PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 149, 389–396 (1975)

"Core" Glycolipid of *Enterobacteriaceae*: Immunofluorescent Detection of Antigen and Antibody[1] (38814)

LOWELL S. YOUNG, KARL R. HOFFMAN, AND PAUL STEVENS
(Introduced by A. F. Rasmussen)

Departments of Medicine and Pharmacology, UCLA Medical School, Los Angeles, California 90024

Investigations of the "rough mutants" of *Salmonella minnesota* have indicated that cell wall "envelopes" of most organisms belonging to the family *Enterobacteriaceae* contain a common "core" glycolipid moiety, 2-keto, 3-deoxyoctonate linked to Lipid A (1, 2). The simplest of these rough mutants, designated the chemotype "Re" of *S. minnesota* R595, has a cell-wall glycolipid that is almost exclusively 2-keto, 3-deoxyoctonate and Lipid A. This moiety possesses significant "endotoxic" properties and studies in both humans and animals suggest that antibody against this antigen protects against shock and death after gram-negative bacteremia (3, 4).

We have recently described the conditions by which highly potent antiserum against the core glycolipid could be produced in rabbits, determined that this antiserum contained precipitating antibody against crude antigens derived from a variety of *Enterobacteriaceae*, and postulated that immunofluorescent detection of core glycolipid antigen might be a useful tool in studying taxonomic relationships between various groups of bacteria (5). In this report we will document the correlation between immunofluorescent detection of this antigen and results obtained by the gel-diffusion technique, detail immunofluorescent methods for measuring antibodies against this antigen in the sera of man and animals, and describe preliminary experiments in which core glycolipid antiserum was used to localize the bacterial antigen in gram-negative bacillary endocarditis.

Materials and Methods. Bacteria. Salmonella minnesota R595, chemotype "Re" (KNV-447) was obtained from Dr. Otto Westphal, Max Planck-Institute für Immunobiologie, Freiburg, Federal Republic of Germany. *Pseudomonas aeruginosa* types I through VII were obtained from Dr. M. W. Fisher, Parke-Davis and Company, Detroit, MI. *E. coli* 014:K7, *E. coli* 08, and *Shigella boydii* were obtained from Dr. Erwin Neter and H. C. Whang, Children's Hospital of Buffalo, N.Y. The remaining *E. coli* serotypes (Table I) were obtained from Dr. Doyle Evans, Sepulveda Veterans Administration Hospital, Sepulveda, CA. *Serratia marcescens* serotype 03 is a clinical isolate typed with monospecific *Serratia* antisera produced against prototype strains obtained from Dr. William H. Ewing of the Center for Disease Control, Atlanta, GA. Clinical isolates of *Aeromonas hydrophila* and *Alkaligenes faecalis* were obtained from the Microbiology Laboratory of the Memorial Sloan–Kettering Cancer Center. *Neisseria meningitidis* Group B and C (reference strains No. HC15 and HC33) were obtained from James C. Feeley of the Special Pathogens Laboratory, Center for Disease Control. *Yersinia enterocolitica* was obtained from Dr. Robert Weaver of the Bacterial Reference Unit, Center for Disease Control. *Edwardsiella tarda* strains were obtained from Dr. George Herman of the Center for Disease Control. Strains of *Bordetella pertussis* were obtained from Eli Lilly and Company and Dr. Robert Weaver. *Achromobacter lwoffi*, *Achromobacter anitratus*, *Alkaligenous odorans*, *Achromobacter violaceum*, and *Moraxella species* were obtained from Dr. M. J. Pickett, Department of Biology, UCLA. The remaining organisms were clinical isolates obtained from the Diagnostic Bacteriology Laboratory of the UCLA Medical Center. Crude bacterial antigen extracts were prepared by boiling washed organisms for 1 hr.

[1] This study was supported by General Development Grant from the National Institutes of Health, G.R.S.G. RR 5354 Grant from the National Institute of Allergy and Infectious Disease, and U.S. Public Health Service Training Grant A1-309.

Copyright © 1975 by the Society for Experimental Biology and Medicine
All rights reserved.

