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Relationship of Structure to Function in Bacterial Endotoxins
IX. Differences in the Lipid Moiety of Endotoxic Glycolipids

AH-KAU NG, R. CHRISTOPHER BUTLER, CHEN-LO H. CHEN, AND ALOIS NOWOTNY*
Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

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Chemical, immunochemical, chromatographic, and endotoxic properties of five chromatographically pure glycolipids were compared. The preparations were extracted by chloroform-methanol from three *Escherichia coli*, one *Salmonella minnesota*, and one *S. typhimurium* Re heptoseless mutant strains. The local Shwartzman skin assay, the nonspecific resistance-enhancing effect, and the *Limulus* assays could not distinguish among the five glycolipids, all five being active in all three assays. Significant differences could be seen when the tumor resistance-enhancing effect of the glycolipids in mice was compared with the nonspecific TA3-Ha murine mammary adenocarcinoma growing in ascites form. Even greater variation was observed in the capacity of the preparations to enhance the nonspecific resistance of mice to virulent *S. typhi* 0901 infections. The data show that the five glycolipids are quite dissimilar in their biological effects. Similarly, thin-layer chromatography and molecular ratio determinations showed that differences exist in the chemical structure of the glycolipids. Accordingly, we claim that not only the polysaccharide but the lipid moiety as well may vary in various gram-negative endotoxin preparations.

It has been claimed that the chemical structure of the lipid moiety (called "lipid A") of all *Salmonella* lipopolysaccharides (LPS) is identical or at least very similar. It was even supposed that this may apply to all *Enterobacteriaceae* (6, 8, 10, 16, 17). We questioned the validity of this generalization (13) since no experimental substantiation of the claim has been presented. In an earlier publication which is relevant to this point, our data indicated that the large variety of chemical differences in the bacterial LPS is not restricted to the polysaccharide region of the LPS, but variations in the lipid moiety also exist (11).

A systematic investigation of this question became possible through the availability of Re mutants of gram-negative bacteria, which synthesize only the lipid moiety of the LPS with 2-keto-3-deoxyoctonate bound to it. This material has been named endotoxic glycolipid (7) since almost all of the characteristic endotoxicity reactions could be demonstrated in it. This glycolipid was isolated by us, using preparative thin-layer chromatography (TLC) as the last step of purification. We obtained and reported for the first time a chromatographically pure endotoxic glycolipid (4). The biological activities, as well as molecular ratios, of such preparations were studied (3, 5). The same procedures were used for the isolation, purification, and biological and chemical studies of a glycolipid from an *S. typhimurium* 1114 Re mutant (11) and, recently, of three similar glycolipids from Re mutants of *Escherichia coli* strains (described below).

In the present study, five different glycolipids were isolated, and their biological and immunochemical activities, as well as chromatographic behavior and chemical composition, were compared.

MATERIALS AND METHODS

The heptoseless Re mutant strains were obtained from the following laboratories. *S. minnesota* R595 was furnished through the courtesy of O. Lüderitz, Max Planck Institute for Immunobiology, Freiburg, West Germany. G. Schmidt of the same Institute donated the *E. coli* F515 strain. B. A. D. Stocker, Stanford University, Stanford, Calif., provided the *S. typhimurium* SL1102 strain. H. G. Boman of the University of Umea, Umea, Sweden, supplied the *E. coli* D21f2 and D31m4 mutants. (The same author carried out comparative studies on those strains as well as on *S. minnesota* R595 [1].)

The bacteria were cultivated in a broth culture medium consisting of 1.5% tryptone (Difco), 0.5% beef extract (Difco), 0.3% sodium chloride, 0.23% Na_2HPO_4, 0.5% yeast extract (Difco), 1% glucose, and 0.02% $MgSO_4 \cdot 7H_2O$. The cells were harvested at late log phase and lyophilized, as reported earlier (7).

Extraction of the endotoxic glycolipid directly

from lyophilized cells was carried out with chloroform-methanol (4:1, vol/vol) by refluxing for 6 h, as described earlier (4). The extracted glycolipid was then precipitated with methanol until the final concentration of chloroform-methanol was 1:2. The precipitate was dissolved in chloroform-methanol (4:1) again, and this procedure was repeated three times. The final glycolipid products were stored dissolved in chloroform-methanol (4:1) at 4 C.

