A HYDROSOLUBLE, ADJUVANT-ACTIVE MYCOBACTERIAL "POLYSACCHARIDE–PEPTIDOGLYCAN". PREPARATION BY A SIMPLE EXTRACTION TECHNIQUE OF THE BACTERIAL CELLS (STRAIN PEUROIS)*

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1. Introduction

Mycobacteria possess several important biological properties as an adjuvant effect in antibody formation (Freund's adjuvant effect) [1] and the induction of experimental arthritis in rats termed adjuvant arthritis [2]. Cell walls from human and non human mycobacterial strains as well as waxes D with a nitrogen-containing moiety (peptido-glycolipids) provoke these reactions [3, 4]. In the different active substances, a polysaccharide (Poly, mainly an arabinogalactan [5]) is linked to a peptidoglycan (PA) [6]. Poly–PA constitutes the hydrosoluble moiety which can be joined by ester linkages to the lipidic part (Lip, mycolic acids as in wax D). We studied previously in detail the nitrogen containing part of waxes D from human origin [6, 7] and we could thus demonstrate a close relationship between the peptidoglycan of an active wax D and the material which constitutes the backbone of mycobacterial as well as other cell walls. Several procedures (saponification [8], acetylation [9]) allowed to obtain the hydrosoluble moiety, but nearly all of them modified more or less the sugars, especially the amino sugars, as established by analytical methods. Only the homogenization of wax D in a buffer medium [10] seemed to yield a "native" Poly–PA, i.e. a hydrosoluble moiety where the sugars remain unchanged during the preparation steps. Our chemical studies strengthened the view that wax D could be some degradation product of the mycobacterial cell walls [11, 12]. This enzymic degradation (autodigestion) might result naturally in a hydrosoluble moiety. This note describes briefly the preparation and partial purification of such a hydrosoluble adjuvant-active fraction from mycobacterial cells.

* 16th communication on waxes D and other mycobacterial constituents.

2. Materials and methods

2.1. *M. tuberculosis var. hominis*, strain Peurois, was obtained from the Pasteur Institute, Paris. The bacterial residues were prepared and their lipids eliminated according to Aebi et al. [13].

2.2. *Preparation of the hydrosoluble adjuvant*

Water-extraction of the Mycobacteria: the bacterial residues from which the lipids have been removed were submitted to a procedure analogous to that used by Wilhelm [14] for the isolation of proteins from human and animal tissues. 100 g were ground and homogenized in 500 ml water with an Ultra-Turrax. After stirring during 5 hr at 20° and centrifugation (4000 rpm at 4°), the supernatant was heated to 80°; ammonium sulfate was added until 40% saturation. After 12 hr at 4° and centrifugation, a precipitate (P_{40}) was obtained and to the supernatant set at 20°, ammonium sulfate was again added until 70% saturation. After 12 hr at 4° and a final centrifugation, a precipitate (P_{70}) and a supernatant (S_{70}) were recovered. P_{40}, P_{70} and S_{70} were exhaustively dialyzed against distilled water and lyophilized.

Further purification was achieved by chromato-

Table 1
Adjuvant activity [4] and arthrogenicity [16] of wax D of human strains and of their hydrosoluble moieties, Poly–PA, obtained by different methods.

Substance obtained from *M. tuberculosis var. hominis*	Adjuvant activity to ovalbumin in guinea-pig					Experimental polyarthritis in rat			
	Dose (μg)	Number of animals		Cutaneous reaction at 24 hr**		Dose (μg)	Number of animals		Severity***
		Tested	Positive	2 (μg)	10 (μg)		Tested	With arthritis	
Controls		16	0	0	0				
Wax Dp_{35}* strain Peurois	200	7	7	17.4	21.1	250	8	8	8+++++
Poly–PA (after saponification of wax Dp_{15}*, strain Peurois)	200	7	7	7.1	9.8				
Poly–PA (after homogenisation of wax Dp_T*, strain $H_{37}R_vS_r$)	200	8	8	11.3	15.6				
"Native" Poly–PA from delipidated bacterial residues of strain Peurois	100	8	8	8.8	12.9	250	6	0	

* For details, see [4].
** Mean diameter of papula (mm).
*** Severity: +++++: arthritis with malformation and ankylosis of the articulations.

graphy 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 as well as by filtration on Biogel P 10 with water as eluent. The fractions were monitored at 220 and 280 nm.

