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Binding of Streptavidin to Bacteria or Fungi and Its Applications in Detecting These Microbes

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***Abstract*: We have investigated the characteristics and utilities of streptavidin-binding to gram-negative and gram-positive bacteria and *Candida* spp. The pre-treatment of these microbes with chemical reagents such as $CHCl_3$, NaOH, and Tween 20 have allowed colorimetric visualization under light microscopy or quantitation on nitrocellulose membranes, using streptavidin/biotinylated alkaline phosphatase conjugates. Analysis of this binding was confirmed by western blot. These binding reactions were due to the specific interaction of streptavidin with biotinylated proteins present in the microbes. Competition assays with free biotin or inhibition by an antibiotin antibody confirmed binding to these proteins. With knowledge of these strongly specific interactions, we attempted to reveal the biotinylated proteins within these microbes using clinical specimens. Using phagocyte-smears from blood, urine, and ascites, these intracellular microbes were easily detected by light microscopy. One of the septic blood samples stained by our technique revealed semi-digested microbial signals despite the absence of a signal with routine staining. This detection system, which combines streptavidin as a probe and biotinylated proteins as a microbial marker, is useful in staining for intracellular bacteria or fungi (e.g., microbial infections in phagocyte-smears).**

***Key words*: Streptavidin, Microbial marker, Biotin-binding proteins, Phagocyte-smears**

Streptavidin was originally discovered as a compound produced by *Streptomycetes* that exhibited antibiotic properties by inhibiting the growth of gram-negative bacteria such as *E. coli*, *Proteus mirabilis*, and *Alcaligenes faecalis* (6, 27). Its mechanism or action was hypothesized to be the inhibition of biotin synthesis (10).

Streptavidin-enzyme conjugates are important tools for revealing biotin-labeled hybridized signals (11, 20) in avidin/biotin detection systems (28). However, frequently they produce non-specific false-positive signals (2, 5, 7, 14, 16, 17, 25) due to their binding to bacterial debris in crude target DNA in bacterial-colony blots. In such gram-negative bacteria as *E. coli* (18) or *Pseudomonas aeruginosa* (17), avidin-binding proteins inside the outer membrane are hypothesized to contribute to the binding of streptavidin. However, this cannot explain these observations seen in other bacteria (7, 25) as inducing non-specific signals. We believe that streptavidin-binding proteins exist commonly in microbes like bacteria and fungi. From this, we employed the specific binding of streptavidin for the detection of clinically important microbes. In this report, we discuss the characteristics of binding and the potential use of streptavidin/biotinylated-alkaline phosphatase conjugates as a means for identifying these microbe-infected specimens.

Materials and Methods

Bacterial strains and Candida *spp.* Strains investigated in this study were kindly provided from the collections of Dr. Hoshina in the Department of Clinical Medicine at Jikei University:

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Abbreviations: Ab, antibody; AP9.5, 0.1 M Tris-HCl, pH 9.5/0.1 M NaCl/10 mM $MgCl_2$; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; bio-ALP, biotinylated alkaline phosphatase; BRL, Bethesda Research Laboratory; BSA, bovine serum albumin; NBT, nitro blue tetrazolium; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PBS-Mg, PBS containing 5 mM $MgCl_2$; PMNs, polymorphonuclear neutrophils; St, streptavidin; St-ALP conjugates, streptavidin-alkaline phosphatase conjugates; TCA-SDS, trichloroacetic acid-sodium dodecyl sulfate.

Pseudomonas aeruginosa HCMJ3080, *Proteus mirabilis* HCMJ3081, *Staphylococcus aureus* HCMJ12732, *Staphylococcus epidermidis* HCMJ12733, *Streptococcus faecalis* HCMJ12970, *Serratia marcescens* HCMJ274, *E. coli* HCMJ11775, *Klebsiella pneumoniae* HCMJ979, *Klebsiella oxytoca* HCMJ980, *Enterobacter cloacae* HCMJ977, *Bacillus subtilis* HCMJ, *Candida albicans* HCMJ01, *Candida krusei* HCMJ02.