Protection of antisera. Antisera against *S. minnesota* R595 chemotype "Re" were produced in rabbits by immunization of albino New Zealand rabbits as previously described (5) with purified glycolipid antigen prepared by the method of Galanos *et al.* (6) using chloroform–phenol–petroleum ether. The composition of the antigen was: Keto-deoxyoctonate (KDO), 14.7% by weight (using the thiobarbituric acid method (7) with sialic acid as the standard), and phosphorus, 1.06% using the method of Chen (8). No heptose could be detected using the cysteine–H_2SO_4 method (9), and no protein or nucleic acids could be detected by uv scanning. Dogs were immunized with a rising dosage schedule of 1 μg/kg iv which was doubled at weekly intervals up to a dose of 256 μg/kg. Antiserum against *E. coli* 014, *S. marcescens* 03, *E. coli* 085:H:9, and *P. rettgeri* 082 was produced by four weekly iv injections of approximately 10^8 heat-killed organisms/ml. Human serum was drawn from patients at least 10 days after documented gram-negative rod bacteremia caused by organisms of the family *Enterobacteriaceae.*

Immunofluorescent detection of antigen on bacterial surfaces. Bacteria for immunofluorescence studies were grown on trypticase soy agar, gently removed by scraping with a moist cotton swab, washed, and boiled for 1 hr at 100°C. The turbidity was adjusted to a barium sulfate standard corresponding to approximately 10^7 organisms/ml. One-tenth milliliter of bacterial suspensions was applied to glass slides, allowed to dry, and fixed in acetone. For direct tests, the NH_4SO_4-precipitated fraction of rabbit antiserum was conjugated to fluorescein isothiocyanate (FITC, Sigma Chemical, St. Louis, MO.) by the method of Cherry using a FITC/protein ratio of 12.8:1 (10). Unbound conjugate was rapidly removed by dialysis ultrafiltration. Antiserum was applied directly to bacteria at a 1:16 or 1:32 dilution and slides were examined with a Leitz Dialux microscope (equipped with Ploem illumination) using an oil-immersion or high-dry fluorite lens, a 200-W (Osram HBO200) mercury arc lamp, and a 500-mm exciter filter. Immunofluorescence was graded from 1+ to 4+ with 1+ being considered significant. For indirect tests a 1:16 or 1:32 dilution of antisera (the dilution chosen because of strong positive homologous reaction with *S. minnesota* R595 organisms and no reactivity at that dilution with pooled normal rabbit serum) was applied for 30 min. The organisms were then gently washed twice with buffer and overlayed with fluorescein-labeled goat anti-rabbit globulin (Antibodies, Inc., Sacramento, CA). Because normal serum contains antibodies against "core" glycolipid, the goat anti-rabbit serum was twice absorbed with washed, boiled heat-killed *S. minnesota* organisms (approximately 10^9 organisms/ml) and diluted 1:16 in McIlvaine's buffer (pH 7.4). All slides were examined by two observers.

Titration of class-specific core glycolipid antibody in human and animal serum. S. minnesota R595 organisms were washed twice in sterile water, boiled for 1 hr, and resuspended at a concentration of 10^8 organisms/ml. Ten-microliter aliquots were dried and acetone fixed for 10 min in each 7-mm well of coated slides (Cel-Line Associates, Minotola, NJ). Dog, human, and rabbit sera were serially diluted in Microtiter plates ("U" Wells, Cooke Engineering), and 30-μl aliquots of each dilutions were added to each well. After incubation for 30 min at 37°C in a moist chamber, the slides were washed gently × 5 with McIlvaine's buffer, blotted dry, and then counterstained with FITC-conjugated antiserum at a dilution of 1:16. Reaction time was again 30 min, followed by washing × 5. The wet slides were then coated with glycerol–McIlvaine's buffer (9:1) covered with 22 × 50-mm coverslips, and each well's content graded for fluorescence, the titer endpoint being the highest dilution associated with 1+ or greater fluorescence.

Goat anti-human IgG and IgM was obtained from Hyland Laboratories, rabbit anti-canine IgG and IgM, and goat anti-rabbit IgG and IgM from Microbiological Associates, Bethesda, MD. These conjugated antisera were twice absorbed with boiled, washed *S. minnesota* organisms to increase test specificity.

Studies with latex particles. Polystyrene latex particles, 0.82 μm diameter (Dow Diagnostics, Indianapolis, IN) were suspended at a final concentration of 10^8/ml in buffer,

boiled for hr with core glycolipid antigen (final concentration 500 μg/ml), washed four times with buffer, and fused to glass slides using a solution of 50:50 methanol/ethanol for 30 min. These slides were then treated like bacteria for detection of antigen.