Preparative thin-layer chromatographic plates were made with silicic acid BioSil A (2- to 10-μm particle size) for the isolation of chromatographically pure glycolipids (4). The solvent system used was chloroform-methanol-water-concentrated ammonium hydroxide (100:80:20:4, by volume).

Determination of the molar ratios of the TLC-separated components was carried out by the procedure described earlier (5). Biological activities of the isolated fractions were also determined as described previously (3).

The tumor resistance of mice was determined by the TD_{50} procedure. TD_{50} indicates the number of viable tumor cells that will cause 50% tumor deaths in the experimental animals. Endotoxin-induced enhanced resistance against this tumor is manifested by increased TD_{50}'s (17).

The nonspecific resistance-enhancing effect of the preparations against viable *S. typhi* 0901 bacterial challenge was measured in mice. The procedure used was described earlier (12a), and the activity of the preparations is expressed as PD_{50}. This means the amount of preparation that reduces the mortality (observed in non-pretreated controls) by 50%.

RESULTS

The results showed that the biological activities of the three *E. coli* glycolipids were similar in the *Limulus* lysate gelation and local Schwartzman skin reactivity. The activities could not be distinguished on a weight basis from each other or from those of smooth *S. minnesota* 1114 or *Serratia marcescens* purified LPS. Comparison of the three *E. coli* glycolipids with the two *Salmonella* glycolipids showed minor differences, the *S. minnesota* R595 being the most active of all five.

We reported earlier that *S. minnesota* R595 glycolipid is more active than smooth endotoxic LPS in inducing necrosis of subcutaneous sarcoma 37 in mice (14). Nigam and associates published findings (2, 9) describing the fixation of such glycolipids to transformed cell membranes. More recently, we measured the resistance of ICR mice against TA3-Ha, a nonspecific tumor line that grows in ascites form and which is derived from a spontaneous mammary adenocarcinoma developed in A/HeHa mice. Previously we reported that an injection of smooth endotoxic LPS could protect the mice from subsequent tumor challenge, and optimal conditions for this protection were elaborated (18).

In the series of experiments reported here, the efficiency of the five Re mutant glycolipids in inducing anti-TA3-Ha resistance was compared. It was found that, whereas *S. typhimurium* 1102 is completely inert in this assay, *S. minnesota* R595 shows a slight protection. The glycolipids of the three *E. coli* Re mutants D21f2, D31m4, and F515 were more active than smooth *E. coli* 0111 or *E. coli* 08 LPS preparations. Table 1 summarizes the results comparing the effectiveness of these glycolipids with a number of smooth endotoxic LPS. The data indicate differences in the biological activity of the glycolipids in this assay.

There was considerable variation in the capacity of the preparations to enhance the nonspecific resistance of mice to virulent bacterial infections. Whereas only 4.3×10^{-4} μg of *S. minnesota* R595 glycolipid was needed to protect 50% of the infected animals, 1.7×10^{-1} μg of *E. coli* F515 glycolipid was necessary to achieve the same degree of protection. There was no correlation between the anti-bacterial and the anti-tumor resistance-enhancing capacities of the preparations. The data are summarized in Table 1.

Whereas the TLC patterns of the two *Salmonella* Re mutants were indistinguishable, as

TABLE 1. *Effectiveness of glycolipids (GL) compared with smooth endotoxic LPS in nonspecific resistance enhancement against TA3-Ha tumor and S. typhi 0901 bacterial infection*

Pretreatment[a]	Resistance to tumor (% mortality)[b]	Resistance to *S. typhi* 0901 (PD_{50})[c]
None	95	
S. marcescens 08 LPS	56	3.1×10^{-3}
E. coli 0111 LPS	60	
E. coli 08 LPS	60	
S. minnesota S1114 LPS	33	1.6×10^{-3}
S. minnesota R5 GL	40	
S. minnesota R7 GL	60	
S. minnesota R595 GL	73	4.3×10^{-4}
E. coli F515 GL	36	1.7×10^{-1}
E. coli D31m4 GL	34	3.9×10^{-2}
E. coli D21f2 GL	44	
S. typhimurium 1102	93	

[a] A 25-μg amount was injected intraperitoneally in ICR mice 24 h before challenge with 3 TD_{50} of viable TA3-Ha tumor cells per inoculum, also given intraperitoneally. TD_{50} = number of viable tumor cells causing 50% mortality.

