Lipoidal Components of Bacterial Lipopolysaccharides: Nature and Distribution of Fatty Acids in *Aerobacter aerogenes*

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The fatty acid distribution of *Aerobacter aerogenes* was studied by comparing the fatty acid composition of the lipoidal component of the endotoxin (lipid A) with the fatty acids of the readily extractable native lipids and total cellular fatty acids. The results for total cellular fatty acids and readily extractable native lipids were generally similar, but both quantitative and qualitative differences exist. In addition, profound differences between these two fractions and lipid A were observed. These differences included fewer fatty acids and lower concentrations of unsaturated and cyclopropane fatty acids in the lipid A. Hydroxy fatty acids persisted in the lipid A. The significance of these differences with respect to mammalian toxicity of endotoxins is discussed.

For many years, it was surmised that in gram-negative bacilli pathogenicity is associated with endotoxins or cell wall lipopolysaccharide complexes. The extent to which such substances are responsible for the pathogenic processes of such organisms is far from clear and in dispute. It is clear that endotoxins produce definite, although nonspecific, pathological effects, including pyrexia, shock, vascular disturbances, and tissue damage. The bulk of currently available evidence indicates that the toxicity of endotoxin complexes resides principally in the lipoidal moiety, the so-called lipid A. If the pathogenic processes produced by microorganisms are to be understood rather than described, a biochemical explanation is necessary, and, in the case of gram-negative bacilli, this would at least include a detailed knowledge of lipid A fractions.

Unfortunately, our knowledge of lipid A compositions is still sketchy at best (as is true of most lipid components of microorganisms), and our knowledge of the function of such substances is virtually nil.

A major obstacle to research in this area has been the difficulty of obtaining adequate amounts of high purity lipid A for meaningful analyses. However, recently several approaches to this problem have been devised; we have found the method of Burton and Carter (2) to be quite satisfactory for this purpose. Following the availability of such methods, a number of studies have appeared presenting data on the composition of lipid A fractions in several gram-negative bacteria, e.g., Homma and Suzuki (5) and Kasai (6).

The following communication presents our studies on *Aerobacter aerogenes* in which we have attempted to characterize the component fatty acids of the lipid A by gas chromatography. These results are compared with the compositions of readily extractable native lipids and with total cellular fatty acids. Comparisons are made with the results of similar studies on various enteric pathogens.

MATERIALS AND METHODS

Bacteria. *A. aerogenes*, strain 62, is a methionine-requiring auxotroph that was employed in a number of studies in our laboratory in recent years. Its characteristics, as well as the procedures used in its isolation, have been described in detail by Shapiro (13).

Medium and culture methods. This organism was grown in the chemically defined medium of Davis and Mingioli (3) to which was added 20 mg of L-methionine per liter. Concentrated methionine and glucose solutions were autoclaved separately and combined with the bulk of the medium after cooling. Multiliter cultures were incubated 24 hr at 37 C in a New Brunswick shaking incubator oscillating at 300 strokes/min. Cells were harvested by centrifugation at 9,500 × *g* for 10 min in a Lourdes model A-2 centrifuge, washed with distilled water, and recentrifuged. The cell pastes were then lyophilized and weighed. The average yield was 0.7 g (dry weight) of cells per liter of growth medium.

Chemicals. Methyl-^{14}C-L-methionine was purchased from New England Nuclear Corp., Waltham, Mass. Chromatographic standards were obtained from Applied Science Laboratories, State College, Pa. All organic solvents were redistilled before use.

Extraction and analysis of readily extractable lipids. Chloroform-methanol extracts containing the readily extractable native lipids of the cells were obtained by the classical method of Folch et al. (4).

The fatty acid contents of materials extracted by this method were liberated by refluxing samples under nitrogen for 5 hr in a mixture of ethyl alcohol, water, and KOH (100:60:9). This hydrolysate was acidified and extracted with pentane. The pentane extract was dried over anhydrous Na_2SO_4, after which the solvent was removed under reduced pressure. The fatty acids were stored in vacuo over P_2O_5. Methyl esters of these fatty acids were made by use of boron trifluoride in methanol by the method of Metcalf and Schmitz (9). The methyl esters were analyzed with a Perkin-Elmer model 154D fractometer fitted with 1-m columns of diethylene glycol succinate (DEGS), butanediol succinate (BDS), or DC-710 silicone. For scintillation counting of radioactivity, individual fatty acids exiting from the gas chromatograph were collected in glass traps cooled to -70 C. These traps were then rinsed with scintillation fluid [3.1 g of Pre-Mix P, (Packard Instrument Co., Inc., LaGrange, Ill.) per liter of toluene] into suitable counting vials and counted in a model 2002 Packard scintillation spectrometer.

Extraction and analysis of total cellular fatty acids. The total fatty acids from whole cells of *A. aerogenes* were obtained by refluxing cells under nitrogen in 20% KOH in 95% ethyl alcohol for 4 hr. The recovery of acids from the hydrolysis mixture, methylation, and gas chromatography were performed with the methods already described.