Suspensions of glycolipid-coated latex particles were incubated with 2-fold serial dilutions of core glycolipid antiserum (0.5 ml) for 1 hr at 37°C and 18 hr at 4°C in order to determine the latex-agglutination titer.

Detection of antigen in cardiac valves. Acute bacterial endocarditis was induced in dogs using the model previously described (11) with the only variation being that *Serratia marcescens* serotype 03 was utilized as the infecting agent. Several cardiac-valve sections were removed within 10 min of death, frozen in an acetone–dry-ice bath, and maintained at −80°C until study. Sections 4 μm thick were prepared on a Harris International Cryostat at −22°C, fixed on glass slides with a 50:50 solution of methanol/ether, and treated with core glycolipid antiserum. Fluorescein-conjugated goat anti-rabbit globulin was then used as a second antibody. As controls, normal cardiac-valve sections were treated with the same series of reagents.

Specificity controls. Multiple absorptions with boiled, heat-killed organisms of *S. minnesota* R595 organisms removed immunofluorescence at all dilutions of antisera. Unconjugated core glycolipid antiserum was also reacted with these bacteria followed by exposure to fluorescein-conjugated antiserum (at corresponding dilutions). Unlabeled antiserum was found to block the attachment of the conjugated antiserum to *S. minnesota* R595.

Results. Antibody against core glycolipid antigen detected by indirect immunofluorescence (IFA). Sera from four rabbits immunized with the purified antigen prepared by the technique of Galanos *et al.* (6) contained precipitating antibodies which were detected by immunodiffusion. This technique, using antigen dissolved in distilled water, has been previously detailed (5). Serial dilution of these antisera followed by exposure to boiled, acetone-fixed *S. minnesota* cells, and a final reaction with FITC-labeled goat anti-rabbit globulin indicated antibody titers of 1:128 or greater. In contrast, a pool of five normal rabbit sera was found to have a titer of less than 1:16. The presence of low titers of natural antibodies against the core glycolipid underscored the necessity to absorb all the second antibodies in the indirect test (i.e., the fluorescein-labeled animal antisera) with boiled heat-killed *S. minnesota* R595 cells. Completeness of absorption was tested on each slide by reaction of the second antibody with *S. minnesota* cells.

Direct immunofluorescence using FITC-conjugated rabbit globulin. Directly conjugated antiserum was found to have a titer of 1:64 or greater in reactions with boiled *S. minnesota* R595 cells. Positive fluorescence was blocked with a 1:64 or less dilution of conjugated antiserum. 1:16 dilution of normal serum failed to block fluorescence observed with directly conjugated antisera.

Presence of core glycolipid antigen as a taxonomic marker. Table I lists 24 strains of *Enterobacteriaceae* found to have the core glycolipid antigen present on their boiled surfaces by either the direct or indirect method. Boiled extracts of each of these organisms all formed at least one precipitin band with core glycolipid antiserum in immunodiffusion tests.

Table II summarizes the core glycolipid immunofluorescence reactions and precipitin test results of organisms not classified among the *Enterobacteriaceae*. It is interesting to note that organisms such as *Aeromonas hydrophila*, *Haemophilus influenzae*, *Listeria monocytogenes*, *Bordetella pertussis*, *Neisseria meningitidis*, and *Yersinia enterocolitica* which are not classified as enteric bacteria were positive by both immunodiffusion and direct immunofluorescent studies for the presence of core glycolipid antigen on their boiled surfaces. Four bacteremic isolates of *Bacteroides fragilis*, an anaerobic gram-negative bacillus which is one of the major components of the fecal flora, were found to be equivocally positive both on gel diffusion and in indirect immunofluorescence. Consistently negative reactions were recorded with *Edwardsiella tarda*, *Ps. aeruginosa* (seven serotypes), *Moraxella species*, and *Alkaligenes fecalis*.

Core glycolipid antibody and type-specific

TABLE I. *ENTEROBACTERIACEAE* REACTING WITH GLYCOLIPID ANTISERUM.
All Strains Reacted By Immunodiffusion and Immunofluorescence.

