Purification of specific precipitinogen and extraction of endotoxin from *Haemophilus influenzae*¹

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SUMMARY After purifying a *Haemophilus influenzae* precipitinogen from endotoxic activity by means of ultracentrifugation, column chromatography (Sephadex 6B), and ion exchange chromatography (DEAE Sephadex A25) a fraction was obtained which still contained a specific precipitinogen that was virtually free of endotoxin. Furthermore, during the chromatographic procedures fractions with a high and a low molecular weight endotoxic activity were found. The limulus lysate test was more sensitive in the high molecular weight fractions and the LD₅₀ in mice in the low molecular weight fractions with endotoxic activity.

The non-encapsulated *Haemophilus influenzae* (HI) is the most common pathogen in the bronchial tree of patients with chronic non-specific lung disease (CNSLD) (Mulder, 1938; Brumfitt *et al.*, 1957). When the effect of *H. influenzae* precipitinogen in skin test and bronchial provocation was studied it was difficult to differentiate between an immunological and a toxic mechanism in the reaction to the test substance (van der Zwan *et al.*, 1975). It has been suggested that endotoxin is present in *H. influenzae* (Branefors-Helander, 1973; Denny, 1974; van der Zwan *et al.*, 1975). A lipopolysaccharide has recently been isolated from *H. influenzae* and defined chemically (Zoon and Scocca, 1975). Therefore a specific precipitinogen, as defined by Omland (1964) and May (1965), without endotoxic activity and also a purified endotoxin is necessary in order to be able to identify the immunological and toxic reaction separately.

A purified precipitinogen is obtained by the methods described below. For extracting lipopolysaccharides from rough growing bacteria the method described by Galanos *et al.* (1969) was used. The limulus lysate test (LLT) was used to measure the endotoxic activity of the extracts, because it is relatively easy to perform and highly sensitive. An *in-vivo* system was required since the bacterial compounds were to be used in man. For that purpose the mortality in mice after potentiation with actinomycin-D was used (Pieroni *et al.*, 1970).

Methods

CULTURE

A rough growing, non-encapsulated strain of *H. influenzae* from a patient with chronic purulent bronchitis (confirmed by The Netherlands National Health Laboratory Service, strain number BBD 5048) was cultured at 37°C on Levinthal plates (Oxoid Ltd). After 24 hours the micro-organisms were transferred to a Microform Fermentor (New Brunswick Scientific Co) containing 12 l Levinthal broth. The inoculum was cultured for 20 hours at 37°C with an aeration of 1 l/min and stirred at 100 rpm. By taking samples from the culture at intervals of four hours it was shown that the pH remained between 7.1 and 7.4. Control of the culture was checked by a Gram stain and by culture on Levinthal and blood-agar plates (Oxoid Ltd). After centrifugation of the culture at 1900 g for 15 minutes the residue was washed three times in 12.5 mmol (6.25 mEq/l) MgCl₂ to remove the remaining broth. The weight of the pellet was considered to be the wet weight of the bacteria.

IMMUNOLOGICAL METHODS

Precipitinogens in the different fractions were identified by immunodiffusion according to Ouchterlony (1962). When the immunodiffusion was positive immunoelectrophoresis was performed by the method of May (1968), which detects antibody to the specific and non-specific antigens of *H. influenzae*. The sera used were from patients with CNSLD who had *H. influenzae* in their sputum. The

¹Dedicated to the late Professor J. R. May

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sera contained either specific or non-specific, or both, precipitins against *H. influenzae* (May, 1968).

ENDOTOXIN DETECTION

Localisation of endotoxin was attempted by staining on the immunoelectrophoresis slide, using Sudan black for the lipid and Schiff's reagent for the saccharide moiety of the endotoxin (Uriel, 1964).

The LLT was used for *in-vitro* activity (Rojas-Corona *et al.*, 1969). The result was read after two hours and considered to be positive when a firm gel had been formed.

In-vivo endotoxic activity was studied in female Swiss mice, weighing between 15 and 25 g, by using the potentiating effect of actinomycin-D (Pieroni *et al.*, 1970). The LD₅₀ was estimated by the method of Reed and Muench (1938). To compare the content of endotoxin in the different preparations end-point titrations were carried out according to Jorgensen and Smith (1974). A reference endotoxin from *Escherichia coli* had an end-point titration at 10⁻⁷ mg/ml solvent.

ENDOTOXIN EXTRACTION

The method of extracting endotoxin, which is specially suited for rough-growing bacteria, was by using a monophasic extraction mixture consisting of aqueous phenol, chloroform, and petroleum ether (Galanos *et al.*, 1969).

The fractionating procedure was as depicted in Fig. 1.

