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Production of Monoclonal Antibody Against the Plasmalemma of Amoeba and its Application in Determining the Role of Membrane Components

Tae In Ahn and Ji-Young Choi

Department of Biology Education
Seoul National University

Monoclonal antibodies (MAbs) reacting with the plasmalemma of *Amoeba proteus* were produced. Specificity of the 3 MAbs was determined by transfer blotting of the SDS polyacrylamide gel. AMS antibody reacted with the mucopolysaccharide bands of the spacer gel, 220 KD and 50 KD proteins of the resolving gel. The major glycoprotein bands (175 KD, 165 KD) and 50 KD protein of the plasmalemma were recognized by AMG antibody. A third, AMP antibody reacted with the 50 KD protein only. In immunofluorescence microscopy of the enzyme treated cells, the antigens of these MAbs were sensitive to proteases, but not sensitive to neuraminidase. In the assay of cell to substratum attachment after binding with the antibody, AMG and AMP antibodies exerted no effect, but AMS hindered the attachment and cell spreading. Thus the effective components of the plasmalemma in cell to substratum attachment appear to be the mucopolysaccharides and 220 KD protein. The membranes of latex particle ingested phagosomes did not show any distinction from the plasmalemma in fluorescence microscopy. Phagosome membranes of amoebae appear to be derived from the plasma membrane without selection in terms of the antigen composition.

KEW WORDS: Monoclonal antibody, Attachment, Plasma membrane, *Amoeba proteus*

A large number of studies have implicated cell-substrate interactions in adhesion, morphology, and movement of vertebrate cells. Similar mechanisms appear to act in free-living amoebae. The requirement of negative substrate charge density for adhesion to substrata is known in *Naegleria* and *Paramoeba* (Preston and King, 1978a, b; Martin, 1987). But the negative charge density exerts little effect on morphology and locomotive rates once sufficient contact between cell and substrate has occurred. Adhesion of *N. gurberi* does not require any specific external cations such as, Ca^{2+} or Mg^{2+}. But Ca^{2+} is required for normal locomotion.

Amoeboid movement is a motion performed by single cells whilst adherent to solid surfaces, and is exhibited by free living protozoa, especially amoebae, by leukocytes and to a lesser extent by embryonic cells, tumor cells and metazoan cells in tissue culture (Bignold, 1987). Particles, such as soot and carmine applied to the surface of amoebae have been reported to move forward relative to the cell body, so that the whole cell surface appears to move in the manner of the tread of a military tank (Bignold, 1987). Amoeboid movement is dependent on a degree of adhesiveness of the outer cell surface for underlying substratum.

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Mediation of specific membrane molecules or the general property of cell surface in adhesiveness is still in question with respect to amoebae. Adhesiveness was reported to be of intermediate intensity, diffuse in distribution and constant with time. With the development of new assays and application of new biochemical assault on the classical problem of cell to substratum adhesiveness, specific membrane adhesion antigens have been described for myogenic and fibroblastic cells (Damsky, *et al.*, 1985).

By producing several monoclonal antibodies against the plasmalemma of amebae, we followed the role of the membrane antigens in adhesion and cellular morphology, and obtained evidence that the antigens mediating cell to substratum attachment distribute all over the surface and that the internalized membranes by endocytosis did not show selectivity in membrane antigens.

Materials and Methods

Cell Culture

Amoeba proteus was cultured in stainless dishes (22 cm × 28 cm × 5 cm) containing modified Chalkley's medium at 23°C (Ahn and Choi, 1985). Amoebae were fed daily with axenically cultured and washed *Tetrahymena* as food organism.

Preparation of Plasmalemma and Membrane Antigen

Plasmalemma of amoebae was prepared by sonication and differential centrifugation as previously described (Ahn and Jeon, 1982). In order to prepare membrane antigens to immunize mice, purified plasmalemma was solubilized in sample buffer of Laemmli (1970) and electrophoresed for 5 hr in 3 mm thick preparatory 7 % SDS polyacrylamide slab gel. After brief staining the gel with Brilliant Coomassie Blue (BCB) or with periodic acid Schiff (PAS) reagent, the bands resolved in spacer gel corresponding PAS I (Ahn and Jeon, 1982) and the 175 KD band in resolving gel were cut out. The proteins in the 175 KD band were electrophoretically eluted (Ahn and Jeon, 1989) and used for immunization. On the other hand the PAS I bands were directly used without elution.