Isolation and characterization of lipid A. The crude cell wall lipopolysaccharide of *A. aerogenes* was obtained with a modification of Westphal's phenol extraction procedure (14). Isolation of lipid A from this crude lipopolysaccharide was achieved by a combination of low-speed centrifugation, ultracentrifugation, and ethyl alcohol fractionation devised by Burton and Carter (2). This procedure yields six fractions, two of which contain lipopolysaccharide. After determination of the incorporation of radioactive methionine methyl carbon into each fraction by methods described above, the two lipopolysaccharide fractions were combined and degraded to release lipid A as described by Burton and Carter (2). Gas chromatographic methods already described were used to characterize this lipid A fraction.

RESULTS

The incorporation of methionine methyl carbon into various lipid fractions was followed in the early stages of this investigation in order to obtain an indication of where cyclopropane acids might be located. It is known that the ring carbon of such acids is derived solely from the methyl group of methionine (11). The distribution of radioactivity among various portions of the bacterial cell are given in Table 1. The bulk of activity was found, as expected, in nonlipoidal fractions. Appreciable activity was also observed in the chloroform-methanol extract (which does not include endotoxin lipopolysaccharide). Fractionation of

TABLE 1. *Percentage yield and specific activities of various cellular fractions of A. aerogenes grown in the presence of methyl-^{14}C-methionine[a]*

Fraction	Percentage yield[b]	Specific activity[c]
Total cellular fatty acids...............	6.0	21,361
Chloroform-methanol extractable lipid...............	9.4	18,805
Cell residue after removal of crude lipopolysaccharide.	55.8	12,350
Crude lipopolysaccharide...........	21.0	12,150

[a] Growth medium contained 1.5×10^6 counts/min of methyl-^{14}C-L-methionine.

[b] Fraction weight dry cell weight.

[c] Counts/min per mg.

TABLE 2. *Characteristics of fractions obtained from crude lipopolysaccharide*

Fraction	Percentage yield[b]	Specific activity[c]
F_1, peptides[a]..............	0.2	4,778
F_2, peptides...............	0.7	9,238
F_3, lipopolysaccharide......	27.0	2,207
F_4, contains F_5 and F_6......	16.1	1,844
F_5, nucleic acids...........	7.1	219
F_6, lipopolysaccharide......	3.3	2,270
Lipid A....................	2.0	1,244

[a] F_{1-6} = fractions obtained during Burton-Carter purification of lipid A.

[b] Fraction weight per dry weight of crude lipopolysaccharide.

[c] Counts/min per mg.

crude lipopolysaccharide by the method of Burton and Carter (2) yielded seven fractions (Table 2). The lipid A fraction which represents the lipoidal content of endotoxin lipopolysaccharide contained relatively little radioactivity, which suggested only a minor content of cyclopropane fatty acids in this fraction, an observation that was born out by subsequent analyses.

The results of gas chromatographic analyses of the fatty acids of various cell fractions are given in Table 3. This study revealed that the total cellular fatty acid content was similar in most respects to that reported earlier (10); however, there are several notable differences. In the previous study, 12 fatty acids were detected, whereas the present investigation revealed 17. The detection of five additional compounds was likely caused by the employment of more selective and sensitive columns and techniques. The additional fractions include small amounts of what appeared to be

C_{13} and C_{15} cyclopropane acids, an equally small amount of what corresponds to a C_{17} normal saturated acid, and a persistent indication of a compound that behaves chromatographically like a C_{19} cyclopropene acid. There have been sporadic but recurrent suggestions of such substances in the literature (11); this is an aspect that merits further investigation. The last compound observed in this study that was not reported in the prior investigation (10) is what appears to be a hydroxy acid, probably hydroxymyristic acid, judging from its chromatographic behavior on BDS, DEGS, and DC-710 silicone columns.

Table 3 also compares the composition of the total cellular fatty acids with the fatty acids of the chloroform-methanol extracted lipids and of lipid A. There are general similarities, but both qualitative and quantitative differences were noted between the total cellular fatty acids and those of the chloroform-methanol extractable lipids, as might be expected. The lipid A, however, differed markedly from both the total cellular fatty acids and the readily extractable lipids with respect to fatty acid content. While many differences are evident, the most interesting ones were the low

TABLE 3. *Fatty acid compositions of total cellular fatty acids, chloroform-methanol extractable lipid, and lipid A of A. aerogenes strain 62*[a]

Fatty acid	Content (%)		
	Total cellular fatty acids	Chloroform-methanol extractable lipid	Lipid A
C_{10} saturated.........	0.1	trace	4.4
C_{12} saturated.........	5.9	0.05	20.2
C_{13} cyclopropane (?)...........	0.2	trace	0
C_{14} unsaturated.......	8.1	3.9	11.5
C_{14} saturated.........	1.5	0	0
C_{15} saturated.........	1.0	1.3	9.8
C_{15} cyclopropane (?)...........	4.2	0	0
C_{16} unsaturated......	30.0	43.1	6.8
C_{16} saturated.........	15.6	25.5	5.5
C_{17} saturated.........	0.9	1.2	0
C_{17} cyclopropane.....	10.1	13.7	5.9
C_{18} unsaturated.......	0.4	0.7	0
C_{18} saturated.........	5.8	8.0	29.8
C_{19} cyclopropane.....	1.5	1.2	0
C_{19} cyclopropane (?)...........	1.3	0.3	0
C_{20} saturated.........	0.7	0	0
C_{14} 3-hydroxy........	12.9	1.0	6.7

[a] Fatty acids were determined by gas chromatography using a BDS column.

concentrations of unsaturated and cyclopropane fatty acids in the lipid A, compared with the total cellular fatty acids and with those of readily extractable lipids, and the persistence of hydroxy fatty acid in the lipid A. It will be noted that the lipid A contains far fewer fatty acids than other fractions of the cell and that quantitative differences are marked.

