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Original Article

Biotinylation of Bacterial Lipopolysaccharide and Its Applications to Electron Microscopy

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We describe a procedure for lipopolysaccharide (LPS) biotinylation using *N*-biotinyl-L-lysine and application of the biotinylated LPS (Bi-LPS) to localization of LPS binding sites and subcellular distribution. Biotinylation of LPS was confirmed by enzyme-linked immunosorbent assay (ELISA), gel immunodiffusion, and immunodot techniques. The biological and toxicological activity of the Bi-LPS was tested by *Limulus* amoebocyte lysate (LAL) assays and histopathological examinations, respectively. Results showed that biotin was conjugated to LPS without disrupting the biological/toxicological activity of the molecule, which indicates that the biotin is directly linked to the polysaccharide portion of LPS. Localization of binding sites and subcellular distribution of Bi-LPS in human platelets and monocytes were studied by electron microscopy using an avidin–biotin–horseradish peroxidase (HRP) or streptavidin–gold method. Platelet surfaces were intensely stained by the reaction product of horseradish peroxidase (HPR) 5 min after incubation, and Bi-LPS was localized in small vesicles and vacuoles of platelets and in the phagocytic vacuoles of monocytes 60 min post incubation. Bi-LPS provides a reliable, stable, and sensitive tool for determination of LPS binding sites and subcellular distribution. (*J Histochem Cytochem 36:1131–1137, 1988*)

KEY WORDS: LPS; Biotinylation; Lysine; Binding site; Subcellular distribution; Electron microscopy.

Introduction

The avidin–biotin complex (ABC) has become an indispensable tool in immunocytochemistry and molecular biology. Avidin is a large (70,000) molecular weight glycoprotein with an affinity constant of 10^{-15} M^{-1} for biotin, a small MW vitamin (26). This affinity is over one million times higher than that of antibody for most antigens. In addition to this high affinity, ABC can be effectively exploited because avidin has four binding sites for biotin, and most proteins can be easily conjugated with several molecules of biotin. This complex has been widely used in electron microscopy for labeling specific cell components, receptors, surface and intracellular antigens (1,27), and in situ hybridization (13,24).

Lipopolysaccharides (LPS), endotoxins from Gram-negative bacteria, are known to be involved in the pathogenesis or induction of endotoxemia and septic shock. LPS consists of polysaccharide-specific antigen, core region, and lipid A. Lipid A (glycolipid) is the endotoxically active center of LPS. Studies on surface binding, membrane incorporation, and subcellular distribution of LPS using radiolabeled (4,14,15,28), FITC-conjugated (16), and horseradish peroxidase (HRP)-conjugated (19) LPS, and LPS–antibody–HRP or gold (9,11,21), have been reported. However, studies on ultrastructural localization of cell surface binding sites and subcellular distribution of LPS are few, although HRP-conjugated LPS has been reported to demonstrate surface binding sites in erythrocytes at the ultrastructural level (19).

Four presently available activated biotin reagents employed for biotinylation of biologically active molecules are biotin-*N*-hydroxysuccinimide ester, 2-iminobiotin-*N*-hydroxysuccinimide ester, maelimidobutyrl biocytin, and biotin-hydrazide. These activated biotin reagents cannot be used directly for biotinylation of LPS because of their chemical effects. Biotin-hydrazide, which is coupled through carboxyl or keto groups (12,14,18), causes loss of fatty acids (2). Biotin-*N*-hydroxysuccinimide ester and 2-iminobiotin-*N*-hydroxysuccinimide ester are coupled through free amino groups (12,14,18), and maelimidobutyrl biocytin through sulfhydryl groups (5). In LPS, neither amino groups nor sulfhydryl groups are present in the polysaccharide moieties. Therefore, a suitable bridging molecule is required to link biotin to LPS.

This report describes a procedure for LPS biotinylation using *N*-biotinyl-L-lysine with lysine as a bridge between LPS polysaccharide moieties and biotin. It also presents examples of avidin–Bi-LPS complex staining in electron microscopy.