2.3. *Characterization of the various constituents*

The amino acid and amino sugar compositions were established with an autoanalyzer after total hydrolysis (6 N HCl; 110°; under vacuum) of 18 hr and 6 hr, respectively. The neutral non amino sugars were determined after hydrolysis (2 N HCl; 1 hr; 110°) qualitatively by paper chromatography (Whatman No. 1; solvent: n-butanol–pyridine–water, 6:4:3, v/v/v) and quantitatively by column chromatography with a Technicon sugar Autoanalyzer. The lipids were investigated by thin-layer chromatography on silica gel after total acid hydrolysis and ether extraction.

2.4. *Biological activities*

Adjuvant activity was determined according to White et al. [4] with a cutaneous reaction to ovalbumin [15]; the arthrogenicity was characterized following Bonhomme et al. [16].

3. Results

3.1. *Preparation of different hydrosoluble fractions and localisation of the Poly–PA*

By a water extraction of the cells of strain Peurois, 1.2% P_{40}, 1.4% P_{70} and 0.9% S_{70} were obtained. Analytical studies allowed to establish the presence of the characteristic constituents of a Poly–PA in fraction S_{70} (Ala:Glu:DAP:NAcGlc:N-glycolylMur, 3:2:2:2:2 (molar ratios); presence of Gal and Ara).