[b] Fifteen to 50 mice per group were used in the tumor resistance assay.

[c] PD_{50} = micrograms of material which causes 50% reduction in mouse lethality of ICR mice injected intraperitoneally with 5×10^8 viable *S. typhi* 0901 cells. The pretreatment was given 24 h before viable challenge, also intraperitoneally. Fifty mice per preparation were used in this assay.

described earlier, the three *E. coli* glycolipids showed significant differences in the intensity and number of chromatographically detectable bands (Fig. 1).

The possibility has been raised (4) that the multiple-band pattern of *S. minnesota* R595 glycolipid, as observed on TLC, is due to the presence of calcium or magnesium cations in these preparations. These cations were removed by ion-exchangers using Amberlite IRC50 or Dowex 50 or Chelex 100. Atomic absorption spectrometry measurements revealed that the calcium content was reduced from 0.68 to 0.05% and that the magnesium content was reduced from 1.82 to 0.19% after ion exchange with Chelex 100. Further purification of *S. minnesota* R595 was carried out by high-voltage paper electrophoresis in pyridine-acetic acid buffer (pH 5.2), which removed noncovalently bound polyamines from the glycolipid. None of the above treatments changed the TLC pattern of the preparation.

The *E. coli* F515 endotoxic glycolipid showed three major fractions by TLC. These were isolated and subjected to biological analysis, which involved *Limulus* lysate gelation and Shwartzman skin tests (3). It was found again, as earlier, that all fractions obtained were equally active in the above assays. These results are identical with previous findings analyzing the various components of *S. minnesota* R595 endotoxic glycolipid.

Finally, the chemical compositions of the glycolipids were compared (5). The molar ratios of hexosamine/phosphorus/2-keto-3-deoxyoctonate/fatty acids were determined (Table 2). The three *E. coli* glycolipids showed 3:5:2:7 molar ratios with relatively minor variations among them. The *S. minnesota* R595 molar ratio for the above components was 2:3:2:6, and the same molar ratio for *S. typhimurium* SL1102 was 2:4:2:7.

DISCUSSION

One should reemphasize here that the TLC system served not only as evidence of chromatographic purity of the preparations, but also as a preparative procedure to obtain chromatographically pure glycolipid preparations. This means that the work presented here was carried out with the purest endotoxic glycolipid preparations hitherto studied (3–5).

Antisera were prepared against *S. minnesota* R595 and *S. typhimurium* SL1102 whole bacteria. The cross-reactivity of these antisera with the five glycolipids described above was investigated, and it was found that all five glycolipids showed cross-reactivity in double-gel diffusion assays. Although all five preparations share one major identical component, there are some