Production of Monoclonal Antibodies

Electrophoretically eluted 175 KD proteins or the cut out bands of spacer gel were injected as antigens. Female BALB/c mice (6-8 weeks old) were injected intraperitonealy with 100 μl of emulsion containing equal volume of antigen solution (300 μg total protein) and Freund's complete adjuvant. Then the mice were boosted at 3-4 week intervals with the same amount of antigen emulsified with Freund's incomplete adjuvant. Two weeks after the 2nd boost injection, mouse spleen cells were obtained and fused with Sp2/O-Ag-14 myeloma cells in incomplete DME using polyethylene glycol (Galfre *et al.*, 1977). Fused cells were plated in 24 well plates and cultured in HAT medium for 7 days, then in HT medium. The hybridoma cells were screened for antibody production after 10-14 days of fusion.

Fused cells producing antibody against the membrane components were first screened by dot blot in 24 well culture plates. Small pieces of nitrocellulose (NC) paper blotted with purified plasmalemma were incubated for 60 min at room temperature with 100 μl of the medium from each hybridoma culture wells, and processed as western blotting (see below). Cells in those positive wells were transferred to 6-well culture plates for further culturing. Hybridoma cells producing specific antibody against the amoeba surface were screened again by immunofluorescence microscopy (see below). Then, the cells producing antibody were cloned after limiting dilution.

Monoclonal antibodies were produced and purified from the ascitic fluid by using the cloned hybridoma. IgG was purified by ammonium sulfate precipitation and chromatography on DEAE-cellulose column. Purified immunoglobins were stored frozen in aliquots at −70°C.

Analytical Gel Electrophoresis and Western Blotting

Laemmli's SDS polyacrylamide gel and buffer systems were used for analytical gel electrophoresis (Laemmli, 1970). After electrophoresis proteins were electrophoretically transferred to NC paper (Towbin *et al.*, 1979) for overnight at 4°C. Air-dried blots were incubated in 2 % (w/v) nonfat dry milk in PBS for 1 hr at room temperature, washed 3 times with 0.3 % Tween 20 in PBS

(TPBS) and stored after air drying. After rewetting in TPBS the blots were incubated with monoclonal antibody for 1 hr, washed 3 times in TPBS, and incubated in anti-mouse-IgG antibody coupled with horse radish peroxidase (1:500 dilution) for 1hr at room temperature (Tsang *et al.*, 1983). The blots were washed twice in TPBS and once in PBS. Then the blots were stained by incubating for 2-10 min in a solution containing 4-chloro-1-naphthol as substrate (Fugita *et al.*, 1986). The color reaction was terminated by washing the blots with distilled water.

Analysis of Cell to Substratum Binding

In order to determine the role of the membrane antigens on the cell to substratum attachment the effect of antibody binding was studied as followings. Healthy amoebae were cleaned with ice-cold Chalkley's solution and incubated with the various concentrations of MAb at 4°C for 30 min. Then the cells were washed 3 times with cold Chalkley's, transferred to a new tissue culture dishes (Falcon plastic) and washed twice with Chalkley's at room temperature. The ability of the cell to substratum adhesion was observed under dissecting scope. Pictures were taken at the black background.

Treatment of Amoebae with Surface Modifying Agents

Modification of the amoeba surface was done as previously described (Ahn and Kwak, 1986). After modification of the cell surface by proteases or neuraminidase amoebae were washed with cold Chalkley's medium and incubated for 30 min at 4°C with various concentrations of ascitic fluid containing monoclonal antibodies, and washed more than 5 times with cold Chalkley's. Then the effect of membrane modification on antibody binding was followed by immunofluorescence microscopy.

Preparation of Phaqosomes for Microscopy

Latex particies (32 μm diameter; Polyscience, Inc. Warrington, PA.) were wet and washed in the Chalkley's, and fed to amoebae (Ahn and Jeon, 1982). Free particles left in the medium were filtered through 100 μm-pore screen and amoebae were suspended in the Chalkley's. The amoebae were picked up individually and mounted on a slide glass using a fine tipped mouth pipette. Then the amoebae were fixed in 50 % acetic acid, flattened and partially ruputred by pressing with a cover glass while freezing on top of dry ice. After flipping off the cover glass the samples were further fixed and permeabilized by incubating at −20°C with methanol for 10 min, then another 10 min in acetone. Then the samples were porcessed for immunofluorescence microscopy by incubating with MAb and fluorescein conjugated 2nd antibody.

Immunofluorescence Microscopy

Amoebae were fixed and permeabilized either by Kim and Jeon (1987) or by incubating for 10 min in 3 % paraformaldehyde and for 1 min in 0.05 % Triton X-100. Thus fixed and permeabilized amoebae in a 24-well plates were successively incubated with MAb and anti-mouse IgG coupled with FITC (1:30 dilution) at room temperature, mounted on a slide glass with a solution containing 10 % p-phenylene-diamine and 90 % glycerol, and examined under the fluorescence microscope. Pictures were taken by 2 min exposure using Kodak Tri-X pan film (ASA400).

