Bacterial lipopolysaccharide and its lipid A component: some historical and some current aspects

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O-antigen and endotoxin

The cell wall of Gram-negative bacteria — such as Salmonella, Shigella, Escherichia and others — contains a firmly bound toxic principle which exerts biological actions mainly after its release from disintegrating cells. It is called endotoxin. Since the work of André Boivin in Paris in the 1930s it has been known that endotoxin is closely related to the species-specific antigen, the O-antigen. Boivin, who introduced the method of extraction by trichloroacetic acid (Boivin & Mesrobeanu, 1933), termed the complex 'antigène glycido-liquipide'. Walter Morgan at the Lister Institute, London, was the first to perform a thorough and detailed study of the purified O antigen of Salmonella and Shigella strains, extracted by his diethyleneglycol method (Morgan, 1937). The water-soluble, high-molecular-weight complex was shown to be composed of a polysaccharide component which carries the serologic species-specificity (O-antigen determinants), a 'conjugated protein' which conferred immunogenicity to the polysaccharide hapten, and a loosely bound kephalin-like lipid (Morgan & Partridge, 1940, 1942). Morgan also developed procedures for the dissociation of the complex, such as the formamide and the phenol methods (Morgan & Partridge, 1941). He demonstrated that the (phosphorous-containing) conjugated protein could be further dissociated to (phosphorous-free) 'simple protein' devoid of immunogenicity. In Morgan's hands the conjugated protein turned out to be an ideal immunogenic carrier, rendering non-immunogenic polysaccharides, like the blood-group-active mucopolysaccharides, by complexing, into powerful saccharide-specific immunogens (Morgan, 1943).

Morgan's interest concentrated mainly on the immunological properties of the polysaccharide component, and he was certainly greatly impressed by the work of the Rockefeller group of Oswald Avery and Walter F. Goebel in New York on the type-specific polysaccharides of Gram-positive Pneumococci. It was, therefore, not surprising that he should include other serologically active polysaccharides in his research activities and this led to his pioneering work in the field of

Friedenreich, V. & Hartmann, G. (1938) Z. Immunitätsforsch. 92, 141–150
Hartmann, G. (1940) Group Antigens in Human Organs, Munksgaard, Copenhagen
Maisonrouge-McAuliffe, F. & Kabat, E. A. (1976) Arch. Biochem. Biophys. 175, 90–113

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†Abbreviations used: KDO, 3-deoxy-D-manno-octulosonic acid, previously known as ketodeoxy-octonico acid; 2-3-OH-14:0, 2- and 3-hydroxymyristate; m.p.d., minimal pyrogenic dose.

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human blood group-active substances in which he became the master.

W. F. Goebel, working on the O antigenic complex of Shigella flexneri in the 1940s, tried to focus on its 'toxic component' (T). He showed, by applying different hydrolytic procedures, that the complex could be dissociated either into toxic polysaccharide and non-toxic protein (Morgan's simple protein) or into non-toxic polysaccharide (hapten) and toxic protein (Morgan's conjugated protein). But the chemical nature of the toxic determinant remained uncertain (Tal & Goebel, 1950).

In the 1950s, our group entered the field, originally from another point of view: the pyrogenicity of the complex (Westphal et al., 1952a,b,c; 1977). Walter Morgan in 1950 kindly provided samples of all defined subunits of his diethylene-glycol extracts, and we found that both his protein-free polysaccharide (as obtained by phenol dissociation) and his conjugated protein exerted high fever-producing activity in rabbits. Applying a variation of the original phenol method, we were then able to prepare purified lipopolysaccharide as the most active pyrogenic principle of Gram-negative bacterial cell walls. Lipopolysaccharide could be split by mild acid into pure polysaccharide (hapten) and the lipid component, called lipid A (Westphal & Lüderitz, 1954). On the basis of biological tests, we proposed in 1953 that the ubiquitous lipid A component was responsible for pyrogenicity as well as for other endotoxic manifestations.