Materials and Methods

Procedure for LPS Biotinylation

A procedure modified from the method reported by O'Shannessy et al. (18) was used for LPS biotinylation. Ten milligrams (10 mg) of LPS (*E. coli*

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0111:B4; Calbiochem, La Jolla, CA) were dissolved in 7 ml 0.1 M sodium acetate buffer, pH 5.25, and oxidized with 1.5 ml 0.05 M sodium periodate at 4°C for 20 min. The reaction was stopped by addition of 1 ml 1.5 M glycerol. After 5 min the reaction product was dialyzed overnight in a piece of Thomas 3787.F25, 8000 MW cutoff dialyzer tubing againsts 0.1 M sodium acetate buffer, pH 5.37, at room temperature. In a 10 ml screw-cap vial, 8.7 ml of the oxidized LPS were added to 25 mg biocytin (Sigma Chemical Company; St. Louis, MO) and dialyzed for 64 hr at 4°C. The biotinylated lipopolysaccharide (Bi-LPS) was stored at 4°C until used.

Confirmation of LPS Biotinylation and Shelf Life

Procedures including double immunodiffusion, immunodot, and ELISA were used to test LPS biotinylation. Five different dilutions (1:2, 1:4, 1:8, 1:16, and 1:32) were prepared from 1 mg/ml Bi-LPS in phosphate-buffered saline (PBS; 0.01 M phosphate, 0.15 M NaCl, pH 7.2). Non-biotinylated LPS and PBS were used as controls. For testing the shelf life of Bi-LPS, samples that had been stored at 4°C for 12 and 22 weeks were used for ELISA and double-immunodiffusion evaluation.

ELISA Technique. Serial dilutions of sonicated suspensions of Bi-LPS and LPS were prepared in 96-well polycarbonate plates. The plates were incubated at 37°C overnight. The supernatant in each well was poured off and blocked with 0.05% gelatin in PBS for 1 hr at room temperature. The wells were washed three times with PBS. One milliliter of 0.05% gelatin in PBS was added to a vial containing 1 ml solution of avidin-alkaline phosphatase (Sigma). The solution was diluted with PBS to a 1:200 ratio. One hundred microliters of this solution were added to each well and incubated on ice for 2 hr. The wells were again washed three times with PBS. One hundred microliters of phosphatase substrate (1.5 mg/ml) (Sigma) were added to each well and incubated for 30 min at room temperature. The reaction was stopped by addition of 50 µl of 3 M NaOH to each well. The plates were then read at 405 nm on a Micro titer reader (MR 600 Microplate Reader; Dynatech).

Ouchterlony Technique (Double Immunodiffusion; DID). From each of the dilutions made for ELISA, 35 µl of the diluted solution were added to the peripheral wells of an immunodiffusion disk (Miles Scientific; Naperville, IL). Thirty-five microliters of diluted avidin–FITC solution (1:10) (Sigma) were added to the central well. Diffusion was allowed to proceed for 48 hr at room temperature.

Immunodot Technique. PVC silica microporous glutaraldehyde-activated plastic sheets (Polysciences, Inc. Warrington, PA) were used. A drop of 10 µl Bi-LPS solution of 1:2, 1:4, and 1:8 dilutions was spot-placed on the plastic sheets and air-dried. The plastic sheets were immersed for 20–30 min in PBS (pH 7.4) containing 1% bovine serum albumin (BSA). The plastic sheets were incubated in a solution containing 1:100 dilution of avidin and biotinylated HRP (ABC kit; Vector Laboratories, Burlingame, CA) at room temperature for 20 min. After three washings in PBS for 15 min, the samples were incubated in a medium containing H_2O_2 and diaminobenzidine tetrachloride at room temperature for 30 min (7,8). The plastic sheets were then washed with PBS and examined.

Limulus *Amoebocyte Lysate Assay*

Endotect (ICN; Cleveland, OH), based on *Limulus* amoebocyte lysate (LAL) reaction with endotoxin, was used to detect the reactivity of Bi-LPS. Non-biotinylated LPS and pyrogen-free PBS were used as controls.

Biological Activity

Five Balb/CJ female mice (20–25 g body weight) per group were given 200 µg of Bi-LPS and LPS in 0.3 ml saline, respectively, by IP injection. Control animals were given saline only. After 48 hr, animals were sacrificed and livers were excised and processed for histopathological examinations.