Escherichia coli (13/13 strains tested)	*Klebsiella pneumoniae*
08: K 27 (F1470-R1)	Type I
08: H 34	Type VI
014: (ATCC 19110)	*Proteus rettgeri:* 082
014: K 7 (SU 4411/41:2163)	*Salmonella* sp.
015	minnesota R595 (Re)
078: H 11	enteritidis
085: H 9	typhimurium
0115	*Shigella boydii* Type III (R3140)
0119: B 14	*Serratia marcescens*
0125: B 15: H 21	03
0125: B 15: H 30	05
0126	07
0126: B 6	
0142	

antibodies against other members of the family Enterobacteriaceae. Indirect immunofluorescence studies were carried out with core glycolipid antiserum and *S. minnesota* R595 and type-specific antisera against *E. coli* 014, *Serratia marcescens* 03, *E. coli* 085:H9, and *Proteus rettgeri* 082. All of these strains reacted strongly with core glycolipid antiserum in titers of 1:16–1:128. As shown in Fig. 1, however, stronger fluorescence was always observed with homologous antiserum (e.g., 014 versus 014 antiserum, panel A). Repeated absorption of homologous antiserum removed not only type-specific fluorescence but fluorescence against core glycolipid antigen. Absorptions with boiled *S. minnesota* R595 failed to remove all of the fluorescence associated with the use of antiserum against *E. coli* 014.

Detection of class-specific core glycolipid antibody in rabbit, dog, and human sera. Natural antibody against antigen was found in normal serum from man, rabbits, and dogs. The highest titers of natural IgG antibody was found in dogs (geometric mean = 92 ± 45.5 SE for nine dogs) while IgM antibody was significantly less (geometric mean = 7 ± 5 SE). In man the predominant class of antibody also appeared to be IgG (mean 39 ± 17 SE) as compared to IgM (mean = 12 ± 11 SE) for eight normals. In rabbits, IgM antibody was more prevalent (mean = 31 ± 16 SE) relative to IgG (mean of 7 ± 6 SE) for three lots of serum pooled from five animals. Sera from immunized rabbits or dogs and sera from nonimmunosuppressed human patients convalescing from enteric bacteremia generally showed 4-fold or greater rises, particularly in titers of IgG antibody.

TABLE II. REACTIONS OF NON-*Enterobacteriaceae* WITH GLYCOLIPID ANTISERUM.
The Figure in Parentheses Indicates if More Than One Strain of Each Species Was Tested.

Organism	Gel precipitation	Immunofluorescence
Aeromonas hydrophila (3)	+	+
Achromobacter lwoffi	---	---
Achromobacter anitratus	---	---
Alkaligenes faecalis	---	---
Alkaligenes odorans	---	---
Bacteroides fragilis (4)	---	---
Bordetella pertussis (4)	+	+
Chromobacter violaceum	---	---
Edwardsiella tarda (3)	---	---
Erwinia sp.	+	+
Flavobacterium sp. (2)	---	---
Hemophilus influenzae (3)	+	+
Listeria monocytogenes (2)	---	---
Neisseria meningitidis		
Group B	+	+
Group C	+	+
Moraxella sp.	---	---
Pseudomonas aeruginosa		
(Fisher types I–VII)	---	---
Yersinia enterocolitica (2)	+	+