It took about 20 years before the hypothesis was shown to be correct, mainly through chemical and biological analyses of bacterial rough mutants (R forms) which were generated from the wild-type smooth organisms (S forms) by S→R mutation (Lüderitz & Westphal, 1966). In these mutants (R₁, R₂,..., Rₕ) blocks at various stages of the multi-step polysaccharide biosynthesis (frequently in the activities of sugar transferases) led to the formation of lipo-oligosaccharides with shorter sugar chains, and these were lacking the typical O-specific sugars, like deoxy- and dideoxyhexoses (Westphal & Lüderitz, 1960).

Lipid A

The most deficient R strains, the Rₕ mutants, were found to synthesize a glycolipid composed mainly of lipid A and 3 molecules of KDO (Galanos et al., 1977). It was possible to split the acid-labile ketosidic KDO linkages by mild acid treatment to arrive at lipid A free of any additional sugar. Free lipid A was thus obtained as a water-insoluble precipitate, soluble in chloroform.

Bacteria taxonomically remote from Enterobacteriaceae may synthesize lipopolysaccharide in which the link between lipid A and the polysaccharide is not mediated by KDO (see Galanos et al., 1977). Here stronger acidic conditions have to be applied which, consequently, either lead to lipid A preparations which are partly degraded or, else, still contain saccharide fragments.

Fig. 1 shows the structure of Salmonella lipid A (Rietschel et al., 1981). This unusual type of phospholipid is built up by a central disaccharide of two β-(1→6)-linked β-glucosamine (GlcN) units, GlcN I (with the reducing end) and GlcN II. The phosphate esters are bound in the 4- (of GlcN II) and the 1- (of GlcN I) positions. P-GlcN-GlcN-P is termed the lipid A backbone.

According to our analyses (Rietschel et al., 1981) the lipoidic character of the molecule is given by substitution of the backbone with 7 mol of long-chain fatty acids: 4 mol of 3-hydroxymyristate (3-OH-14:0) and 3 mol of non-hydroxy-
ated fatty acids, i.e. 1 mol each of 12:0, 14:0 and 16:0 respectively, and additional small amounts of 2-0H-14:0. The exact position of the seven acyl ester groups have hitherto been only partly identified. The backbone contains two amino groups substituted by 3-0H-14:0, and, besides the two phosphate-substituted hydroxy groups, another four hydroxy groups, of which position 3 in GlcN II forms the (ketosidic) link to the specific polysaccharide via KDO.

There is evidence that the two ester-bound 3-0H-14:0 acids are linked directly to the backbone, substituting two of the three available hydroxy groups. The normal fatty acids, in contrast, appear to be linked at the 3-hydroxy groups of the 3-0H-14:0 residues; 14:0 (and 2-0H-14:0) substitute the ester-bound 3-0H-14:0, while preliminary evidence indicates the 12:0 and 16:0 acids to be ester-linked to the two amide-bound 3-0H-14:0. In this way lipid A is characterized by a high content of a rather unusual structure of long-chain fatty acid esters of 3-hydroxy-myristate. Thus only the 3-hydroxylated acids would substitute the backbone directly, two in amide and two in ester linkage, while the non-hydroxylated (and 2-0H-14:0) would be ester-linked to the 3-0H-14:0 groups (H. W. Wollenweber & E. T. Rietschel, unpublished work).

These conclusions are in perfect agreement with the structure evaluated recently for a lipid A precursor molecule (Lehmann, 1977). Temperature-dependent mutants defective in KDO synthesis produce an incomplete lipid A containing the backbone substituted by 4 mol of 3-0H-14:0, two in amide, two in ester linkage. The other fatty acids are absent. The further steps in lipid A biosynthesis (which are blocked in the mutant) include the transfer of KDO (Munson et al., 1978) and the addition of the non-hydroxylated fatty acids.

It should be mentioned that Salmonella lipid A, like R-core and O-polysaccharide chains, exhibits microheterogeneity. Lipid A is partially substituted by amino compounds, such as 4-amino-~-arabinose (at GlcN II) and phosphoethanolamine (at GlcN I) which are linked to the phosphate groups of the backbone (Mühlradt et al., 1977). The amino and hydroxy groups of these substituents (head groups) are unsubstituted (see Fig. 1).