Localization of Binding Site and Intracellular Distribution in Human Platelets and Monocytes

Peripheral blood mononuclear cells (PBMC) were purified by Ficoll–Hypaque gradient density centrifugation of heparinized venous blood from healthy donors (5). The layer containing PBMC was collected in RPMI 1640 medium (Gibco Laboratories; Grand Island, NY) and used without washing so that platelets could also be retained. Cells were exposed to 100 µg/ml of Bi-LPS or LPS in RPMI 1640 medium at 37°C for 5 and 60 min. At the end of each incubation, cells were fixed immediately in 1% glutaraldehyde–1% paraformaldehyde at room temperature for 30 min. After three washings in 0.1 M sodium cacodylate buffer (pH 7.5), cells were stored in the same buffer in a refrigerator until further processing.

Avidin-biotin-horseradish peroxidase complexes (Vector) or streptavidin–gold conjugates (20 nm) (Bethesda Research Laboratory; Bethesda, MD) were used to label the Bi-LPS bound to cell surfaces of platelets and monocytes. HRP was developed by incubating in a diaminobenzidine–H_2O_2 medium (7,8). Cells incubated with Bi-LPS or LPS for 60 min were treated with 0.3% H_2O_2 for 30 min to abrogate the endogenous peroxidase activity and then with 0.2% saponin in 0.1 M sodium cacodylate buffer, pH 7.5, for 2–3 hr before incubation in avidin–biotin–HRP in Tris-saline (pH 7.6) with 0.2% saponin at 4°C for 24 hr. HRP was revealed by the aforementioned procedure (7).

All cell samples were post-fixed in 2% osmium tetroxide at 4°C for 2–3 hr, dehydrated in a series of graded ethanol solutions and propylene oxide, and embedded in Epon (Poly/Bed; Polysciences, Warrington, PA).

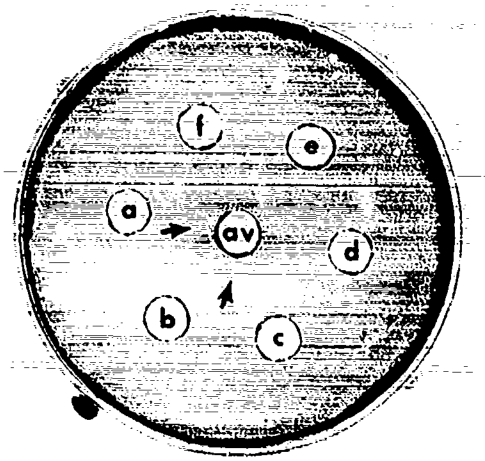
Table 1. *Results from Bi-LPS dilutions using double immunodiffusion (DID) and ELISA techniques*[a]

	Dilutions											
	1		1:2		1:4		1:8		1:16		1:32	
Samples	A	B	A	B	A	B	A	B	A	B	A	B
Bi-LPS (12 wks)	–	+	–	+	+	+	+	+	+	+	+	–
Bi-LPS (22 wks)	–	+	–	+	++	++	+++	+++	++	++	+	–
LPS	–	–	–	–	–	–	–	–	–	–	–	–
PBS	–	–	–	–	–	–	–	–	–	–	–	–

[a] A, ELISA. B, double-immunodiffusion technique: +++, strong fluorescence or precipitation line; ++, moderate fluorescence or precipitation line; +, weak fluorescence or precipitation line; –, no precipitation line.

Figure 1. Ouchterlony immunodiffusion disk used for confirmation of LPS biotinylation. (a) and (b) wells contained Bi-LPS of various dilutions and storage times; (c) well contained non-biotionylated LPS; (d) well contained PBS only; (e) and (f) wells contained 0.1 M and 0.05 M sodium acetate, respectively; central well (av) contained 1 mg/ml FITC-conjugated avidin. The crescent line (arrows) between the central and peripheral wells indicates the reaction product of Bi-LPS with avidin.