Results

Characterization of the Monoclonal Antibodies

By dot blot analysis 5 positive clones reacting with the amoeba plasmalemma were selected. After limiting dilution, 3 clones were confirmed to react with the cell surface components by immunofluorescence microscopy and western blotting. Then these 3 clones were injected into the peritoneal cavities of BALB/c mice, monoclonal antibodies were obtained from the ascitic fluids. Specificity of these antiboiies were determined by SDS-PAGE and transfer blotting of the plasmalemma (Fig. 1). The MAb reacting with the PAS staining bands resolved in the specer gel, the 220 KD and the 50 KD band in resolving gel was designated AMS. The second MAb reacting with 175 KD, 165 KD and 50 KD band was named AMG. The 175 KD and 165 KD bands were not stained well enough with PAS reagent in this study. But these are the major glycoprotein bands of the plasmalemma (Ahn and Jeon, 1982). The 3rd

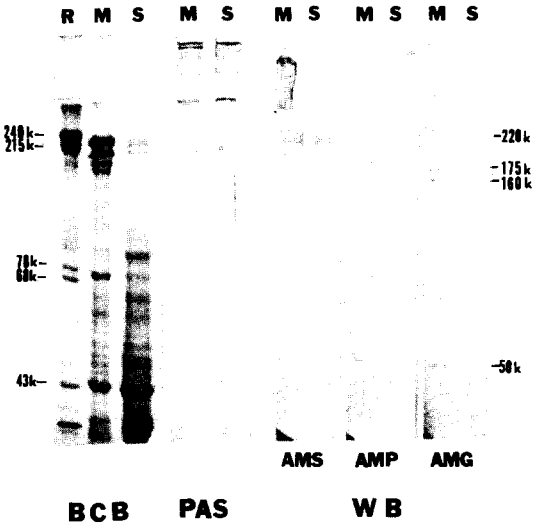

Fig. 1. Specificity of the monoclonal antibodies shown by SDS PAGE and western blotting (WB). Crude preparation of the plasma membranes (M) and water soluble proteins (S) were run in parallel and stained by Brilliant Coomassle Blue (BCB) and periodic acid schiff reagent (PAS). Note AMS antibodies recognized PAS stained components of the spacer gel, 220 KD and 50 KD proteins. AMP antibodies reacted with 50 KD protein only. AMG antibodies recognized 175 KD, 160 KD glycoprotein bands (Ann and Jeon, 1982) and 50 KD proteins.

monoclonal antibody which reacted with the common 50 KD protein only was designated as AMP.

Binding of these 3 MAbs on to the plasmalemma of amoebae was visualized by fluorescence microscopy (Fig. 2, row C). Thus the antigens reacting with the antibodies appeared to distribute all around the cell membrane with even intensity. Modification of the plasmalemma with neuraminidase did not significantly affect on the binding of the 3 MAbs (Fig. 2, row N). But tryptic digestion of the plasmalemma diminished the fluorescence of AMS and AMP (Fig. 2, row T). The antigens of the 3 MAbs were significantly reduced after digesting with proteinase K (Fig. 2, row P). Thus the antigenic determinants of these 3 MAb appeared to be the polypeptide moiety of the molecules. In this experiment, live cells after enzymatic modification were used for immunofluorescence microscopy. Thus the reduction of the fluorescence was brought by the loss of binding sites on the surface of the amoebae.

Role of the Membrane Antigens on Cell to Substratum Attachment

In order to determine the roles of the antigenic determinants on cell to substratum atachment, amoebae were incubated with each MAb at 4°C, washed with cold Chalkley's solution and observed at room temperature after transferring to a new Falcon dish. As shown in Figure 3, binding of AMG or AMP did not interfere cell to substratum attachment (Fig. 3 B. D). These amoebae showed the normal morphology and locomotion (cf. Fig. 3 A). But AMS bound cells could not either adhere or spread. These amoebae were all rounded up forming filopod like projections on the surface. Such a morphology was prolonged for more than 5 hours. None of the MAb bound amoebae at any concentration of the antibodies formed aggregate among the amoebae even after mild centrifugation in a conical centrifuge tube. But polyclonal antiserum produced from rabbit could aggregate amoebae readily (Ahn and Kim, unpublished result).

Distribution and the Fate of Surface Antigens on Phagocytosis

Amoebae were incubated with 32 μm latex particles to induce phagocytosis for an hour and washed the free particles. Then the distribution and the fate of endocytosed antigens were chased by immunofluorescence microscopy (Fig. 4). Immediately after phagocytosis all of the phagosomes were fluorescent irrespective to the MAbs. Such a high intensity of fluorescence was visible until the phagosomes were 2 h old. Hereafter the antibody bound fluorescence of phagosomes diminished gradually. Thus the phagosome membranes appeared to be made directly from the plasmalemma without any selectivity among the membrane antigens of the 3 MAbs. Then the antigens on the phagosomal membranes disappeared from the phagosomes without detectable preference.