EXTRACT PREPARATION

The bacteria were disrupted by ultrasonic disintegration (MSE) as described by May (1968) using a buffer of 12.5 mmol (6.2 mEq/l) MgCl₂ to stabilise the endotoxin in its high molecular weight form (Greer *et al.*, 1973). Ultracentrifugation was carried out at 200 000 *g* for 30 minutes to remove all particle-bound endotoxin. Since the supernatant still had considerable endotoxic activity a further separation was tried by means of chromatographic procedures.

Fig. 1 Diagram of fractionating procedures.

CHROMATOGRAPHY

Column chromatography was carried out using Sepharose 6B as a carrier (column diameter 2.5 cm, volume 450 ml). The supernatant obtained after ultracentrifugation was concentrated in a diaflowmeter with a filterpore of 10 m μ to a volume of 3.5 ml. This concentrate was centrifuged at 2000 *g* for 20 minutes to remove denaturated material and was brought on the column. The eluate was sampled by means of a LKB fraction collector and measured at 276 nm by an Uvicord (LKB). After chromatography the eluate was pooled as follows: fraction I ($\pm$ 60 ml), fraction II ($\pm$ 36 ml), fraction III ($\pm$ 132 ml), and fraction IV ($\pm$ 78 ml), (Fig. 2). The place of the absorption maxima of the fractions were reproducible for different batches, although the sharpness of the peaks differed slightly. In addition ion exchange chromatography was performed using DEAE-Sephadex A25 (Davies *et al.*, 1974). The pooled fractions were dialysed during 48 hours against running tap-water and lyophilised.

Fig. 2 Absorption maxima after passage of particle-free cytoplasmic fraction of *H. influenzae* via a column with Sepharose 6B.

CHEMICAL ANALYSIS

The fractions were characterised according to their protein, lipid, and carbohydrate content. The amount of the protein in the various fractions was estimated by the method of Lowry (Lowry *et al.*, 1951). The lipid content of the unsaturated moiety was assessed as described by Hoeflmayr (Hoeflmayr and Fried, 1967). For the estimation of saturated fatty acids the fractions were extracted in chloroform and dried in a nitrogen stream. For the thin-layer chromatography Kieselgel (Merck Ltd) was used (thickness 0.25 mm). A mixture of petroleum ether 40/60 and acetone in a ratio of 85:15 v/v (Merck Ltd) was used for development. The results were read with ultraviolet light (350 nm) and graded from - to + + +. Anthrone reagent was used for detecting carbohydrate, which was quantified in amounts equivalent to galactose (Toennies and Kolb, 1964). When repeated the results varied by 5% to 12% for the protein and lipid content and by 5% to 18% for the carbohydrate.

Evaluation of procedures and results

PURIFICATION OF PRECIPITINOGEN

The whole *H. influenzae* bacterium showed a high endotoxic activity (Table 1) and was obviously negative in the precipitation tests. Since endotoxin is a component of the inner cell wall (de Petris, 1967) ultrasonic disintegration and ultracentrifugation were performed and a particle-free cytoplasmic fraction (CF) obtained. This was still considerably contaminated with endotoxin but it contained the specific and non-specific antigen, as was shown in the IEF (Table 1).

Since it could be expected that the endotoxin had a high molecular weight (Lüderitz *et al.*, 1966) column chromatography was carried out. Fig. 2 shows the elution pattern of 3.5 ml of the concentrated CF when brought on a Sepharose B6 column.

Table 1 Endotoxic activity (measured in vivo and in vitro) and precipitinogen content in the lyophilised *H. influenzae* fractions expressed in μ g of *E coli* endotoxin equivalents

	Fractionating procedures										
	None	Chlorof. ether phenol extract	Ultracentr.	Sepharose 6B				DEAE-Sephadex A25		Sepharose 6B DEAE Sephadex A25	
	Bacteria	Endotoxin	CF	Fr. I	Fr. II	Fr. III	Fr. IV	Specific	Non-specific	III/S	III/AS
LD ₅₀ in mice + actinomycin	2.5	25	0.2	2	0.5	0.03	0.02	0.07	2	0.003	0.014
End titration limulus assay	100	1000	1	1000	0.1	0.1	0.1	0.1	0.1	0.001	0.1
Precipitins											
Specific	—	—	+++	+	+	+++	—	+++	+	+++	+
Non-specific	—	—	+++	++	+++	++	+	—	+++	—	+++

Each of the four main absorption maxima (I/IV) was pooled and tested for endotoxic activity and the presence of specific and non-specific precipitinogen. The main endotoxic activity was concentrated in fraction I, whereas fractions II-IV had a much lower activity. The main content of specific precipitinogen was found in fraction III. This indicated that toxicity does not depend on the presence of specific precipitinogen.