After the structural elucidation of Salmonella lipid A, the nature of lipid A from other bacterial genera and families has become apparent. The data obtained, regarding constituents and partial structures, allow certain conclusions on structural constancy and variability of the lipid A principle (Lüderitz et al., 1978; Rietschel et al., 1981). The lipid A backbone is the most conservative feature in lipopolysaccharide. It is found in Enterobacteriaceae, plant pathogens, certain anaerobics and photosynthetic bacteria. Some variation was encountered regarding the nature of the head groups: besides phosphoethanolamine and 4-amino-arabinose, additional phosphate, GlcN, or arabinoside substituents were identified. The amide-bound fatty acids are without exception in the 3-hydroxy-d-form, but variable in chain length. Greater variation was found in the ester-bound acids: non-hydroxylated, L-2- and D-3-hydroxylated, straight and branched fatty acids with chain lengths from 10 to 20 carbon atoms were identified (Galanos et al., 1977; Wilkinson, 1977).

A lipid A structurally completely different from enterobacterial lipid A was found to be synthesized by Rhodo
pseudomonas viridis and palustris and Pseudomonas diminuta and vesicularis (Fig. 2). This lipid is devoid of phosphate and GlcN. It contains 2,3-diamino-2,3-dideoxy-0-glucose substituted with amide-linked 3-0H-14:0 and acetyl groups (Weckesser et al., 1979).

The variation in structure of lipid A from different sources offers interesting implications. It was found that they may differ in biological activity from highly active (pyrogenicity, lethality) in the case of enterobacterial lipid A to the other extreme of complete inactivity in the case of Rh. viridis (Lüderitz et al., 1978). Comparative investigations of lipid A of different bacterial origin may, thus, lead to the recognition of structure-function relationships and to the identification of biologically active substructures in the molecule, as in the polysaccharide component with respect to serological specificities (Lüderitz et al., 1966).

The natural, although restricted, diversity in the structure of lipid A seems to represent an additional useful tool regarding questions of taxonomy and evolution, including evolutionary aspects of the endotoxic principle in bacteria. As an example, a phylogenetic link between certain species of Rhodopseudomonas and Pseudomonas has been suggested on the basis of their similar, unusual lipid A component (Fig. 2). It is obvious that all components of bacterial lipopolysaccharides, the O chain, the R core, and notably, lipid A, may represent ideal taxonomic markers (Nikaido, 1970; Rietschel & Lüderitz, 1980).

Standard lipopolysaccharide and lipid A preparations

In lipopolysaccharides and lipid A, charged groups play an important role. They include various groups such as the phosphate esters of lipid A, or KDO (in the polysaccharide). In the usual preparative methods used, these charges are neutralized by various counterions (Na+, K+, Mg2+ or Ca2+) or by organic bases. Their proportion within the same preparation largely depends upon the bacterial culture conditions. In our Institute, Galanos & Lüderitz (1975) introduced a method by which uniform salt forms of lipopolysaccharide and lipid A can be produced. It consists essentially of an electrodialysis by which the original mixed salt form of the endotoxin is converted into the free acid which, in turn, is continually being neutralized by one defined cation. Thus, the pure Na+ and Ca2+ salts as well as triethylamine or spermidine salts were obtained. It turned out that cations, although making up only a few percent of the preparations, have great influence on their physicochemical and biological properties. While the (monovalent) triethylamine salt form is of relatively low particle size and highly water soluble, the divalent cations, by establishing cross-links, confer higher particle sizes and lower water-solubility; the Ca2+ salt, for example, forms precipitates.

The salt form reflects also on endotoxic activities. As an example: while the triethylamine salt of lipopolysaccharides and lipid A exerts rather high acute toxicity, is highly pyrogenic in rabbits (m.p.d. i.v. <0.001 /kg) and does not interact with the complement system, the Ca2+ salts on the other hand are much less toxic, of much lower pyrogenicity (m.p.d. in the order of 0.1 /kg), but do strongly interact with complement (Galanos, 1975; Galanos & Lüderitz, 1976). Many discrepancies in this field between various research groups may now be explained on that basis! We strongly emphasize the need to use only standardized endotoxic preparations in order to obtain repro-

\[ \text{Fig. 2. Representation of the lipid component of some Pseudomonas and Rhodopseudomonas strains} \]

The exact link to KDO is still unknown.
ducible results. A procedure for the manufacture of a stand- 
adardized lipopolysaccharide has recently been published (Galanos et al., 1979). In earlier studies on the chemical basis of endotoxins the fact that lipid A was only sparingly soluble in water has hampered clearcut results and conclusions. The tri- 
ethylamine salt, now available, is easily water-soluble (without any additional carrier) and can be produced in standardized form, as can any given bacterial lipopolysaccharide.