Detection and observation of microbes stained with streptavidin and biotinylated alkaline phosphatase under light microscopy. Suspensions of freshly cultured bacteria or *Candida* were made in distilled water. The microbes were then smeared on a slide glass and fixed by gentle flaming. The fixed cells were immersed in chloroform for 10 min, then in a solution of 0.5 M NaOH/1.5 M NaCl for 3 min, and finally for 3 min each in series of graded ethanols (70, 85, and 95%). After air-drying, the slide was horizontally placed in a humidified chamber onto which 100 μl of streptavidin (4 μg/ml, Amersham) in phosphate-buffered saline (PBS: 10 mM $NaPO_4$, pH 7.4/0.137 M NaCl) was loaded and allowed to incubate for 20 min. Then, the slide was washed twice for 5 min each in a coplin jar containing 50 ml of PBS. The slide was removed from the coplin jar and a 100 μl aliquot of biotinylated alkaline phosphatase (bio-ALP) (4 μg/ml, E.Y. Lab) in PBS containing 5 mM $MgCl_2$ was loaded onto the horizontally placed slide. This was allowed to incubate in the humidified chamber for an additional 10 min. The slide was then washed twice for 5 min with AP9.5 (0.1 M Tris-HCl, pH 9.5/0.1 M NaCl/10 mM $MgCl_2$). Finally BCIP (5-bromo-4-chloro-3-indolyl phosphate, 167 μg/ml, Sigma) and NBT (nitro blue tetrazolium, 330 μg/ml, Sigma) in AP9.5 were added as substrates and allowed to react for 10 min in the dark to give a color change. The stained smear was rinsed in a coplin jar with PBS, air-dried, and examined under light microscopy.

Quantitative staining of microbes fixed on nitrocellulose membranes using streptavidin and bio-ALP. Several concentrations of cell suspensions (10^5–10^9) were prepared by diluting strain stocks (10^9) suspended in PBS. Cellular suspensions were trapped on a nitrocellulose membrane (Whatman) using a Minifold-I apparatus (Schleicher & Schuell) and air-dried completely. The membrane was placed in a dish containing 3MM paper (Whatman) saturated with chloroform for 10 min. It was then placed face-up in a similar chamber containing 3 MM paper saturated with 0.5 M NaOH/1.5 M NaCl for 5 min. The membrane was then thoroughly rinsed with PBS and subsequently air-dried. The microbes-fixed nitrocellulose was immersed in PBS/0.5% (v/v) Tween 20 for 30 min. The membrane was then incubated for 30 min in PBS (50 ml per 25 mm^2 sheet) containing streptavidin (4 μg/ml), after which a 10 min wash with PBS/0.25% (v/v) Tween 20 was performed. The membrane was then immersed in 50 ml per sheet of AP9.5 containing bio-ALP (4 μg/ml). For color development, substrates were added to the solution and allowed to incubate for at least 10 min. Substrate reactions were stopped with a PBS rinse. Competitive binding experiments were also conducted using goat anti-biotin antibody (α-bio Ab, Vector), streptavidin and free biotin (Vector). A streptavidin-alkaline phosphatase conjugate (BRL for BlueGENE™) was used to detect the presence of biotinylated protein in microbes and colorimetric changes were observed.

Western blot analysis. Microbial extracts were obtained with TCA-SDS (trichloroacetic acid-sodium dodecyl sulfate) according to the method of Osborn et al (23). Microbial proteins were isolated using an SDS-PAGE plate (PAG-10/20™, Daiichi Chemical) and transferred to nylon membranes (Immobilone™, Daiichi Chemical) as described in the procedure of Laemmli (19). The membrane was reacted with streptavidin and bio-ALP as described above for colorimetric analysis. Another blotted membrane was incubated with α-bio Ab (10 μg/ml in PBS containing 1% (w/v) BSA) for 30 min before being treated with streptavidin and bio-ALP. Additionally, human whole blood or phagocyte fractions were obtained by the following dextran method and incubated with *S. aureus* ($\sim 10^9$/ml) for 20 min at room temperature. Cell precipitations were obtained by centrifugation at 3,000 rpm for 10 min. Proteins from the phagocytic cells were isolated and analyzed by the western blotting method described above.

