KOR. JOUR. MICROBIOL. August, 1994, p. 280~284 Vol. 32, No. 4

A Possible Role of Lipopolysaccharides in the Prevention of Lysosome-Symbiosome Fusion as Studied by Microinjection of an Anti-LPS Monoclonal Antibody

Choi, Eui Yul

Department of Genetic Engineering, Hallym University, Chunchon, Korea 200-702

Lack of lysosomal fusion with symbiosomes in symbiont-bearing *Amoeba proteus* may be due either to the presence of a component in the symbiosome membrane or to the absence of a component needed in the fusion process. Using monoclonal antibody as a probe, lipopolysaccharides were identified as symbiosome-membrane components contributed by symbionts and were found to be exposed on the cytoplasmic side of the membrane. In order to test whether lipopolysaccharides may play a role in the prevention of lysosome-symbiosome fusion, the antilipopolysaccharides antibody was microinjected and processed for double immunostaining in conjuction with anti-lysosome antibody as a lysosome-fusion indicator. Microinjection of the anti-LPS antibody caused symbiosomes to fuse with lysosomes, suggesting that X-bacterial lipopolysaccharides could be 'fusion-preventing' factors.

KEY WORDS □ monoclonal antibodies, microinjection, lysosome fusion, lipopoysaccharides, symbiosis

X-bacteria are unidentified Gram-negative bacteria that spontaneously infected the D strain of *Amoeba proteus* in the laboratory and established a stable symbiosis within a few years (17). At present, each amoeba harbors about 42,000 bacteria, which are enclosed by membraneous vesicles called symbiosomes, and the host and symbionts are mutually dependent for survival. It has been found that the host and symbionts synthesize and exchange a few proteins between them (18).

One of the unanswered questions is how X-bacteria avoid destruction by amoebae that are rich in lysosomal enzymes and digest any other ingested living organisms. Although a number of studies regarding the prevention of lysosomal fusion in symbiosis and pathogenicity have been conducted, little is known about the mechanism for the inhibition of lysosomal fusion or identity of the components involved in the fusion inhibition (22). The lack of lysosomal fusion with symbiosomes in xD amoebae may be due either to the presence of a component in the vesicle membrane or to the absence of a component needed in the fusion process.

In the previous study, we produced monoclonal antibodies (mAbs) against the symbiosome membranes and identified the antigen as lipopolysaccharides (LPS) on the symbiosome membranes (4). Since X-bacterial LPS were found to be located on the cytoplasmic side of the symbiosome membranes, it has been suspected that LPS may be involved in the prevention of lysosome-symbiosome fusion. Thus, it was of interest to see if the LPS play any role in the inhibition of lysosomal fusion. To test the possibilty, anti-LPS mAb was purified and microinjected into amoeba cytoplasm. The result suggested that LPS might play a role in the prevention of the symbiosome-lysosome fusion.

MATERIALS AND METHODS

Amoebae

Two strains of *Amoeba proteus*, D and xD were grown in a modified Chalkley's solution (19) with axenically cultured *Tetrahymena pyryformis* as food organism. Amoebae were grown in Pyrex baking dishes (34×22×4 cm), feeding every day. *Tetrahymena* were grown in 2% proteose peptone, 0.2% liver concentrate, and vitamin concentrate (11).

Isolation of lysosomes

Amoebae (5-m*l* packed cells) were harvested and lysed by homogenization in 5 m*l* of the homogenizing buffer. The lysate was filtered through a 45-μm-pore-size nylon screen to remove unbroken cells and large pieces of plasma membranes. The filtrate (5 *ml*) was placed on top of 10~50% linear gradient of Percoll in a 50-*ml* tube and centrifuged for 30 min at 10,000 ×*g* and 1-*ml* fractions were pulled from the top of the tubes. The fractions were combined together, diluted 5 times with the homogenizing buffer, and collected by centrifugation for 10 min at 15,000×*g*.