Because the separation was only partial another method was used. Immunoelectrophoresis followed by lipid and saccharide staining suggested that the charge of the lipopolysaccharide moiety was negative, as opposed to that of the positively charged specific precipitinogens (Fig. 3). Therefore ion exchange chromatography was carried out (Davies *et al.*, 1974), using a cytoplasmic fraction.

The first eluate (CP/S) contained only the positively charged fraction. The second eluate (CP/AS) with the negatively charged fraction contained also components of the positively charged fraction. This contamination was slightly variable. When fraction III obtained by column chromatography was used in the ion exchange chromatography instead of the CF fraction a fraction (III/S) was obtained. This showed a very low endotoxic activity (Table 1) while still containing a high concentration of the specific precipitinogen as demonstrated in the immunodiffusion. It showed a reaction of identity with *H. influenzae* extract prepared as described by May (1968) (Fig. 4). It could be that endotoxin itself is a precipitinogen. Therefore endotoxin, prepared as described by Galanos *et al.* (1969), was tested in the immunodiffusion against sera with specific or non-specific precipitins against *H. influenzae*. No precipitate was formed. Therefore

Fig. 4 Lines of identity between May antigen (two opposite wells) and purified *H. influenzae* antigen (two wells in between, bottom left). Centre well contains serum of a patient with mainly specific *H. influenzae* precipitins.

we concluded that endotoxin did not contribute to the precipitate as shown in Fig. 4.

COMPARISON OF METHODS USED FOR ENDOTOXIN DETECTION

In vitro the LLT shows a high endotoxic activity for the whole bacterium, the high molecular weight fraction I, and the extracted lipopolysaccharide (Table 1). The lower molecular weight fractions contain less endotoxic activity.

The *in-vivo* test confirmed the *in-vitro* findings.

Fig. 3 From top to bottom: lipid moiety in Sudan black staining, lipopolysaccharide moiety in PAS staining, and precipitinogen content of *H. influenzae* antigen after immunoelectrophoresis.

After a separation via DEAE-Sephadex A25 the *in-vivo* test confirmed that the main lipopolysaccharide activity is found in the negatively charged fraction, as was suggested by staining techniques. Endotoxin of *E. coli* (Jorgensen and Smith, 1974) is used as a standard; the endpoint in the titration in the LLT is found at 10^{-7} mg.

The LD₅₀ for *E. coli* endotoxin in mice after potentiation with actinomycin-D is 10^{-6} mg (Pieroni *et al.*, 1970). If the amount of lyophilised material used in both experiments is expressed in mg necessary to obtain an endotoxic activity equivalent to 1 µg *E. coli* endotoxin the sensitivities can be compared (Table 2). In the high molecular weight fractions the LLT is more sensitive than the *in-vivo* method, whereas for the low molecular weight fractions the reverse is found.

Table 2 Amount of the various fractions expressed in mg lyophilised material/ml solvent containing an endotoxic activity equivalent to 1 µg *coli* endotoxin as estimated in the limulus lysate test (LLT) compared with the LD₅₀ in mice after potentiation with actinomycin-D

Compounds	LLT	Mouse LD ₅₀	LD ₅₀ /LLT
Bacterium	0.01	0.4	40
Endotoxin	0.001	0.04	40
Cytopl. fraction	1	5	5
Fraction I	0.001	0.5	500
Fraction II	10	20	2
Fraction III	10	30	3
Fraction IV	10	5	0.5
Specific fraction	10	15	1.5
Non-specific fraction	10	0.5	0.05

The results of the chemical analysis and the molecular weight of the fractions are summarised in Table 3. It was shown that fraction I, II, and III had an increasing protein/carbohydrate ratio. The low molecular weight fraction IV, however, had a low protein/carbohydrate ratio. Unsaturated lipids were detected in the bacteria and in fraction IV, whereas the saturated fatty acids were mainly visualised in the bacteria, fraction I, the fraction IV.

Table 3 Molecular weight of the fractions obtained in column chromatography Sepharose 6B. Content of protein and carbohydrate expressed as a ratio. Unsaturated lipid in percentage of original amount in dry weight. Saturated fatty acid graded from 0 to +++ in thin-layer chromatography

Procedures	Molecular weight	Protein (%)	Protein/carbohydrate	Unsaturated lipids	Saturated fatty acid
Bacterium		54	2.1	4	+++
Sepharose 6B					
Fraction I	≥ 340000	27	2.4	ND	+++
Fraction II	210000	30	3.4	ND	+++
Fraction III	44000	52	11.6	ND	+++
Fraction IV	≤ 23000	7	1.8	2	+++
DEAE-Sephadex A25					
Specific fraction III S		24.5	2.9	ND	+++
Non-specific fraction III AS		33	2.3	ND	+++

ND = not detectable.