Figure 2. Confirmation of LPS biotinylation using the immunodot technique. Bi-LPS of various concentrations (a and f, 1 mg/ml; b, 0.5 mg/ml; c, 0.25 mg/ml), LPS (d), and PBS (e) were spot-placed on PVC-silica and treated as described in the text for visualization. The presence of HRP reaction product is indicative of biotinylation, and the intensity of staining correlates with various concentrations. Positions d and e, showing no staining, are non-biotinylated LPS and PBS, respectively.



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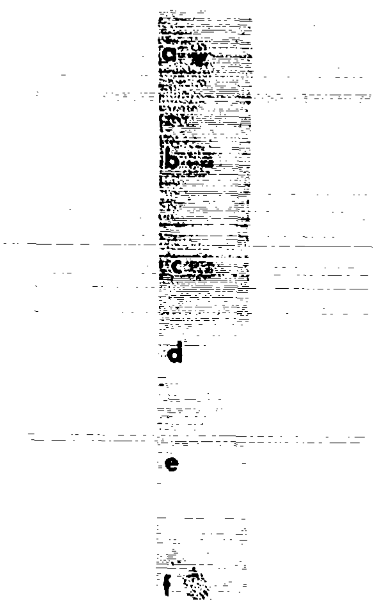

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Ultra-thin sections prepared with a diamond knife were lightly stained in lead citrate and examined in a JEOL 100 CX transmission electron microscope.

Results

Results from the tests of Bi-LPS samples using ELISA and immunodiffusion techniques indicated that positive reactions were found only in 1:4, 1:8, and 1:16 dilutions. No reactions were observed in the ELISA samples without dilution or at 1:2 dilution and in the samples diluted at 1:32 for the immunodiffusion test (Table 1). As indicated by the formation of a crescent avidin–biotin–LPS complex, Bi-LPS remained reactive after being stored for 12–22 weeks (Figure 1). Similar results from the same samples were obtained by using an immunodot procedure (Figure 2, Table 2).

Gelation was observed in both Bi-LPS and LPS using *Limulus* amoebocyte lysate. This indicated that the reactivity of Bi-LPS was retained after the biotinylation procedure. Gelation was not formed by pyrogen-free PBS (Table 3).

Results from the histopathological examination indicated scattered foci of slightly to moderately swollen hepatocytes with either increased cytoplasmic granulocytic (cloudy swelling) or many intracytoplasmic microvacuoles (vacuolar degeneration) and mild coagulative necrosis in animals treated with LPS and Bi-LPS, respectively (Figure 3). No pathological alterations were observed in livers of control animals which were given saline only (Figure 4).

Five minutes after incubation with Bi-LPS, the surfaces of platelets were intensely stained by the reaction product of HRP or labeled by gold grains (Figures 5A and 5B), whereas the cell surfaces of the monocytes were labeled by a few gold grains and HRP reaction product (Figures 6A and 6B). No staining or labeling was observed on the cell surfaces of both platelets and monocytes in the samples incubated with non-biotinylated LPS. Incorporated Bi-LPS was observed in platelets 60 min and in monocytes 5 min after incubation. The reaction product of HRP, indicating incorporated LPS, was observed in small vesicles and vacuoles of platelets (Figure 7). In monocytes, Bi-LPS was localized in many large phagocytic vacuoles as previously reported (10) (Figures 8 and 9).

Table 2. *Results from immunodot tests using PVC-silica*[a]

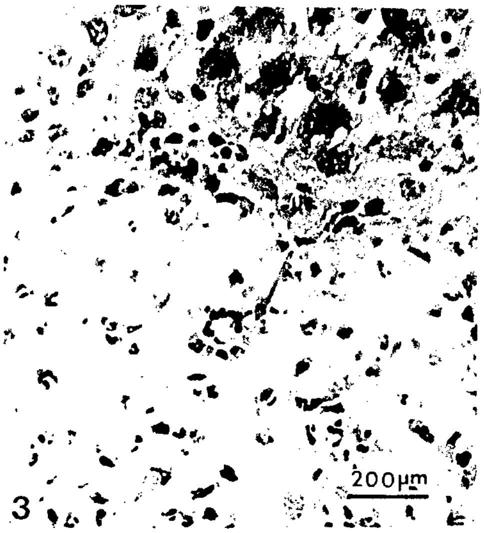
Samples	Dilutions 1	1:2	1:4
PBS	–	–	–
LPS	–	–	–
Bi-LPS (22 wks)	+ + +	+ +	+

[a] + + +, strong HRP staining; + +, moderate; +, weak; –, no reaction.