Detection of bacteria or fungi in situ *from infected smears using streptavidin-alkaline phosphatase conjugate*. A suspension of *S. aureus* ($\sim 10^8$/mouse) was injected abdominally into mice which were humanly sacrificed after 8 hr. Infected ascites and blood (from which PMNs were isolated) were collected from these mice. Experimentally infected human cells were prepared by using whole blood incubated with the same bacterial strain ($\sim 10^9$/20 ml) at 37 C for 60 min. From these samples, phagocytic cell fractions were separated using a 6% (w/v) dextran method (21). The ascites and phagocytic cell fractions were centrifuged at

800 rpm (400×*g*) to obtain cell precipitates. The precipitates were then suspended in a small volume of PBS (~10^5/100 μl) and smeared on a 2% gelatin (Difco)-coated slide. Septic blood samples obtained from clinical specimens were treated in a similar manner. Human bloods with *Candida* spp. infected cells were prepared by the method of Seki et al (24) and phagocytic cells were smeared on coated slides. Infected urine samples were smeared directly onto coated slides. All smears were allowed to air-dry completely before being immersed in Carnoy's fixative ($CHCl_3$: EtOH : CH_3COOH = 6 : 3 : 1) for 10 min after which they were again air-dried. The samples were then immersed in 70 mM NaOH for 3 min and immediately dehydrated in a series of graded ethanols (70, 85, and 95%) for 3 min each. The bacteria or *Candida* smears were then treated with streptavidin alkaline phosphatase conjugate in accordance with BRL's *in situ* hybridization manual (3). Slides were counter-stained with Naphthol Blue Black (Sigma) (a microspatula of powder/200 ml of PBS or water) and examined under light microscopy.