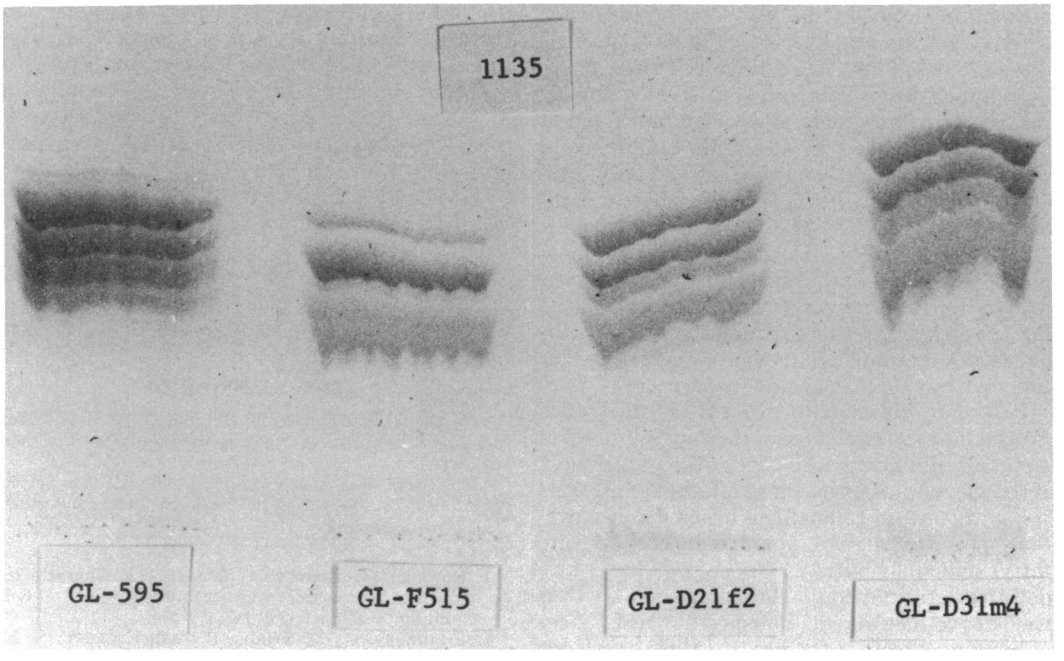

FIG. 1. *TLC pattern of glycolipids. All bands visible on this plate are approximately equally active in five endotoxicity reactions (4).*

TABLE 2. *Chemical composition[a] of the endotoxic glycolipids from Re mutants*

Component	*S. minnesota* R595		*S. typhimurium* SL1102		*E. coli* F515		*E. coli* D31m4		*E. coli* D21f2	
	Wt (%)	Molar ratio	Wt (%)	Molar ratio	Wt (%)	Molar ratio	Wt (%)	Molar ratio	Wt (%)	Molar ratio
Hexosamine	10.72	1 (2)[b]	7.59	1 (2)	10.16	1 (3)	11.51	1 (3)	10.72	1 (3)
Phosphorus	2.58	1.40 (3)	2.72	2.07 (4)	2.80	1.61 (5)	3.10	1.67 (5)	3.00	1.61 (5)
2-Keto-3-deoxy-octonate	13.67	0.98 (2)	8.78	0.92 (2)	8.64	0.64 (2)	9.67	0.53 (2)	8.05	0.63 (2)
Fatty acids	43.01	2.71 (5)	38.05	3.50 (7)	35.59	2.43 (7)	37.50	2.28 (7)	33.59	2.18 (7)

[a] Analyzed by methods described previously (3, 5).
[b] Rounded-off molar ratio.

minor components present in some but not in all five glycolipids. Details of these investigations will be published separately (A. K. Ng, C. H. Chen, C. M. Chang, and A. Nowotny, J. Gen. Microbiol., in press).

For the explanation of the differences observed in the TLC patterns of the various glycolipids, one has to consider first that differences in the fatty acids may exist. The presence or absence of polar or nonpolar fatty acids in the glycolipids may determine chromatographic behavior of the separated components. Minor quantitative differences in the fatty acid distribution among visible bands were found earlier when *S. minnesota* R595 glycolipids were analyzed. It is a likely possibility that the glycolipid bands differ from each other in the molar ratios of the fatty acids ester or amide bound to hexosamines; they may also differ qualitatively in their fatty acid composition. Finally, the glycolipids may differ from each other in components hitherto not sought. Although the preliminary data seem to indicate that the phosphorylated hexosamine backbone is a common feature for the lipid moiety of all endotoxins so far investigated, there is no convincing evidence yet available that would exclude variations within this backbone structure.

It is more difficult to explain the difference in the anti-bacterial and the anti-tumor resistance-enhancing activities of the glycolipids. If the glycolipids exert these effects by interacting with certain cells of the host's defense system, this interaction may require a specific fatty acid distribution. We do not have any definite data that could show convincingly that the above-mentioned nonspecific resistance-enhancing effects of the glycolipids are dependent exclusively upon the presence or absence of fatty acids. As stated earlier, we cannot exclude the possibility that functional groups other than fatty acids may be involved in the formation of the various active sites of the endotoxic molecules (12, 13). This may be particularly true for biological activities reported here. The presence or absence of such functional groups or structural features may render endotoxic preparations active in one and inert in another biological parameter, whereas the fatty acid composition of the compared preparations may be quite similar. That the above assumptions were correct has been shown by very recent findings of Skidmore et al. (15), who reported that the B cell mitogenic effect of their endotoxic preparation in "unresponsive" C3H/HeJ mice depends upon the extraction procedure used for obtaining the endotoxin. Although the preparations compared were equally active in some endotoxicity parameters, they were quite different in mitogenicity (15).

In conclusion, the data of the present study confirm that the lipid moiety of endotoxin, as studied by using chromatographically pure endotoxic glycolipids of various heptoseless Re mutants, is neither chemically nor biologically identical, although similarities are quite obvious.