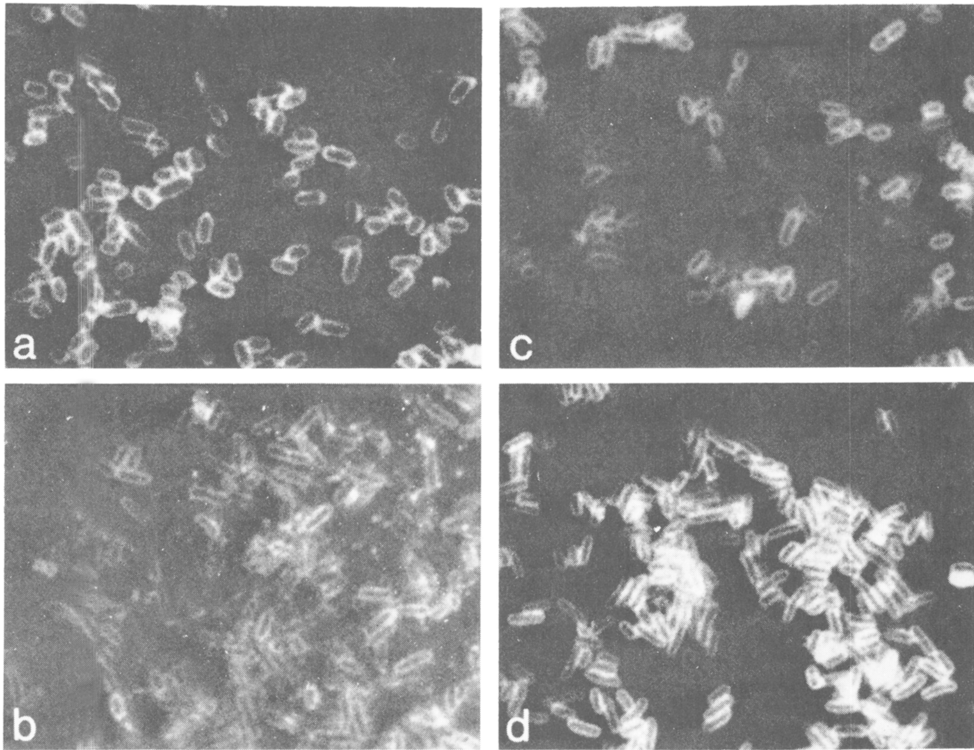

FIG. 1. Indirect immunofluorescence demonstrating the reaction of antisera against *E. coli* 014 with (a) *E. coli* 014 (b) *S. minnesota* R595. Homologous antiserum always showed brighter fluorescence, as core glycolipid antisera reacted less strongly with (c) *E. coli* 014 than (d) *S. minnesota* R595. (Magnification ×1200.)

Detection of antigen and cardiac valves. Dogs dying of endocarditis due to *Serratia marcescens* 03 were found to have brightly fluorescing valvular bacterial vegetations when the latter were exposed to core glycolipid antiserum by either the direct or indirect technique (Fig. 2). In these sections, taken from animals infected less than 3 wk, the fluorescence was associated with intact bacilli and could not be observed in the inflammatory exudate or within phagocytic cells. Attempts to detect core glycolipid antigen in lungs, liver, or kidney tissues from these animals, particularly sections from areas of hemorrhagic infarction, were unsuccessful.

Latex particle studies. Core glycolipid antigen was found to coat latex particles as detected by indirect immunofluorescence, but these particles would not agglutinate even at the lowest dilutions of antiserum.

Discussion. The use of immunofluorescence technique to detect core glycolipid antigen or antibody could be readily anticipated in view of the ability to measure antibodies against the antigen by other serologic techniques. These studies confirm that the core glycolipid of *Enterobacteriaceae* is an antigenic structure widely distributed among many gram-negative bacteria as well as some gram-negative coccal organisms as well. Just as the so-called common antigen (CA) of Kunin is a consistent taxonomic marker (12), every organism classified among the family *Enterobacteriaceae* that we tested reacted strongly with core glycolipid antiserum and there was complete correlation with immunodiffusion test results. It is clear, however, that this antigen is found in some organisms (e.g., meningococci) that have endotoxic activity but are not *Enterobacteriaceae* (13) and is absent from some bacilli with well-known endotoxic properties (e.g., *P. aeruginosa*).

Among the organisms which reacted most strongly with Re antiserum both in the IFA