DISCUSSION

The increasing number of lipid components detected in previously investigated organisms (as techniques become more refined and discriminating) shows that in spite of all the studies that have gone before, we are still uncertain about the composition, let alone the function of microbial lipids. This investigation also shows that studies of the overall fatty acid content of whole cells, while significant in itself, is not very informative regarding specific lipoidal fractions; i.e., the distribution of fatty acids is far from homogeneous in both physical and chemical fractions.

In this study, we were particularly interested in whether the composition of the lipid A (i.e., the lipoidal component of "endotoxin") differed in any distinctive way from the overall cellular lipids. We have found that there are marked qualitative and quantitative differences that were described above. However, these differences are not very remarkable with respect to potential mammalian toxicity. The general concept of gram-negative endotoxins has been that they are somehow peculiarly injurious to mammals, that toxicity is largely due to the lipid portion of the endotoxin (lipid A), and that, therefore, lipid A must have some particularly offensive aspect with regard to mammalian physiology. It has been repeatedly reported that gram-negative rods are rich in cyclopropane fatty acids which are not found in mammals (7, 11); the question has arisen as to whether these unique compounds might be prominent in the lipid portions of bacterial endotoxins. In this study, we have shown that at least in *A. aerogenes* this is not the case; that, indeed, the reverse is so in that the lipid A of this organism contains little cyclopropane acid and an abundance of innocuous fatty acids.

We chose to begin our studies of fatty acid distribution among lipid A and other cellular fractions in the enteric group by using *A. aerogenes*. There were several reasons for choosing this organism: (i) it is a typical member of this group; (ii) its lipid content and metabolism have been described in considerable detail; (iii) it is known to contain significant amounts of cyclopropane acid, particularly C_{17}, which could be

readily followed with isotope techniques such as described above (10); and (iv) it is readily grown and handled with considerable safety compared with the more pathogenic species. It should be noted, however, that, as extensive clinical experience at this institution and elsewhere has shown, *A. aerogenes* itself is fully capable of initiating pathogenic processes in humans, particularly when they are suffering from malignancies and other debilitating diseases or undergoing prolonged antibiotic therapy; in such cases it produces typical "endotoxin shock" (8, 15). Indeed, the F_3 fraction of the organism employed in this investigation produced a mean temperature rise in rabbits of 1.8 C when administered parenterally at a level of only 1.0 $\mu g/kg$ of body weight.

Our findings with *A. aerogenes* are in various ways congruent with observations reported for *Escherichia coli* (2), *Serratia marcescens* (1), and *Proteus* P18 (12). As these reports suggested, we noted in *A. aerogenes* that cyclopropane acids were less prominent in lipid A than in other lipid fractions while hydroxy acids persist. Indeed, it has been suggested that an absence of cyclopropane acids and a relatively high content of hydroxy acids may be distinctive aspects of lipid A fractions (7). We did not observe such a sharp distinction in *A. aerogenes*; we did find a decrease in cyclopropane fatty acids compared with other fractions and a persistence of hydroxy acid in the lipid A. The concentration of hydroxy acid in lipid A was not major and did decrease compared with total cellular fatty acids, although it was nearly seven times higher than what was found in the readily extractable lipid. Whether this is characteristic only of *A. aerogenes* or whether the distinction between lipid A fatty acids and other cellular lipids is not as clear-cut as previously suggested is yet to be determined.

Eventually, we are interested in extending the methods and concepts developed in the studies of these peripheral enterics to the understanding of the more troublesome endotoxin-producing pathogens. Following the lead of the studies already described, we have made some preliminary examinations of species of both *Salmonella* and *Shigella*; with these species we have obtained results quite similar to those reported for *A. aerogenes*. We have not as yet observed any striking differences between any of the lipid A preparations examined, nor has anyone else. This is consonant with the fact that endotoxin preparations from whatever organism when administered to experimental animals produce similar effects and not the specific diseases attributed to the individual organisms from which the endotoxins

were produced. Indeed, "endotoxins" from what are commonly regarded as nonpathogenic gram-negative rods also produce effects similar to those associated with endotoxins from pathogenic species. Endotoxins are as a class offensive to mammals and produce a common recognizable syndrome, but, in contrast to exotoxins, they do not produce different clinically recognizable diseases.

This commonality of physiological effect is in harmony with what we have so far been able to determine with respect to the chemical nature of endotoxin lipid A fractions. While there are discernible differences between such fractions from different species, these differences are minor and unremarkable.

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