Purification of lipopolysaccharides

LPS were purified from X-bacteria according to Darveau and Hancock (7). Purified LPS were subjected to SDS-PAGE in 14% polyacrylamide gel with the Laemmli buffer system (21). After electrophoresis, the gel was cut into two parts, one was silver stained for detecting LPS (15, 25) and the other half was electrophoretically transferred to nitrocellulose membrane and processed for immunoblotting (24).

Production and purification of mAbs

The mAbs to bacterial LPS (4) and lysosomal membrane proteins (6) were obtained as described previously. For the production of larger amount of mAbs, ascites fluids were obtained from mice injected with hybridoma cells. To induce tumors, BALB/c mice were first injected with 0.5 *ml* of pristane (2,6,10,14-tetramethylepentadecane). For purification of mAbs, 5 *ml* of ascites fluids were centrifuged for 10 min at 15,000×*g*, and the supernatant was diluted 4 times with PBS. MAbs were precipitated by adding an equal volume of saturated ammonium sulfate for 30 min with a gentle stirring and collected by centrifugation for 10 min at 7,000×*g*. The pellet was suspended in PBS and dialyzed extensively against PBS. A protein-A column (1-*ml* packed volume) was prepared and washed extensively with PBS. The dialyzed solution was centrifuged for 30 min at 12,000×*g* to remove insoluble aggregates and the supernatant was applied to the column. The column was washed with PBS until the absorbance of unbound protein came down the the background level and antibodies were eluted with 0.1 M glycine-HCl (pH 2.5). The antibody solution was neutralized with the addition of 1 M Tris and concentrated by spinning in Centricon-30 (Amicon) to make final concentration of 5 mg/*ml*.

Biotinylation of mAbs

Biotinylation of protein-A purified mAbs was performed according to Boorsman *et al.* (1). BNHS (Biotin-N-hydroxy-succinimide, Molecular Probes) was dissolved in dimethylformamide (DMF) at 10 mM concentration. The reaction was initiated by adding 10 *μl* of the BNHS solution to 1 *ml* of mAb solution (1 mg/*ml* in 0.1 M sodium hydrogen carbonate) and proceeded for 1 hr at room temperature. The excess of BNHS was removed by dialysis overnight at 4°C. For indirect immuno fluorescence microscopy of biotinylated mAbs, FITC-conjugated streptoavidin (Molecular Probes) was used at a concentration of 10 *μg/ml*.

Indirect immunofluorescence microscopy

Amoebae were collected in Syracuse watch glasses and the medium was removed by aspiration. Cells were fixed with cold methanol (−20°C) for 5 min, washed 3 times with PBS, and treated with culture fluids of hybridoma cells (1 : 1 dilution in PBS) or ascites fliud (1 : 50 dilution in PBS) for 3 min. After washed with PBS 3 times, amoebae were incubated in goat anti-mouse IgG antibody (1 : 50 in PBS) for 30 min at room temperature. The labeled amoebae were washed 3 times with PBS and mounted in a solution containing 90% glycerol, 1 mg/*ml* p-phenylenediamine, and 10% PBS (23), and observed with a Leitz epifluorescence microscope.

Microinjection of mAbs

For the preparation of agar-coated coverslips, 0.6% agar in the Chalkley's solution was boiled, filtered, and poured onto clean glass coverslips (17). Twenty amoebae were picked up with a fine-tipped pipette and placed on top of an agar-coated coverslip (22×40 mm) in 4 groups of 5 amoebae. Excess medium was removed to make amoebae firmly attached. To remove aggregates formed during concentration and storage, the antibody solutiom was filtered through a 0.25-*μ*m-pore-size filter unit (West Coast Scientific). The antibody concentration of the filtrate was adjusted to 5 mg/*ml* before injection. About 5×10^{-5} *μl* of antibody solution (approximately 1/50 of amoeba cell volume) was injected into an amoeba using a micropipette mounted on a de Founbrune micromanpulator. After 3 hr, microinjected amoebae were fixed with 3% paraformaldehyde in PBS, permeabilized with cold ethanol, and treated with goat anti-mouse IgG antibodies conjugated with Texas Red to detect LPS. Then, to localize lysosomes, the cells were treated with biotinylated anti-lysosome mAb and stained by treating with streptavidin conjugated with FITC.