**Lipid A as the endotoxic component**

There are a large number of endotoxic activities for which the lipid A component of lipopolysaccharide could be pin-pointed as the active centre carrying the responsible ‘endotoxic’ deter- 
inants (Table 1). The question, then, remains, as to how far, besides the salt form, additional polysaccharide components may have modifying influences on any of these activities. Generally it is fair to say that the activities listed in Table 1 can be elicited by lipid A free of polysaccharide (or oligosaccharide). However, one must be careful in generalizing in this way, as will be apparent from other aspects considered below.

**The tumour-necrotizing activity of endotoxin**

One of the most interesting and intriguing properties of endotoxin is its tumour-necrotizing activity. This has been known for almost one hundred years (see Westphal et al., 1977) and was the subject of many investigations, including clinical trials, especially in cases of sarcomas. Various mouse models were introduced, such as the transplantable sarcomas S 180 or Meth A, the latter a methylcholanthrene-induced fibrosarcoma. We confirmed the pioneering findings of Shear (Shear, 1943; Hartwell et al., 1943) that purified bacterial lipopolysaccharide is the active principle in eliciting necrosis in sarcoma-bearing mice. In 1961, together with Erwin Neter in Buffalo (Mihich et al., 1961), we demonstrated that lipid A had tumour-necro- 
tizing properties in S 180 sarcoma-bearing mice. But other investigators reported that they were unable to confirm our findings.

In the meantime, and since we have water-soluble and standardized lipid A preparations at hand, we have repeated such studies in Meth A-bearing CBF mice. The results clearly show that doses (10–100 μg intraperitoneally) of lipid A, similar to that of lipopolysaccharide curing 100% of the animals, were either almost inactive or led to tumour enhancement (Westphal et al., 1979) and this effect was even aggravated if lipid A was injected twice within 2–4 days. After lipid A injections the tumour looks inflamed (hyperaemia), but there is only little or no subsequent haemorrhage, in distinct contrast to treatment with lipopolysaccharide. However, if animals at a certain tumour size (7–9 mm diameter) are first treated with lipid A, followed 2–4 days later with lipopolysaccharide (10–50 μg i.p.), we observe a more drastic haemorrhage compared to lipopoly- 
saccharide alone, and followed by 100% cure. From an ongoing histological analysis (N. Freiberg, unpublished) it becomes clear that lipopolysaccharide treatment proceeds in (at least) two steps: first a rapidly occurring tumour-localized hyperaemia (within hours), followed later by cellular breakdown, necrosis and rejection (within days). It turns out that free lipid A (10–25 μg) produces only hyperaemia in the tumour, whereas lipopolysaccharide produces both hyperaemia and necrosis.

In our earlier studies on lipid A as an inducer of S 180 sarcoma necrosis, owing to problems of solubility of our preparation at the time, we used lipid A complexed with low-molecular-weight dextran as water-solubilizing (dispersing) carrier (Mihich et al., 1964). We have repeated studies on Meth A tumour-bearing mice: while free lipid A (triethylamine salt) is indeed almost inactive, lipid A complexed with dextran or suitable dextran derivatives is nearly as active as lipopolysaccharide. This fact seems to explain the difference in tumour-necrotizing activities of pure lipid A, polysaccharide-complexed lipid A and whole bacterial lipopolysaccharide. Further elucidation of these findings may lead to a more differentiated approach to ‘therapy’ in which the two steps will be optimized more selectively. Endotoxin-mediated tumour necrosis is one of the few known examples where bacterial lipopolysaccharide and pure lipid A exert different activities, probably due to different fates in vivo on the basis of different lipophilic/hyrophilic character.