Discussion

Gram-negative bacteria are commonly used in skin and bronchial provocation tests (Barr *et al.*, 1965; Brown, 1934; Hajós, 1963; Hajós and Hajós, 1964; Herxheimer, 1951; Hirdes, 1969; Ricci *et al.*, 1967). Seemingly, however, no thought was given to the possibility that the reaction could be due to endotoxin rather than an antibody-antigen mediated reaction. For detecting endotoxin the LD₅₀ in mice after actinomycin-D was used as an *in-vivo* method.

Despite the limitations of the LLT's non-specificity (Elin and Wolff, 1973; Stumacher *et al.*, 1973; Sullivan and Watson, 1974; Wildfeuer *et al.*, 1974; Weckesser *et al.*, 1974) its usefulness is well established (Cooper *et al.*, 1971; Jorgensen and Smith, 1973; Levin *et al.*, 1970). To what extent particle size and electrophoretic mobility of compounds were related to endotoxic activity was unknown. The purification of the *H. influenzae* precipitins gave us the opportunity to find out.

To separate the specific precipitinogen from endotoxin we used the following characteristics of the latter: (1) its localisation in the cell wall, (2) its high molecular weight, and (3) its electrophoretic mobility (Fig. 1). After centrifugation the supernatant proved to be contaminated with water-soluble free endotoxin (Jorgensen and Smith, 1974), probably due to the ultrasonic disruption. The cell wall itself was completely removed from the cytoplasmic fraction. The highest concentration of endotoxin was found in fraction I after gel filtration by Sepharose 6B. The fractions II-IV, however, still had a considerable endotoxic activity. Since the molecular weight of these fractions is below that of the endotoxin (Table 3) when prepared by the phenol-water extraction (Lüderitz *et al.*, 1966) this activity is probably the result of fragments of the endotoxin, as described by Marx *et al.* (1968).

Interestingly, the sensitivity for the endotoxin in the high molecular weight fractions, as found in the *H. influenzae* bacteria and fraction I, was about 10^3

times higher in the LLT when compared with the toxicity in mice. This discrepancy was not found in the low molecular weight fractions II and III and only partly found in fraction IV (Table 1).

This suggests that fragmentation of the endotoxin molecule results in a diminished endotoxic activity in mice. Nowotny *et al.* (1975) studied a polysaccharide-rich fraction from *Salmonella* endotoxin with an average molecular weight of about 10 000 which gave neither a mortality in mice nor a positive LLT. Ng *et al.* (1974), however, found a glycolipid from rough growing bacteria with a molecular weight between 15 000 and 18 000 with a high endotoxic activity when measured in the LLT and in mice.

The clotting reaction in the LLT is probably an enzymatic process (Young *et al.*, 1972). The component in the lipopolysaccharide responsible for the reaction is unknown. The toxic effect in animals is caused mainly by 'lipid A' (Milner *et al.*, 1971), but polysaccharides play an important role as well (Westphal and Lüderitz, 1954).

Tables 1 and 3 show that a compound present in fraction IV with an average molecular weight of about 23 000 was still toxic for mice. The positive LLT and the potentiating effect of actinomycin-D suggest an endotoxic activity. The fraction had a very low protein/carbohydrate ratio, but some lipid compounds and unsaturated fatty acids were detectable (Table 3). The glycolipid demonstrated by Ng *et al.* (1974) might be present in this fraction and be responsible for the clotting reaction. Since we were dealing with unpurified endotoxic preparations it was impossible to correlate the chemistry with either mortality in mice or clotting properties in the LLT.

The separation obtained with the DEAE-Sephadex showed a difference in sensitivity of both test methods in the positively charged and the negatively charged fraction, which was partly caused by the insolubility of the former. This difference suggests that compounds difficult to resolve should be tested *in vivo* and in the LLT.

Conclusion

To discriminate between an immunological and a toxic mechanism a specific precipitinogen and endotoxin had to be prepared from *H. influenzae*. A combination of ultracentrifugation, column chromatography (Sephadex 6B), and ion exchange chromatography (DEAE-Sephadex A25) resulted in a bacterial extract that contained the specific precipitinogens and a very low endotoxic activity. Aqueous phenol, chloroform, and petroleum ether extraction were performed to obtain endotoxin.

Both the LLT and the mortality in mice after

actinomycin-D potentiation proved to be sensitive and reproducible methods for detecting endotoxic activity in bacterial compounds. It was shown that the low molecular weight fractions from *H. influenzae* still contained endotoxic activity. The LLT proved to be more sensitive in the high molecular weight fractions, whereas the *in-vivo* method was more sensitive in the low molecular weight fractions. We can offer no explanation for this phenomenon. The use of both methods together seems justified.

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