RESULTS AND DISCUSSION

The rationale for the microinjection experiment was that, if the injected anti-LPS mAb abolished the ability of the LPS to prevent lysosomal fusion, symbiosomes in injected cells would fuse with lysosomes. Consequently, the lysosome-symbiosome fusion complex could be formed. If the injected mAbs did not affect the ability of LPS, the symbiosome would not fuse with lysosomes. In this case, lysosomes and symbiosomes would be detected as separate identities. Thus, the fusion events could be monitored by double immunofluorescence staining in conjuction with anti-lysosome mAb as a lysosome-fusion indicator (6).

In order to check the specificities of the antibodies used in this study, X-bacterial LPS and

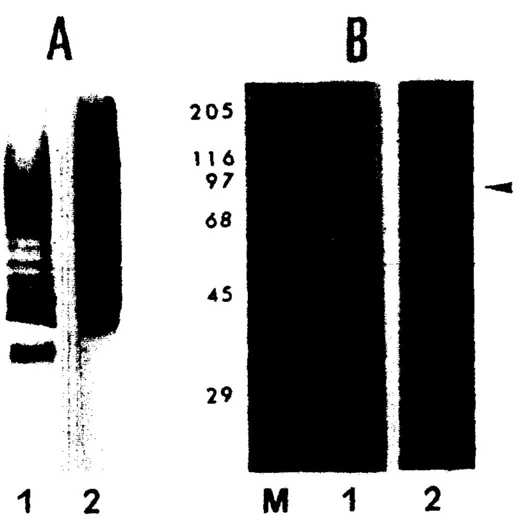

Fig. 1. *Immunoreactivities of anti-LPS and anti-lysosome mAbs.*
(A) Purified X-bacterial LPS visualized by a silver-staining method (lane 1) and the corresponding immunoblot probed with anti-LPS mAb (lane 2). (B) Amoeba lysosomal proteins separated by SDS gel electrophoresis (lane 1) and the corresponding immunoblot probed with anti-lysosome mAb. The anti-lysosome mAb specifically recognized a protein band of 90 kDa. The arrowhead indicates the position of the immunoreactive band.

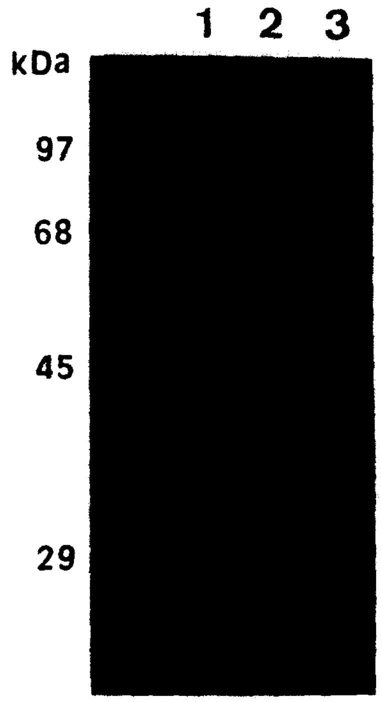

Fig. 2. *An SDS polyacrylamide gel of purified mAbs.*
Lane 1, anti-LPS mAb; 2, anti-lysosome mAb; 3, anti-HSP 60 mAb. The anti-HSP 60 mAb was generated against groEL protein of X-bacteria and confirmed to be present in X-bacteria cytoplasm. Thus, it was used as a control antibody for microinjection.

amoeba lysosomes were isolated, separated by SDS PAGE, and immunobloted (Fig. 1). For microinjection, the two mAbs and anti-HSP 60 mAb, a control antibody for microinjection (5), were first purified by protein-A affinity chromatography, and then concentrated (Fig. 2). Since both the anti-lysosome mAb and anti-LPS mAb were obtained from mouse, both of them were recognized by the same secondary antibody after double immunofluorescence staining. Thus, the anti-lysosome mAb was biotinylated after purification to be distinguished from the injected anti-LPS mAb.