The localization of lipopolysaccharide action on the tumour is reminiscent of investigations about the anatomical site of high-dose toxin action in normal experimental animals in which Tonutti (1949, 1950) showed that hyperaemia and haemorrhage in hormonal glands is governed by the respective trophic 
hormone. Hypophysectomised animals do not show toxin- 
mediated haemorrhage of the glands. If such animals are given toxin plus trophic hormone, the haemorrhagic reaction concentrates in the target gland of that trophic hormone—it can be directed into other glands by concomitant injection of toxin and the adequate trophic hormone. This raises an interesting question, whether sarcomas may be a target for hormone action? It is well known that endotoxin activates the hypo- 
physis for trophic hormone production and release, with a subsequent stimulation of the respective gland (see Westphal et al., 1952b). It also raises the question whether there may be a difference between lipopolysaccharide and lipid A in their capacity to stimulate the hypophysis.

We have demonstrated that some endotoxic properties are not prerequisites for tumour-necrosis. For example, prostaglandin synthetase inhibitors, such as indomethacin, which inhibit lipopolysaccharide-induced prostaglandin biosynthesis, do not interfere with the lipopolysaccharide-induced tumour necrosis. On the contrary, they even enhance necrosis, and the animals tolerate the rather high doses of lipopolysaccharide much better (almost no weight loss etc.) (Westphal et al., 1979; see also Brunda et al., 1980). This leads to additional pharmacological approaches in an attempt to minimize toxic ‘side effects’ of lipopolysaccharide, such as those evolved by certain prostaga- 
ldins. In this context another aspect is of interest: the finding was made at the Sloan–Kettering Cancer Centre, New York, that high (toxic) doses of lipopolysaccharide induce the pro- 
duction of an endogenous mediator (called TNF) that causes tumour regression and necrosis without toxic side effects (Carswell et al., 1975).

The first step of endotoxic action on tumours appears to be non-immunologic in nature (Jones, 1980; Jones et al., 1980; see also Overgaard & Overgaard, 1979). Whether further events leading to necrosis and rejection involve immunological components is presently under discussion. CBF mice, once completely cured from a Meth A tumour by lipopolysaccharide
treatment, are highly resistant against another challenge with large numbers of Meth A cells; they develop macrophages that are cytotoxic toward Meth A tumor cells (H. Taskov & M. L. Lohmann-Matthes, unpublished work; see also Hibbs et al., 1977).

For the chemist and biochemist the question is, what are the necessary signals (determinant structures) that stimulate the various steps of the process, and can these phases be induced more selectively, under less 'heroic' conditions (with fewer side effects)? Endotoxin-mediated tumor necrosis is one of many known endotoxic manifestations (see Table 1), but still offers a wide field of research, and much clinical interest, in which various disciplines should interact for a final elucidation of the mechanisms of this very impressive phenomenon.

Homage to Walter Morgan

When our group in Göttingen, during the Second World War, started to work on enterobacterial O antigens, with the idea of creating urgently needed, better vaccines, we asked experts in the field, like Professor Hans Schmidt, chief of research of the Behring-Werke in Marburg, for his advice. Hans Schmidt answered: 'I think I cannot do better than to let you have a copy of a copy of a copy of a microfilm with two articles from the Lister Institute, London, written by Walter Morgan, and smuggled into Germany via Switzerland. . . . Here every possible question appears to be answered as far as presently available techniques allow'. For years these classical publications of the Morgan group on enterobacterial O antigens were something like a bible for us. They are written in such a concentrated, yet modest fashion that, reading them again and again, they always furnish more and more insight into the very complex matter. Without his basic work, all that followed would have had to start at the very beginning. Today the authors wish to thank Walter Morgan for the establishment of a clear base to the field, on which every successor was (and is) able to build up further chapters of the story on solid ground, not to speak about the continuous interest and friendship of Walter Morgan that we have enjoyed since!

'Little (only) Walter did not hit
To leave to others still a bit' . . .

Morgan, W. T. J. (1943) Br. J. Exp. Pathol. 24, 41–49
Tomutti, E. (1949) Die Pharmacie 4, 441
Tomutti, E. (1950) Dtsch. Z. Chr. 264, 61

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