ADJUVANT ACTIVITY OF MONOMERIC BACTERIAL CELL WALL PEPTIDOGLYCANS.

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SUMMARY

We have previously shown that lysozyme solubilized cell walls of Mycobacteria or Nocardiae can replace whole mycobacterial cells or Wax D in Freund's complete adjuvant and it was found quite recently that hydrosoluble peptidoglycans, free of neutral sugars,are also adjuvant active. We show now that the simplest fragment tested - the disaccharide tetrapeptide (I) - increases circulating antibodies to ovalbumin and induces a delayed hypersensitivity toward this antigen. Similar compounds obtained from the basal layer of the cell wall of E. coli are also active. Thus the immunoadjuvant activity of soluble cell wall peptidoglycans is a property of the monomeric unit and is not restricted to acid fast bacteria.

INTRODUCTION

Freund's complete adjuvant contains whole mycobacterial cells as essential component. Many efforts have been made toward the identification of the chemical structure which, in the mycobacterial cell, is responsible of the adjuvant activity.

In 1958, White et al. (1) have shown that Wax D of human strains of Mycobacteria, i.e. Wax D which consists of a mycolate of an arabinogalactan covalently linked to a fragment of peptidoglycan, can replace mycobacterial cells; Wax D preparations from other strains which do not contain a peptidoglycan moiety are inactive.

As active Wax D preparations have a composition similar to that of the cell wall, it was not astonishing to find that pure cell wall preparations are also able to replace whole mycobacterial cells in Freund's adjuvant (2, 3). Adam et al. (3, 4) then showed that hydrosoluble products, obtained by lysozyme treatment of purified cell walls, were more active than Wax D and cell walls: among these, the best defined has a molecular

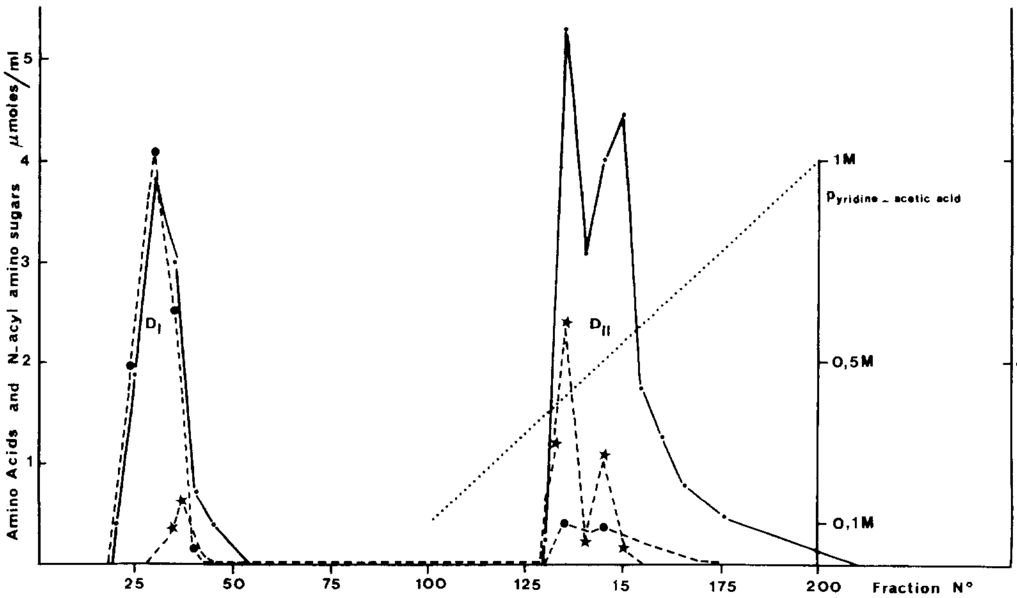

Fig. 1

DE 32 elution profile of fraction B from M. smegmatis. 350 mg were applied to a column (2 x 32 cm) of DE 32. The column was washed with equilibration buffer (500 ml, 0.1 M pyridine-acetic acid pH 7.3) then developed with a linear 0.1 M pH 7.3- 1 M pH 5 gradient of pyridine-acetic acid buffer (500 ml).
5 ml fractions were collected and •——• amino acids ●- - - -● neutral sugars and ✱- - -✱ acyl amino sugars determined as previously described (3, 15).

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weight of approximately 20.000 daltons and consists of an arabinogalactan linked to a peptidoglycan; it was called WSA (Water Soluble Adjuvant). Similar products were also obtained by Migliore et al. (5), Hiu (6) and Amar et al. (7) from Mycobacteria. They can also be obtained from Nocardia strains (4) and are free of the toxic effects observed with whole mycobacterial cells(8).