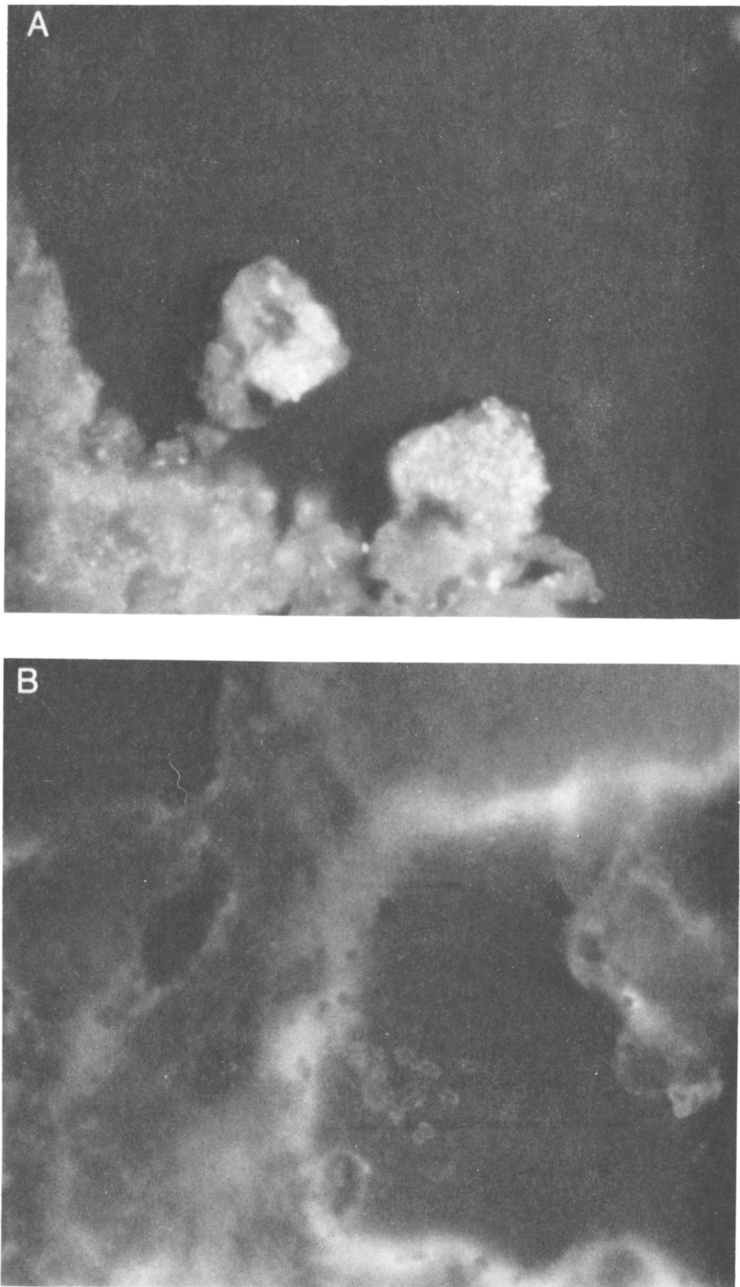

FIG. 2. Immunofluorescent (indirect technique) detection of core glycolipid antigen in the cardiac vegetations of a dog infected with *Serratia marcescens* 03. Discrete bacillary structures are identified at low (A, ×516) and high (B, ×1200) magnification.

test and in gel diffusion was *Bordetella pertussis*. The well-known adjuvant effect of pertussis immunization may, in part, derive from the fact that it shares the core glycolipid antigen with a number of other gram-negative bacteria. Similarly, the adjuvant effect of prior immunization with a gram-negative bacterium on subsequent immuni-

zation with another gram-negative organism (serologically unrelated as defined by agglutination of the O-specific antigen) may possibly be explained on the basis of the sharing of a common antigen (i.e., core glycolipid) by apparently unrelated organisms.

One of the technical problems encountered was the detection of substantial amounts of natural antibody against core glycolipid. This is not unexpected in view of the finding that normal serum contains antibodies or substances which may inactivate or bind endotoxins (14, 15). The significance of this finding is that in order to perform antibody titrations or antigen by the indirect technique it becomes necessary to absorb natural antibodies from the reagents used during the second antibody reactions. However, the double-antibody procedure has the advantage of detecting antiglycolipid antibodies by antibody class and offers the possibility of relating the prognosis and clinical events associated with gram-negative septicemia to class-specific antibody titers. This may be important inasmuch as the hemagglutination technique appears to emphasize the presence of IgM antibodies over IgG and we have not found the tube agglutination technique a reliable method for detecting core glycolipid antibody using either whole bacterial cells or antigen-coated latex particles. It is interesting with respect to the latter observation that core glycolipid is like the common antigen (CA). Though the latter antigen can be detected by immunofluorescence, high-titered antiserum does not agglutinate CA-coated latex particles (16).