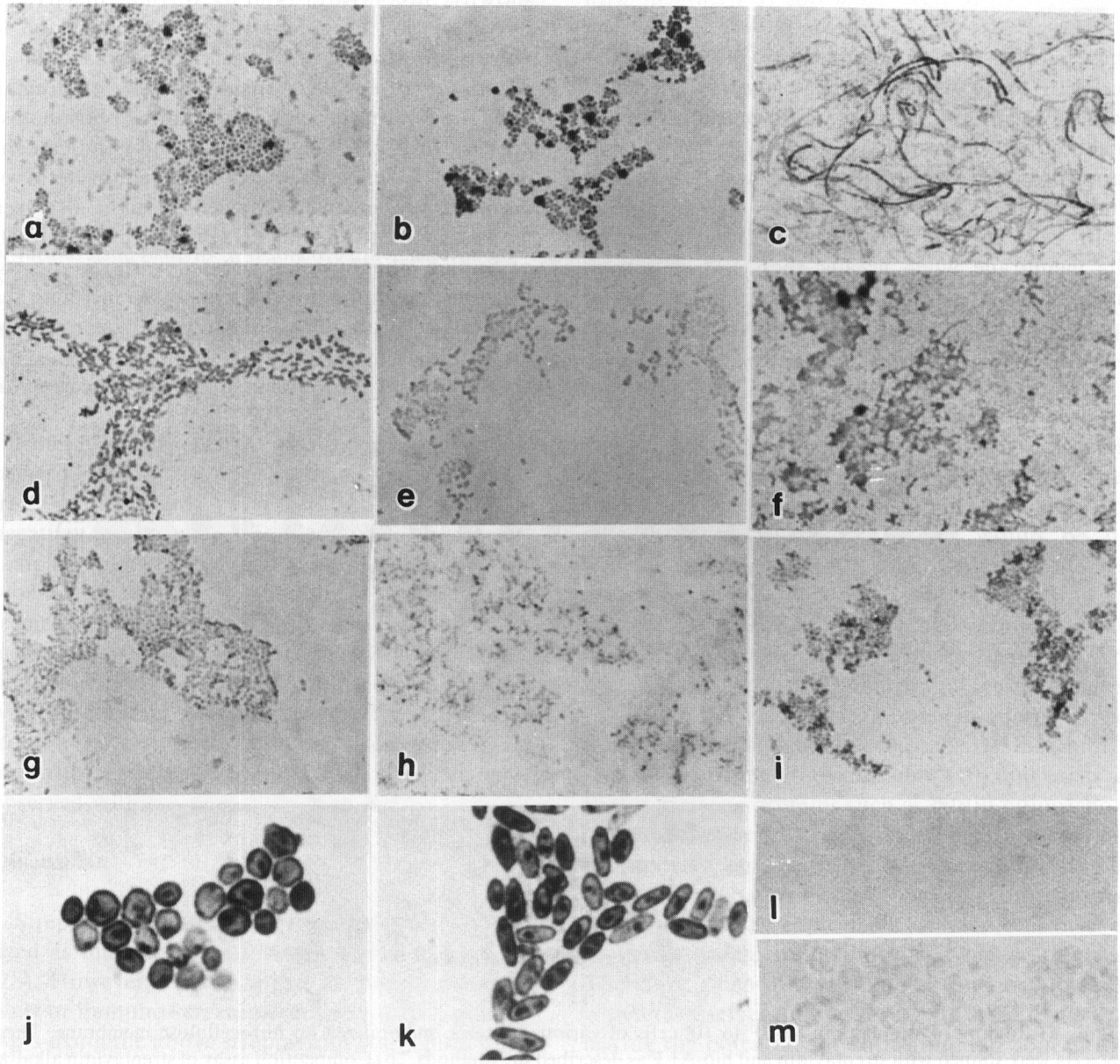

Fig. 1. Microscopical view of gram-negative or gram-positive bacteria and *Candida* spp. stained by streptavidin and bio-ALP. The following microbes were visualized with the pre-treatment as described in the methods. a) *Staphylococcus aureus*, b) *Streptococcus faecalis*, c) *Bacillus subtilis*, d) *Klebsiella pneumoniae*, e) *E. coli*, f) *Pseudomonas aeruginosa*, g) *Enterobacter cloacae*, h) *Serratia marcescens*, i) *Proteus mirabilis*, j) *Candida albicans*, and k) *Candida krusei*, were treated with streptavidin and bio-ALP. l) *K. pneumoniae* and m) *C. albicans* treated by bio-ALP (4 μg/ml) without adding streptavidin.

Results

Under light microscopy (Fig. 1), both gram-negative and gram-positive bacteria and *Candida* spp. could be visualized using streptavidin and bio-ALP conjugates. In the case of gram-negative bacteria, especially *Pseudomonas aeruginosa* (Fig. 1f), disruption of cell morphology was observed when compared to gram-positive bacteria and *Candida*. These gram-negative cells, which appeared larger than normal when observed by light microscopy, seemed to have burst due to treatment with $CHCl_3$ or NaOH. As shown in Fig. 1, l and m, these bacteria or *Candida* could not be visualized by bio-ALP only without initial streptavidin treatment. The amount of streptavidin bound to microbial biotinylated proteins (Fig. 2a) was reliably quantitative provided the number of cells was between 10^5 and 10^8. The signal was virtually undetectable at $< 10^5$ cells and the microbes would not adhere to the membrane when greater than 10^9 cells were used. This was observed regardless of the microbial species. No species-specific differences relative to the binding characteristics of streptavidin were observed. This binding was inhibited with streptavidin, α-bio Ab, and biotin (Fig. 2b). Western blot analysis of microbial extracts (Fig. 3a) on the distribution of streptavidin-binding proteins revealed distinctive differences among microbial species. This was demonstrated by the presence or various band patterns from staining with streptavidin and bio-ALP. The specific binding of streptavidin was blocked by pre-treatment with α-bio Ab (Figs. 2b, 3b-i). Similar results were obtained with streptavidin pre-treatment (data not shown). Streptavidin binding was not inactivated by acetylation (Fig. 3b-ii). The data from the *in vitro* or *in vivo* experiments in which bacteria or *Candida* were taken inside mouse or human phagocytes using streptavidin alkaline phosphatase are seen in Fig. 4. Figure 4, a, b, and e indicate the detection of experimentally ingested bacteria or *Candida* present inside the cells. This is also seen by western blotting analysis (Fig. 3c) using extracts of human phagocytes incubated with *S. aureus*. Phagocyte components did not cross-react with the streptavidin alkaline phosphatase conjugates.