In the process of purification of WSA by filtration on Sephadex G 50 a fraction containing smaller products, with a very low content of neutral sugars was obtained. This fraction is also adjuvant active (3). The constituents of this fraction were purified and Adam, Amar et al.(9) were able to prove that peptidoglycan fragments completely free of neutral sugars are at least as active as WSA in replacing whole mycobacterial cells, Wax D or cell walls in Freund's adjuvant. Analogous active fractions, but still containing 11 to 13 % neutral sugars have been prepared by Migliore-Samour and Jollès (10). Recently Nauciel et al (11) have reported that polymeric peptidoglycans of some Gram negative bacteria can induce hypersensitivity and Nguyen-Dang et al.(12) have found cell walls of Corynebacterium parvum and B. megaterium active in the Jerne test.

We now report that monomeric peptidoglycans of M. smegmatis and of E. coli are also immunoadjuvant, using ovalbumin as antigen.

MATERIAL AND METHODS

Water Soluble Adjuvant fractions (WSA) were obtained by lysozyme digestion of cell walls or whole bacilli according to methods previously described (3,4). The soluble fragments were first separated by gel filtration on Sephadex G 75 and the lower molecular weight compounds (fraction B) were purified by chromatography on DE 32 (Whatman) (Fig.1) and fraction D_{II} treated with Myxobacter AL_1 amidase (13). The incubation mixture was filtered on Sephadex G 25 and purified by high voltage electrophoresis. Preparative electrophoresis was carried out on Whatman n°3 MM paper at 60 v/cm for 1 hour in pH 3,9 pyridine, acetic acid, water (23:6:971) buffer under varsol in a Savant apparatus.

The cell wall peptidoglycan of E. coli was prepared according to the method of Pelzer (14), then digested with lysozyme and the soluble compounds were fractionated on Sephadex G 50 in 0,1 N acetic acid.

Analyses were performed and degree of polymerisation of the fractions was determined according to Ghuysen et al. (15).

Adjuvant activity was determined according to the method used by White et al. (1) , with ovalbumin as antigen (Table II).

RESULTS

350 mg of fraction B of the products solubilized by lysozyme from purified cell walls of M. smegmatis were chromatographed on DE 32. The second peak D_{II} (250 mg) (Fig. 1) is almost completely free of neutral sugars (less than 3 %).

200 mg of the material of peak D_{II} were treated with 20,000 units of Myxobacter AL_1 enzyme and filtered on a Sephadex G 25 column (2 x 60 cm). Three peaks were obtained: S_I, S_{II}, S_{III} of which S_{II} and S_{III}

Table I

Composition of peptidoglycans obtained by enzymatic hydrolysis of Mycobacterial cell walls.

Fraction	Molar ratios relative to DAP				
	Neutral sugars	Ala	Glu	DAP	Aminosugars (GlcNH$_2$/Mur 1:1)
S_I	0.15	1.8	1.16	1	1.9
S_{II}	0	1.45	0.98	1	1.8
S_{III}	0	1.45	0.97	1	3
S_{E1} [x]	0	1.9	1.02	1	2
S_{E2} [x]	0	1.9	1	1	1.9
S_{E3} [x]	0	1.5	0.98	1	1
S_{E4} [xx]	0	2	1	1	2

[x] In these fractions about half of the DAP is converted to mono-DNP-DAP by dinitrophenylation, in S_{E3} one third of the Ala is N-terminal.

[xx] In S_{E4} all the DAP is converted to mono-DNP-DAP by dinitrophenylation.

are completely free of neutral sugars and contain only Ala, Glu, DAP, glucosamine and muramic acid (Table I).

Dinitrophenylation shows that S_{III} is essentially a mixture of monomers and S_{II} and S_I contain respectively dimers, trimers and higher oligomers of the basal unit .From S_{II}, three pure products S_{E1}, S_{E2} and S_{E3} were obtained by preparative high voltage electrophoresis at pH 3,9. S_{E1} and S_{E2} are dimers of the disaccharide tetrapeptide subunit. They differ only by their electrophoretic mobility, probably due to a difference in the number of amide groups. S_{E3} is a disaccharide heptapeptide, S_{E4} is a pure sample of disaccharide tetrapeptide obtained from M.smegmatis and characterized by Wietzerbin-Falszpan (16).