In order to see if microinjection of an anti-LPS mAb cause fusion between lysosome and symbiosome, xD Amoebae were microinjected with the anti-LPS mAbs or anti-HSP 60 mAb solution. To monitor the lysosome fusion event, the cells were processed for double immunostaining to localize lysosomes and symbiosomes. In xD amoebae injected with an anti-LPS mAb, some of the symbiosomes showed positive staining with the anti-lysosome mAb, indicating that they had fused with lysosomes (Fig. 3 B & b). However, in the amoebae injected with an anti-HSP 60 mAb, as a control, none of the symbiosomes stained with the anti-lysosome mAb (Fig. 3 A & a). The result of double immunostaining experiments in conjuction with microinjection of the mAbs indicated that X-bacterial LPS appeared to be involved in the prevention of lysosomal fusion.

A fair number of studies have been reported regarding the inhibition of lysosomal fusion in human pathogens (2, 3, 8, 9). So far, no known factor inhibiting lysosomal fusion has been identified. Furthermore, little is known about the mechanism. Some polycations and sulfatide lipids have been known to inhibit lysosomal fusion (10, 12). However, the mechanism of these inhibitors is now controversial (13, 14). The microinjection experiments showed that the anti-LPS mAbs injected into xD amoeba cytoplasm appeared to let symbiosomes fuse with amoebalet symbiosomes fuse with amoeba lysosomes. One interpretation of the results in that X-bacterial LPS are involved in the prevention of lysosomal

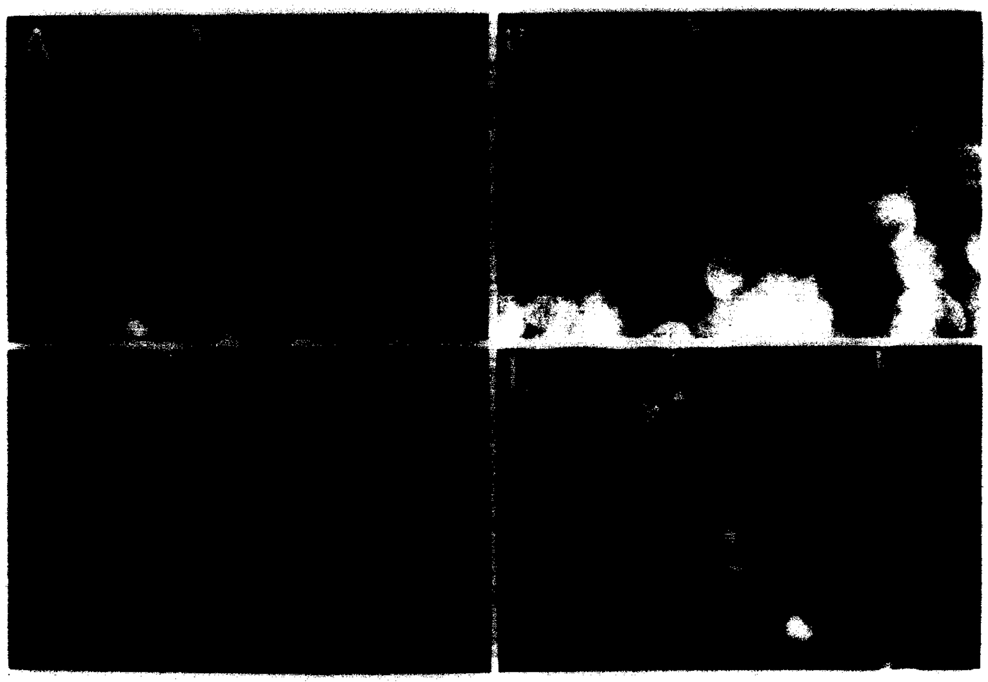

Fig. 3. *Symbiosome-lysosome fusion after microinjection of anti-LPS mAb.*
Indirect immunofluorescence micrographs of amoebae injected with a control mAb (A, a) and anti-LPS mAb (B, b). A and B, stained for symbiosomes; a and b, stained for lysosomes. It has been seen that symbiosomes became fusible with lysosome only when the anti-LPS mAb was microinjected into amoeba cytoplasm. The arrows indicate the position of symbiosomes (magnification, 1100 X).