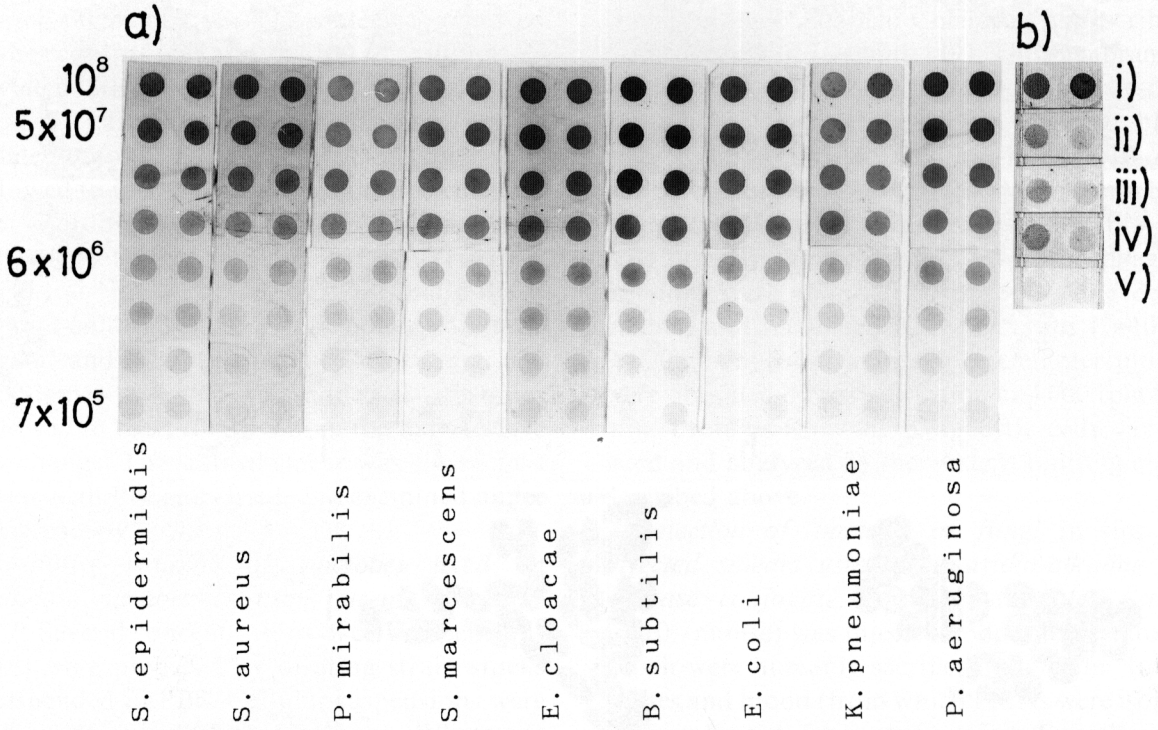

Fig. 2. a) Binding of streptavidin to 10^5 to 10^8 cells of various bacteria immobilized on nitrocellulose membrane. These bacteria were stained with streptavidin and bio-ALP as described in "Methods." b) Competition assay of streptavidin alkaline phosphatase conjugates with α-bio Ab, biotin, and streptavidin. Approximately 10^7 cells of *S. aureus* (right side) and *K. oxytoca* (left side) were blotted respectively according to the above methods, these cells were incubated with streptavidin alkaline phosphatase conjugates (4 μg/ml) and α-bio Ab (3 μg/ml), biotin (10 μg/ml) or streptavidin (4 μg/ml), and then detected as described in "Methods." i) Streptavidin alkaline phosphatase conjugates, ii) streptavidin alkaline phosphatase/streptavidin, iii) streptavidin alkaline phosphatase/α-bio Ab, iv) streptavidin alkaline phosphatase/biotin, and v) bio-ALP.