Table 3. *Results of* Limulus *ameobocyte lysate assays*[a]

Samples	Dilutions 1	1:2	1:4	1:8	1:16	1:32
Bi-LPS (22 wks)	+	+	+	+	+	+
Bi-LPS (12 wks)	+	+	+	+	+	+
LPS	+	+	+	+	+	+
PBS	–	–	–	–	–	–

[a] +, gelation; –, no gelation reaction.



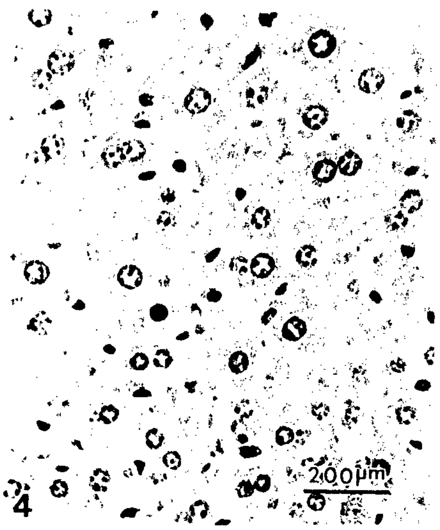


Figure 3. Liver of mouse treated with Bi-LPS or LPS. Pathological changes including necrosis, sinusoidal distension, and vacuolation of hepatocytes are evident 48 hr after injection with Bi-LPS or LPS. Original magnification × 732.

Figure 4. Liver of normal control animal. The hepatic tissue displays normal morphology. Original magnification × 732.

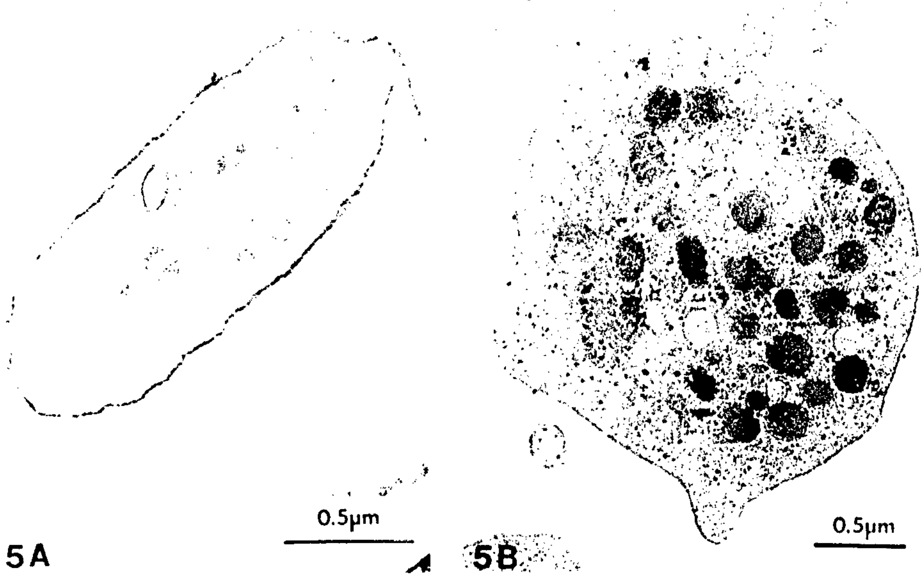


Figure 5. Localization of surface LPS binding sites in platelets using Bi-LPS and avidin–biotin–HRP or streptavidin–gold (20 min). **(A)** HRP reaction product representing LPS binding sites is localized on the cell surface 5 min after incubation. Original magnification × 43,200. **(B)** A few gold grains are seen on the surface of the cell 5 min after exposure to Bi-LPS. Original magnification × 29,960.