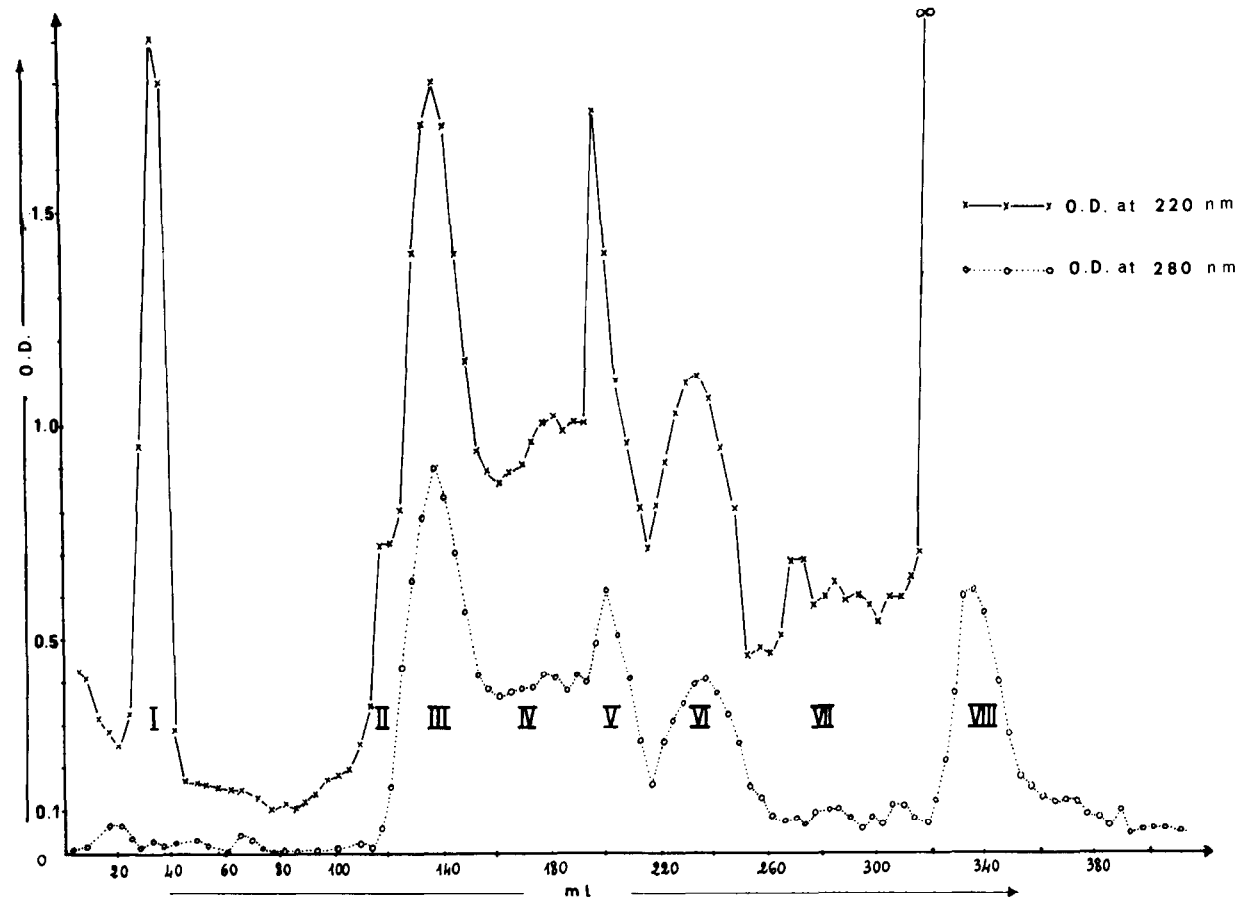

Fig. 1. Chromatography on DEAE-cellulose (62 × 2.5 cm) of fraction S_{70} (500 mg) obtained by water-extraction of mycobacterial cells, strain Peurois (see Methods).

3.2. *Further purification of Poly–PA (S_{70})*

Fig. 1 indicates the further purification of 500 mg S_{70} from strain Peurois on DEAE-cellulose. Eight peaks (I–VIII) were characterized. Peaks III and IV contained the Poly–PA; they were separately submitted to filtration on Biogel P-10. Two fractions could again be obtained: one (a) containing mainly a polysaccharide (poly-glucose) and the other (b) corresponding to the Poly–PA.

3.3. *Analytical data concerning the Poly–PA*

The sedimentation constant S_{20} of the hydrosoluble Poly–PA (S_{70}), peak III/b, was 1.9 compared to 0.65 for PA obtained by acetylation and around 2 for Poly–PA obtained by saponification. The amino acid and amino sugar contents were 6.2 and 7%, respectively. On the basis of 3 residues of Ala/mole, a molecular weight of 14,800 was calculated. The neutral reducing sugars (Ara, Gal, Man) constituted the remaining part of S_{70}, as the lipid content was lower than 0.5%.

All these analytical data are in favour of a close relationship between the hydrosoluble Poly–PA described in this note and the Poly–PA obtained by saponification of wax D [7].

3.4. *Biological activity of the hydrosoluble Poly–PA [15, 16]*

Table 1 indicates that the "native" hydrosoluble Poly–PA obtained by our mild extraction technique possessed an adjuvant activity when added to Freund's incomplete adjuvant with an antigen (ovalbumin). The

hydrosoluble fractions previously obtained by chemical procedures were not regularly active, probably because the sugars were more or less modified. Our "native" Poly–PA did not elicit the arthritis-inducing effect observed with wax D.

4. Conclusion

This note describes the preparation by a mild extraction technique of a hydrosoluble adjuvant-active substance (polysaccharide–peptidoglycan) from the cells of the human mycobacterial strain Peurois. These studies were achieved quite independently from those just reported by Adam et al. [17]; their hydrosoluble polysaccharide fraction was obtained by a different procedure requiring the preparation of purified *cell walls* (from *M. smegmatis*) which were further digested by hen lysozyme. Previously Misaki et al. [18] also used cell walls (strain BCG) for the preparation of a mucopeptide obtained by pronase digestion, capable of eliciting resistance of mice to staphylococcal infection but failing to induce or provoke the delayed hypersensitivity.

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