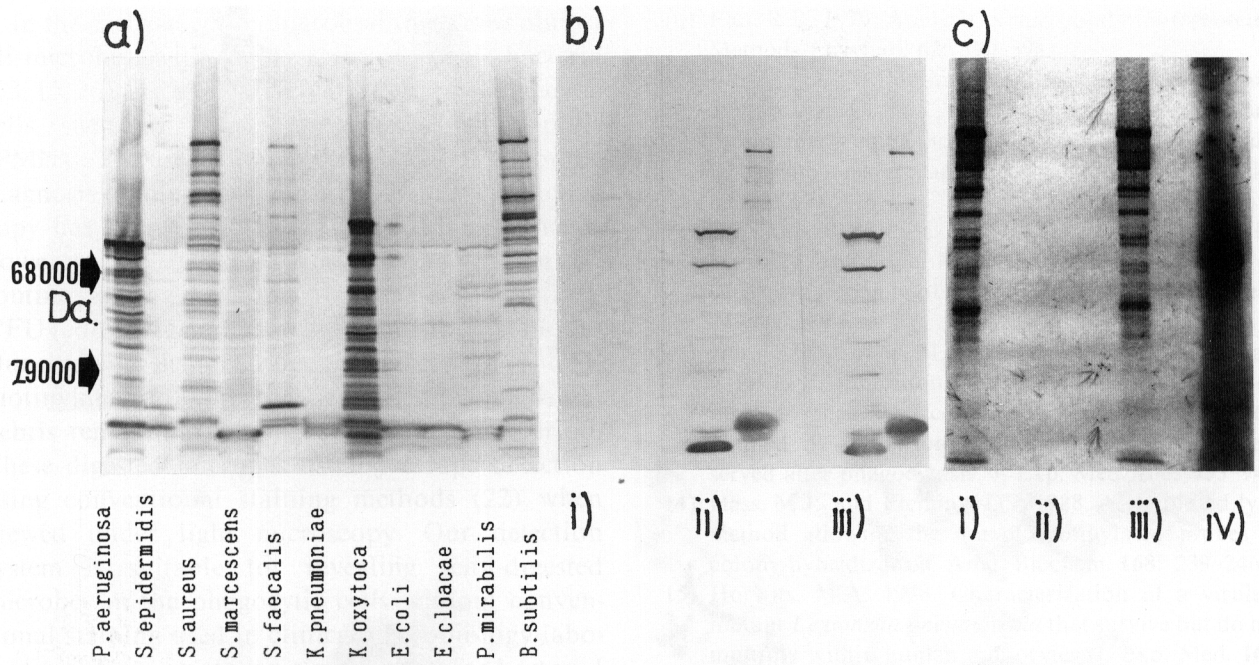

Fig. 3. Detection of streptavidin-binding proteins by western blot analysis using streptavidin and bio-ALP. a) Distribution of various band patterns by distinctive differences among microbial species. b) Blocking of streptavidin-binding proteins by pre-treatment of α-bio Ab. i) After treatment with α-bio Ab, *S. aureus* or *K. oxytoca* extract was detected as described in "Methods." ii) After acetylation (the membrane was incubated with 0.8% (w/v) acetic anhydride in 0.1 M triethanolamine for 20 min), these same bacterial proteins were detected as described in "Methods." iii) Without any pre-treatment, these proteins were detected. c) Cross-reaction of streptavidin to phagocytes-components. Each lane shows i) *S. aureus*, ii) phagocytes pellet, iii) phagocytes fractions incubated with *S. aureus*, iv) whole blood.