Discussion

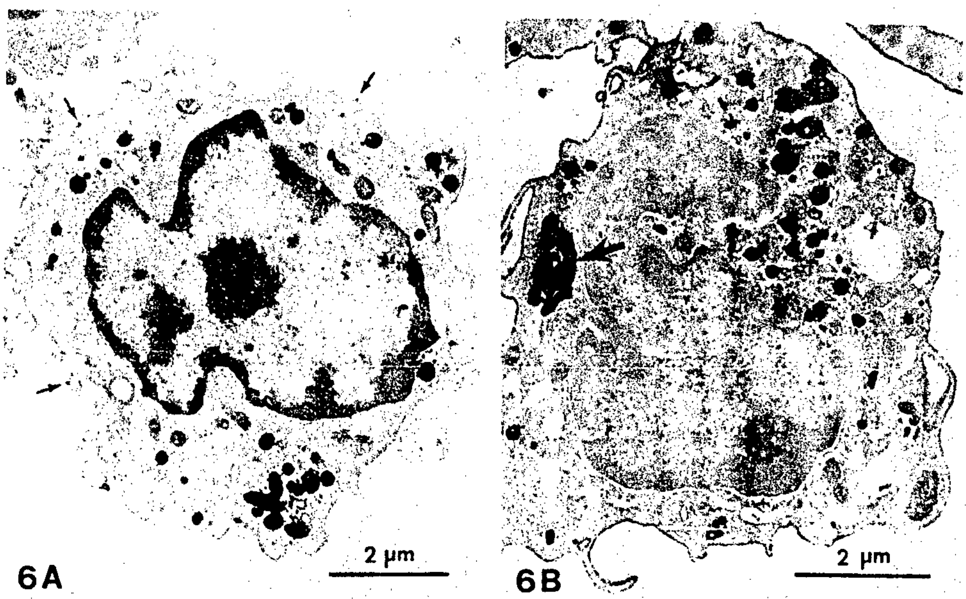
In this study, we have developed a technique for biotinylation of LPS from *E. coli* (B4) using lysine as a bridge. This provides a simple and rapid procedure for synthesizing stable Bi-LPS for ultrastructural localization of surface binding sites and subcellular distribution. The hydroxyl groups on the carbohydrate moieties are the sites for biotin attachment. The glycosides in the polysaccharide portion of LPS contain vicinal hydroxyl groups in a gauche conformation. Oxidation of glycosides by periodate yields dialdehyde, which can react with α-amino group of lysine whose ε group is linked to biotin (*N*-biotinyl-L-lysine or biotinyllysine). Biotinylation was confirmed by the results from all the tests using double immunodiffusion, ELISA, and immunodot methods. Importantly, our results also indicated that the biological/toxicological activities of Bi-LPS were retained, as shown by the pathological alterations in the liver after administration of Bi-LPS. The retention is due to the use of an activated biotin reagent and lysine, which was used as a linker between the carbohydrate moieties and biotin without disrupting lipid A, the biologically active center of LPS. Although the percentage of biotinylated LPS molecules was neither determined nor implied, we have shown by the use of four different methods that at least a sizeable quantity of the amount of LPS used was biotinylated. Other than a slight difference in the pH of sodium acetate buffer in which LPS was dissolved and in which the samples were dialyzed, there was no other relevant difference between the 12- and 22-week-old samples.

LPS has a micellar size of several millions in aqueous solution, as a result of aggregation of each amphipathic LPS molecules (20) depending on pH. The stacking of LPS particles (6,22,23) and the transition of LPS particles from disks to vesicles (6) have been reported. The degree of dispersion of LPS (availability of Lipid A) is believed to affect its ability to attach to the wells. Therefore, the negative reactivity at high concentrations using the ELISA technique may have been due to the non-availability of the lipid moieties

Figure 6. Localization of surface binding sites in monocytes by Bi-LPS and streptavidin-gold or avidin-biotin-HRP. Monocytes are characterized by many peroxidase-positive granules in the cytoplasm. (A) Gold grains are seen attaching to the cell surface 5 min after incubation. Original magnification × 10,000. (B) The cell surface is distinctly stained by HRP reaction product after a 5-min incubation with Bi-LPS. Arrow indicates ingested LPS bilayers. Original magnification × 11,300.