fusion and that they are the fusion-preventing factors. Recently, Joiner *et al.* (20) demonstrated that the mechanism of fusion inhibition is likely to reflect a modification of the vacuole membrane at the time of its formation, as opposed to the opinion that a soluble fusion inhibitor is secreted by the parasite. The result further strengthens the view that the inhibition of lysosome-symbiosome fusion in amoeba-bacteria symbiosis is caused by a component on symbiosome membranes, such as X-bacterial LPS.

One approach to confirm that X-bacterial LPS are fusion-preventing factors is to clone X-bacterial genes for the synthesis of LPS. Once the genes are available, *E. coli* transformed with a plasmid inserted with the cloned genes could be tested for the survival inside amoeba cytoplasm after induced phagocytosis.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Hallym University, 1992.

REFERENCES

1. **Boorsma, D.M., J.V. Bommel, and J. Vanden Heuvel,** 1986. Avidin-HRP conjugates in biotin-avidin immunoenzyme cytochemistry. *Histochem.* **84**, 333-337.
2. **Chan, J., T. Fusihara, P. Brennen, M. McNeil, and S.J. Turco,** 1989. Microbial glycolipids: Possible virulence factors that scarvenge oxygen radicals. *Proc. Nat. Acad. Sci. USA* **86**, 2453-2457.
3. **Chang, K.P. and D.M. Dwyer,** 1976. Multiplication of human parasite (*Leishmania donovani*) in phagolysosomes of hamster macrophages *in vitro*. *Science* **193**, 679-681.
4. **Choi, E.Y. and K.W. Jeon,** 1992. Bacterial-endosymbiont-derived lipopolysaccharides on amoeba symbiosome membranes. *J. Protozool.* **39**, 205-210.
5. **Choi, E.Y., G.S. Ahn, and K.W. Jeon,** 1991. Elevated levels of stress proteins asssociated with bacterial symbiosis in *Amoeba proteus* and soybean root nodule cells. *Biosystems* **25**, 205-212.
6. **Choi, E.Y., K.J. Kim, and K.W. Jeon,** 1992. Lysosomal membrane proteins of *Amoeba proteus* as studied with monoclonal antibodies. *J. Protozool.* **39**, 671-677.
7. **Darveau, R.P. and R.E.W. Hancock,** 1983. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium*

