15] Hiu, I.J. (1972) Nature New Biol. 238, 241.
[16] Aebi, A., Asselineau, J. and Lederer, E. (1953) Bull. Soc. Chim. Biol. 35, 661.
[17] Tanaka, A. (1963) Biochim. Biophys. Acta 70, 483.
[18] Jollès, P., Migliore, D. and Bonhomme, F. (1968) Immunology 14, 159.
[19] Dische, Z. (1949) J. Biol. Chem. 181, 379.
[20] Fiske, C.H. and Subbarow, Y. (1925) J. Biol. Chem. 66, 375.
[21] Calkins, V.P. (1943) Anal. Chem. 15, 762.
[22] Eegriwe, E. (1932) Z. Anal. Chem. 89, 123.
[23] Bonhomme, F., Boucheron, C., Migliore, D. and Jollès, P. (1966) Compt. Rend. Série D, 263, 1422.
[24] Migliore, D., Augier, J., Boisvert, H. and Jollès, P. (1971) J. Bacteriol. 107, 548.
[25] Maral, R., Werner, G., Migliore-Samour, D. and Jollès, P. (1973) unpublished results.