Figure 7. Intracellular localization of LPS in platelets using Bi-LPS and avidin-biotin-HRP. LPS bilayers stained by HRP reaction product (arrows) are observed in the vacuoles and vesicles of platelets 60 min after incubation. Original magnification × 72,000.

Figure 8. Intracellular localization of LPS in monocytes using Bi-LPS and avidin-biotin-HRP. Sixty minutes after incubation, LPS bilayers that are intensely stained by HRP reaction product are seen in many phagocytic vacuoles (arrows). Original magnification × 10,000



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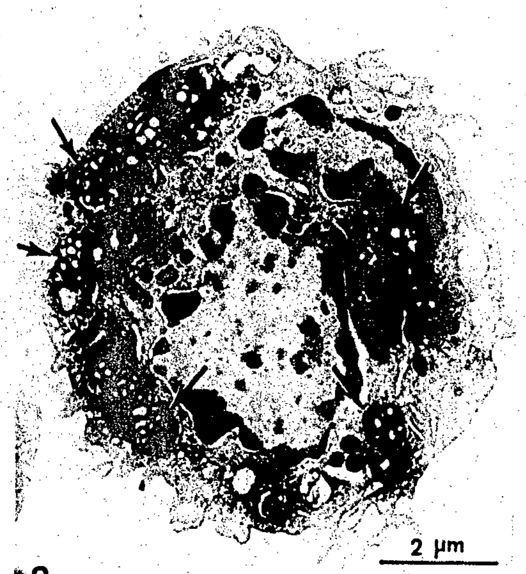

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of LPS to serve as anchors to the wells. Our results have shown that sonication of low-concentration samples for a few minutes increased the relative dispersion of lipid moieties and enhanced the positivity of test results. This is in agreement with the reported results of Ogawa and Kanoh (17). Also, at high concentrations, the double-immunodiffusion technique did not show a positive reaction until after 72 hr. This may be due to that large micellar formation mentioned above obviously impeding diffusion. These results indicate that sonication and serial dilution are necessary to limit micelle formation and aid diffusion through the gel. LPS has been revealed (23) by conventional TEM, seen as stacked and/or bilayer structures. But on the surface of intact bacteria, the lipid-A portion of the LPS which causes the stacking is still embedded in the cell walls of the bacteria, leaving the polysaccharide portion hanging freely outside. Therefore, unless an antibody specific to the polysaccharide is used, the structure of the latter could not be revealed by conventional TEM. However, using the Bi-LPS technique the polysaccharide moieties on the surface of intact bacterial can be revealed.

As shown in Figures 5A, 6B, 8, and 9 with respect to the intensity and clarity of surface and intracellular staining, LPS-biotin-avidin-HRP complex has proven to be more advantageous and of greater analytical value than radiolabeled, FITC-conjugated, and HRP-conjugated LPS. Bi-LPS has at least two advantages over LPS-HRP conjugates which were previously used to localize LPS receptors or binding sites on the surface of erythrocytes (16). First, avidin- or streptavidin-linked HRP, colloidal gold, ferritin, or hemocyanin can be used as markers for localization of surface binding sites or receptors at the light or ultrastructural level. Second, Bi-LPS provides more intense staining than LPS directly linked to a marker, since avidin-biotion-HRP can amplify staining. Moreover,

Figure 9. Intracellular localization of LPS in monocytes using Bi-LPS and avidin-biotin-HRP. LPS bilayers (large arrows) stained by HRP reaction product are seen in vacuoles with a limiting membrane (small arrows). Original magnification × 160,000.

LPS binding sites on the cell surface can also be analyzed quantitatively by labeling with colloidal gold or ferritin (25).

In conclusion, Bi-LPS provides a reliable and sensitive means for study of LPS binding sites and subcellular distribution because of its smaller size and easier intracellular diffusion as compared to other LPS probes.

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17. COSATI CODES

FIELD	GROUP	SUB-GROUP

18. SUBJECT TERMS *(Continue on reverse if necessary and identify by block number)*
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19. ABSTRACT *(Continue on reverse if necessary and identify by block number)*

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT
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