We believe that the applications of the techniques described in this paper go beyond exploring taxonomic relationships and quantitative assessments of antibodies in gram-negative septicemia. The antigen can be localized in the intact bacteria contained within valvular vegetations of animals dying of acute gram-negative endocarditis. The immunofluorescence technique can be used to study more chronic bacterial infections or inflammatory processes which may be triggered or mediated by hypersensitivity to enterobacterial antigens, as has been suggested for ulcerative colitis or chronic interstitial nephritis (17, 18). Conflicting results have been reported in studies of the latter condition in which detection of CA was sought in renal tissue by FA technique (19). Type-specific antigen has been found to persist in renal experimental infections (20), and the use of techniques to detect core glycolipid antigen may have relevance to the late sequelae of chronic gram-negative bacillary infections inasmuch as the Lipid A moiety possesses biologic activity (21) whereas the biologic activity of CA is uncertain.

Summary. The Re chemotype mutant of *Salmonella minnesota* R595 has a cell-wall glycolipid composed principally of 2-keto, 3 deoxyoctonate and Lipid A, which is an antigen widely shared by *Enterobacteriaceae*. High-titered antiserum against this antigen can be conjugated with fluorescein isothiocyanate for direct detection of this antigen in heterologous bacteria and staining of bacteria in tissue. Alternatively, the indirect immunofluorescence technique can be used for antigen detection on bacterial surfaces and in tissues, and this method can quantitate glycolipid antibody in mammalian sera. The latter may be particularly useful in serologic studies because, although the glycolipid antigen is a surface antigen and purified extracts can be used to coat latex particles, high-titered antisera will not agglutinate bacteria or coated latex particles.

The expert technical assistance of Shahnaz K. Raza is gratefully acknowledged.

1. Lüderitz, O., Staub, A. M., and Westphal, O., Bacteriol. Rev. **30**, 192 (1966).
2. Lüderitz, O., Galanos, C., Risse, H. J., Ruschmann, E., Schlecht, S., Schmidt, G., Schulte-Holthansen, H., Wheat, R., and Westphal, O., Ann. N.Y. Acad. Sci. **133**, 349 (1966).
3. McCabe, W. R., Kreger, B. E., and Johns, M., N. Engl. J. Med. **287**, 261 (1972).
4. McCabe, W. R., J. Immunol. **108**, 601 (1972).
5. Young, L. S., and Stevens, P., Experientia **30**, 192 (1974).
6. Galanos, C., Lüderitz, O., and Westphal, O., Eur. J. Biochem. **9**, 245 (1969).
7. Aminoff, D., Biochem. J. **81**, 384 (1961).
8. Chen, P. S., Toribara, T. Y., and Warner, H., Anal. Chem. **28**, 1756 (1956).
9. Dische, Z., J. Biochem. **204**, 983 (1973).

10. Cherry, W. B., *in* "Manual of Clinical Microbiology" (J. E. Blair, E. H. Lennette, and J. P. Truant, eds.), p. 693. Williams & Wilkins, Baltimore (1970).
11. Keys, T. F., Sapico, F., Touchon, R., Barenfus, M., and Hewitt, W. L., Amer. J. Med. Sci. **263,** 103 (1972).
12. Whang, H. Y., and Neter, E., Experientia **29,** 69 (1973).
13. Jennings, H. J., Hawes, G. B., Adams, A., and Kenny, C. P., Can. J. Biochem. **51,** 1347 (1973).
14. Ho, M., and Kass, E. H., J. Lab. Clin. Med. **51,** 297 (1958).
15. Von Eschen, K. B., and Rudbach, J. A., J. Infect. Dis. **129,** 21 (1974).
16. Neter, E., and Whang, H. Y., *in* "Cellular Antigens" (A. Nowotny, ed.), p. 14, Springer-Verlag, New York (1972).
17. Perlmann, P., Hammarstrom, S., Lagercrantz, R., and Campbell, D., Proc. Soc. Exp. Biol. Med. **125,** 975 (1967).
18. Aoki, A., Imamura, S., Aoki, M., and McCabe, W. R., N. Engl. J. Med. **281,** 1375 (1969).
19. Schwartz, M. M., and Cotran, B. S., N. Engl. J. Med. **289,** 830 (1973).
20. Thomsen, O. E., and Hjort, T., Acta Pathol. Microbiol. Scand. Sect. A. **81,** 474 (1973).
21. Lüderitz, O., Galanos, C., Lehmann, V., Nurminen, M., Rietschel, E., Rosenfelder, G., Simon, M., and Westphal, O., J. Infect. Dis. **128** (Suppl.), S17 (1973).

Received: Dec. 16, 1974. P.S.E.B.M., 1975, Vol. 149.