Furthermore, we were able to detect many signals of bacterial origin in the phagocyte-smears prepared from septic bloods (Fig. 4, c and d). However, negative results were obtained when these same samples were stained using gram- or methylene blue-method. Many signals in PMNs (Fig. 4d) seemed to represent semi-digested bacteria. Our method also detected an obviously *Candida*-like fungi in a specimen obtained from a dialysis patient with ascites (Fig. 4f). A urine smear was obtained from a patient with culture-proven gonococcal urethritis in which bacteria-like particles were observed within a phagocyte (Fig. 4g).

Discussion

Streptavidin-biotin enzyme conjugates are utilized as the standard of avidin/biotin technology (28). However, hybridization on bacterial-colony blots or immuno-screening on bacteriolytic plaque-blots using the above streptavidin conjugates often results in false-positive signals (2, 5, 7, 14, 16, 17, 25). These observations were confirmed by our results that specific binding of streptavidin is due to microbial biotinylated proteins exposed by chemical treatments such as chloroform, NaOH, and certain detergents. Bacterial or fungal cell-walls could be damaged by these chemicals used for pre-treatments. By these treatments, the permeability of streptavidin into these microbes would be increased, and consequently streptavidin can bind to these microbial proteins. Since the binding of streptavidin alkaline phosphatase conjugates to these microbes was inhibited by biotin, α-bio Ab, and streptavidin, we considered that the characteristics of streptavidin binding to these microbes was due to specific biotinylated proteins. In fact, biotin is widely distributed in microbes (10) and exists as biotin carrying proteins such as biotin holocarboxylase synthetase in *E. coli* (12). These proteins are biotinylated covalently in lysine residues (1). In light of this, bacterial or fungal rich biotinylated proteins might be exploited as a microbial marker to probe using streptavidin enzyme conjugates. Therefore, attention was paid to phagocytic cells, which themselves did not react with streptavidin, as having the potential for collecting and concentrating microbes (4) containing these biotinylated proteins. We believe this may provide an important opportunity to screen infectious microbes in phagocytes as a tool for the diagnosis of bacterial or fungal infections.