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LITERATURE CITED

1. **Bowman, H. G., and D. A. Monner.** 1975. Characterization of lipopolysaccharides from *Escherichia coli* K-12 mutants. J. Bacteriol. 121:455-464.
2. **Brailovsky, C., M. Trudel, R. Lallier, and V. N. Nigam.** 1973. Growth of normal and transformed rat embryo fibroblasts. Effects of glycolipids from *Salmonella minnesota* R mutants. J. Cell Biol. 57:124-132.

3. **Chen, C. H., C. M. Chang, A. M. Nowotny, and A. Nowotny.** 1975. Rapid biological and chemical analyses of bacterial endotoxins separated by preparative thin-layer chromatography. Anal. Biochem. 63:183–194.
4. **Chen, C. H., A. G. Johnson, N. Kasai, B. A. Key, J. Levin, and A. Nowotny.** 1973. Heterogeneity and biological activity of endotoxic glycolipid from *Salmonella minnesota* R595. J. Infect. Dis. 128:S43–S51.
5. **Chen, C. H., and A. Nowotny.** 1974. Direct determination of molar ratios of various chemical constituents in endotoxic glycolipids in silicic acid scrapings from thin-layer chromatographic plates. J. Chromatogr. 97:39–45.
6. **Galanos, C., O. Lüderitz, and O. Westphal.** 1971. Preparation and properties of antisera against the lipid-A component of bacterial lipopolysaccharides. Eur. J. Biochem. 24:116–122.
7. **Kasai, N., and A. Nowotny.** 1967. Endotoxic glycolipid from a heptoseless mutant of *Salmonella minnesota*. J. Bacteriol. 94:1824–1836.
8. **Lüderitz, O.** 1970. Recent results on the biochemistry of the cell wall lipopolysaccharides of *Salmonella* bacteria. Angew. Chem. Int. Ed. Engl. 9:649–750.
9. **Nigam, V. N.** 1975. Effect of core lipopolysaccharides from *Salmonella minnesota* R mutants on the survival times of mice bearing Ehrlich tumor. Cancer Res. 35:628–633.
10. **Nigam, V. N., D. Malchow, E. T. Rietschel, O. Lüderitz, and O. Westphal.** 1970. Die enzymatische Abspaltung langkettiger Fettsäuren aus bakteriellen Lipopolysacchariden mittels Extraktion aus der Amöbe von *Dictyostelium discoideum*. Z. Physiol. Chem. 351:1123–1132.
11. **Ng, A. K., C. M. Chang, C. H. Chen, and A. Nowotny.** 1974. Comparison of the chemical structure and biological activities of the glycolipids of *Salmonella minnesota* R595 and *Salmonella typhimurium* SL1102. Infect. Immun. 10:938–947.
12. **Nowotny, A.** 1969. Molecular aspects of endotoxic reactions. Bacteriol. Rev. 33:72–98.

12a. **Nowotny, A.** 1969. Basic exercises in immunochemistry. Springer-Verlag New York, Inc., New York.

13. **Nowotny, A.** 1971. Chemical and biological heterogeneity of endotoxins, p. 309–329. *In* G. Weinbaum, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. 4. Academic Press Inc., New York.
14. **Nowotny, A., S. Golub, and B. Key.** 1971. Fate and effect of endotoxin derivatives in tumor-bearing mice. Proc. Soc. Exp. Biol. Med. 136:66–69.
15. **Skidmore, B. J., D. C. Morrison, J. M. Chiller, and W. O. Weigle.** 1975. Immunological properties of bacterial lipopolysaccharide (LPS). 2. Unresponsivenss of C3H/HeJ mouse spleen cells to LPS-induced mitogenesis is dependent on method used to extract LPS. J. Exp. Med. 142:1425–1435.
16. **Westphal, O.** 1960. Récentes recherches sur la chimie et la biologie des endotoxines des bactéries à gram négatif. Ann. Inst. Pasteur Paris 98:789–813.
17. **Westphal, O., and O. Lüderitz.** 1954. Chemische Erforschung von Lipopolysacchariden gramnegativer Bakterien. Angew. Chem. 66:407–417.
18. **Yang, C., and A. Nowotny.** 1974. Effect of endotoxin on tumor resistance in mice. Infect. Immun. 9:95–100.