strains. *J. Bacteriol.* **155**, 831-838.
8. **Draper, P. and R.J.W. Rees,** 1970. Electron-transparent zone of Mycobacteria may be a defence mechanism. *Nature* **228**, 860-861.
9. **Draper, P., and R.J.W. Rees,** 1993. Electron-transparent zone that surrounds *mycobacterium lepraemurium* inside host cells. *J. Gen. Microbiol.* **77**. 79-87.
10. **Draper, P., P. Hart, R. D'Arcy, and M.R. Young,** 1979. Effects of anionic inhibitors of phagosome-lysosome fusion in cultured macrophages when the ingested organism is *Mycobacterium lepraemurium. Infect. Immun.* **24**, 558-561.
11. **Goldstein, L. and C. Ko,** 1976. A method for mass culturing of large free-living amoebae. *Methods Cell Biol.* **13**, 246-255.
12. **Goren, M.B., P. Hart, R. D'Arcy, M.R. Young, and J.A. Amstrong,** 1976. Prevention of phagosome-lysosome fusion in cultured macrophages by sulfatides of *Mycobacterium tuberculosis. Proc. Nat. Acad. Sci. USA* **73**, 2510-2514.
13. **Goren, M.B., A.E. Vatter, and J. Fiscus,** 1987. Polyanionic agents as inhibitors of phagosome-lysosome fusion in cultured macrophages: Evolution of an alternative interpretation. *J. Leukocyte Biol.* **41**, 117-122.
14. **Hart, P., R. D'Arch, and M.R. Young,** 1988. Polyanionic agents inhibit phagosome-lysosome fusion in cultured macrophages; A reply to the suggestion of Goren, Vatter, and Fiscus to the contrary. *J. Leukocyte Biol.* **43**. 179-182.
15. **Hitchcock, P.J. and T.M. Brown,** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide. *J. Bacteriol.* **154**, 269-277.
16. **Jeon, K.W.,** 1970. Micromanupilation of amoeba nuclei. *Methods Cell Biol.* **4**, 179-194.
17. **Jeon, K.W.,** 1982. Intergration of bacterial endosymbionts in amoebae. *Int. Rev. Cytol.* **14** (suppl), 29-47.
18. **Jeon, K.W.,** 1992. Macromolecules involved in the amoeba-bacteria symbiosis. *J. Protozool.* **39**, 199-204.
19. **Jeon, K.W. and M.S. Jeon,** 1976. Endosymbiosis in amoebae: Recently established endosymbionts have become required cytoplasmic component. *J. Cell. Physiol.* **89**, 337-344.
20. **Joiner, K.A., S.A. Fuhrman, H.M. Miettinen, L.H. Kasper, and I. Mellman,** 1990. *Toxoplasma gondii*; Fusion competence of parasitophorus vacuoles in Fc receptor-transfected fibroblast. *Science* **249**, 641-649.
21. **Laemmli, U.K.,** 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature* **227**, 680-685.
22. **Moulder, J.W.,** 1985. Comparative biology of intracellular parasitism. *Microbiol. Rev.* **49**, 298-337.
23. **Platt, J.L. and A.F. Michael,** 1983. Retardation of fading and enhancement of intensity of immunofluorescence by *p*-phenylenediamine. *J. Histochem. Cytochem.* **31**, 840-842.
24. **Towbin, H., T. Staehelin, and J. Gordon,** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Nat. Acad. Sci. USA* **76**, 4350-4354.
25. **Tsai, C.M. and C.E. Frisch,** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal. Biochem.* **119**, 115-119.
26. **Young, M.R. and Hart, P., R. D'Arcy,** 1986. Movements and other distinguishing features of small vesicles identified by darkfield microscopy in living macrophages. *Exp. Cell Res.* **164**, 199-210.

(Received May 10, 1994)
(Accepted June 13, 1994)

초 록: 리소솜과 공생낭의 융합저해에서의 Lipopolysaccharide의 역할에 관한 연구

최의열 (한림대학교 자연과학대학 유전공학과)

공생 아메바에서 리소솜과 공생낭 간에 융합이 저해되는 이유로서는 먼저 이들 공생낭의 막에 어떤 특별한 인자가 존재하여 융합을 저해하거나 또는 융합 과정에 필수적인 어떤 요소가 이들 공생막에는 부족하여 융합이 일어나지 않는다고 유추해 볼 수 있다. 단일 클론 항체를 추적물질로 사용하여 이들 인자나 구성요소를 알아내는 과정에서, lipopolysaccharides가 공생 박테리아에 의하여 생산되어 공생낭의 막에 삽입된다는 것을 확인하였으며 이들이 공생막상에서도 세포질 방향으로 노출되어 있다는 것을 알아내었다. 따라서 이들 lipopolysaccharides가 리소솜과 공생낭간의 융합 저해에 관여하는 가를 알아보기 위하여 이들에 대한 단일크론 항체를 공생 아메바의 세포질에 미세주사하여 보았다. 주사된 아메바에서는 공생낭과 리소솜간의 융합이 일어나는 것으로 미루어 보아, 아마도 lipopolysaccharides는 융합저해 요소 중의 하나로 사료되어 진다.