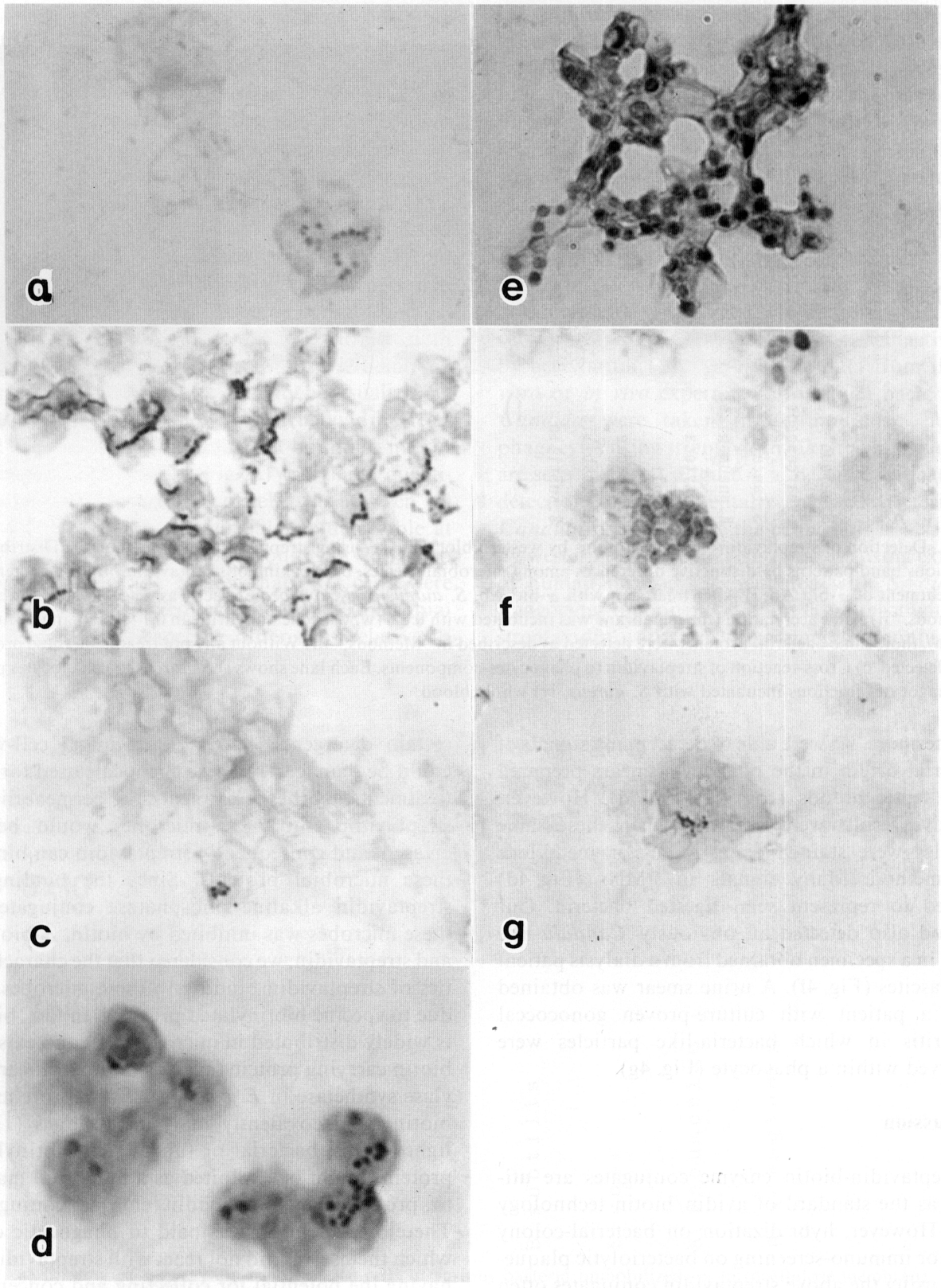

Fig. 4. Detection of the microbes in the phagocyte-smears using streptavidin alkaline phosphatase conjugates. Experimentally or clinically intracellular bacteria were stained with streptavidin alkaline phosphatase as previously described. a) *S. aureus* in mouse phagocytes. b) *S. aureus* ingested by human blood PMNs. c) Intracellular unknown bacteria from septic blood. d) Semi-digested bacteria from septic blood. e) Experimentally ingested *C. albicans* in human blood PMNs. f) *Candida*-like fungi in ascites from a dialysis patient. g) Intracellular bacterial signals in a gonococcal urethritis.

In the early stages of microbial infections almost all microbes, with the exception of some bacteria (13, 15, 26), are immediately digested by phagocytic cells such as polymorphonuclear neutrophils (PMNs). The usefulness of buffy-coat smears for the diagnosis of microbial infections with light microscopy has been illustrated (4, 8, 9). It is difficult, however, to detect intracellular bacteria by routine staining if the bacterial load is less than 10^5 CFU (colony-forming units) per milliliter of blood. Our study has demonstrated the presence of residual biotinylated proteins from semi-digested microbial debris remaining in phagosomes in some period. These digested microbes usually escape detection using conventional staining methods (22) when viewed under light microscopy. Our detection system is suitable for revealing semi-digested microbes in the phagocytic cells without conventional staining used in clinical microbiology laboratories. This simple staining method for bacterial or fungal biotinylated proteins in infected phagosomes utilizing streptavidin enzyme conjugates may contribute to the rapid and accurate clinical diagnosis of infected specimens.

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