Peptidoglycan fractions of E.coli obtained by digestion of 20 mg of the basal layer with lysozyme were filtered on a column of Sephadex G 50 (2.5 x 80 cm) in 0.1 N acetic acid. Three peaks C_1, C_2 and C_3 were obtained which contain as major constituents the peptidoglycan aminoacids and aminosugars. C_1 is a mixture of oligomers, C_2 a mixture of dimers and C_3 is a mixture of the disaccharide tetra- and tripeptides.

TABLE II : Adjuvant activity of bacterial peptidoglycans.

Fraction tested	Dose (μg/animal)	Humoral antibody (μg/ml) Animal number 1	2	3	4	Mean	Delayed hypersensitivity Challenge dose 10 μg	100 μg
0 (FIA)	0	600	660	680	420	590	10 E	4 I
M. butyricum (FCA)	50	4000	2400	3600	2800	3200	13 I	16-6
M. smegmatis								
dimer of the disaccharide tetrapeptide S_{E1}	25	5680	4000	3960	4000	4410	9 I	22-4
S_{E2}	25	2800	5200	4120	4400	4135	10 I	25-8
disaccharide heptapeptide S_{E3}	25	6000	4200	3340	4800	4585	12 I	22-8
disaccharide tetrapeptide (I) S_{E4}	25	5200	4000	2800	3600	3850	8 I	19-2
E. coli								
basal layer	50	2000	2760	2200	1600	2140	5 I	18-7
C_1	25	1600	1360	800	1200	1240	6 I	11 I
C_2	25	1040	2000	2480	2080	1900	12 I	11-2
C_3	50	5600	4960	4240	4560	4840	15 I	22-5

Hartley female guinea pigs weighing 300-350 g were injected in both hind-foot pads with 0.1 ml of a water-in-oil emulsion made of equal parts of a solution of ovalbumin (50 mg/ml in saline) and either Freund's complete adjuvant or Freund's incomplete adjuvant, completed as indicated with peptidoglycan fractions. To obtain the sera, the guinea pigs were killed 21 days after the injection. The adjuvant activity is measured as μg/ml of antigen-antibody complex at the equivalente point. The complex is estimated with the Folin reagent, with ovalbumin as standard.

Animals were challenged for delayed hypersensitivity 28 days after the injection. The resulting reactions were measured 48 hr after the injection.

E = Erythema, I = Induration ; the first number is the diameter of erythema, the second is the diameter of necrosis.

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\begin{array}{l}
\text{N-acetylglucosamine} \xrightarrow{\beta\ 1-4} \text{N-glycolylmuramic acid} \\
\qquad\qquad\qquad\qquad\qquad\qquad\downarrow \\
\qquad\qquad\qquad\qquad\qquad\quad \text{L-Ala} \\
\qquad\qquad\qquad\qquad\qquad\qquad\downarrow \\
\qquad\qquad\qquad\qquad\qquad\quad \text{D-Glu} \overset{\alpha}{-} NH_2 \\
\qquad\qquad\qquad\qquad\qquad\qquad\downarrow \\
\qquad\qquad\qquad\qquad\quad \text{meso-DAP} - NH_2 \\
\qquad\qquad\qquad\qquad\qquad\qquad\downarrow \\
\qquad\qquad\qquad\qquad\qquad\quad \text{D-Ala}
\end{array}
$$

I

Results of adjuvanticity tests are described in Table II, which shows that all the peptidoglycan fragments,even the monomeric unit (I) can replace mycobacterial cells in Freund's adjuvant; they induce both an increase in circulating antibodies toward ovalbumin and delayed hypersensitivity toward this same antigen.

CONCLUSIONS

Results reported above show that the disaccharide tetrapeptide (I),a subunit of the cell wall peptidoglycan,can replace whole mycobacterial cells in Freund's adjuvant as concerns stimulation of antibody production and induction of delayed hypersensitivity.

Analogous fractions prepared from E.coli cell walls, where muramic acid is N-acetylated and D-Glu and meso-DAP are not amidated have the same activity. Further work is under way to determine the minimal structural requirements for adjuvant activity.[x]

Nguyen-Dang et al. (12) have reported that cell walls of B. megaterium are adjuvant active in the Jerne test. They have found that hydrosoluble fragments obtained after lysozyme digestion are inactive in the same test (17). The apparent discrepancy of these results with ours, might be explained by the difference in experimental conditions.

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[x]Preliminary results of an experiment under way show that neither the disaccharide nor the tetrapeptide are adjuvant active.

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