Discussion

The results show that amoebae have some discrete components on the plasmalemma which are mediating cellular attachment on to the substratum. Among the antigens binding with AMS, 50

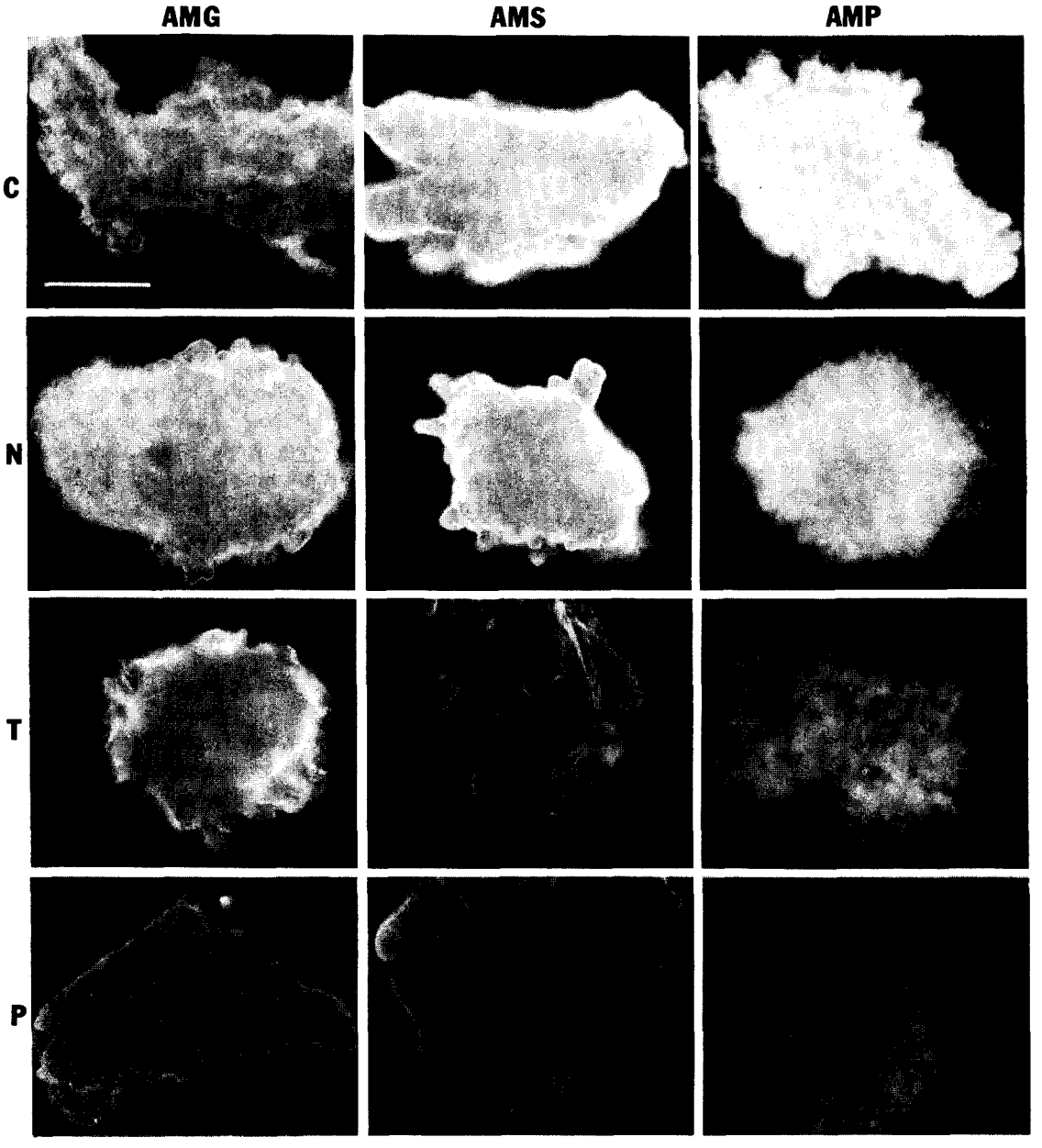

Fig. 2. Immunoflourescence micrograph of amoebae after treating with neuraminidase (row N), trypsin (row T) or proteinase K (row P). Cells in row 'C' were untreated control. Samples of each lane were processed with the monoclonal antibodies marked on top. Note the fluorescence of the cells in row 'P' is very little for all 3 antibodies. Trypsin removed the flourescence of AMS and AMP. The bar in C/AMG represents 100 μm for all pictures.

KD protein which was also recognized by AMP could not be the active component in the cell to substratum attachment since the adherence was not interfered by 50 KD protein-specific AMP binding. Then the effective component of the cell to substratum adherence could be the PAS stained

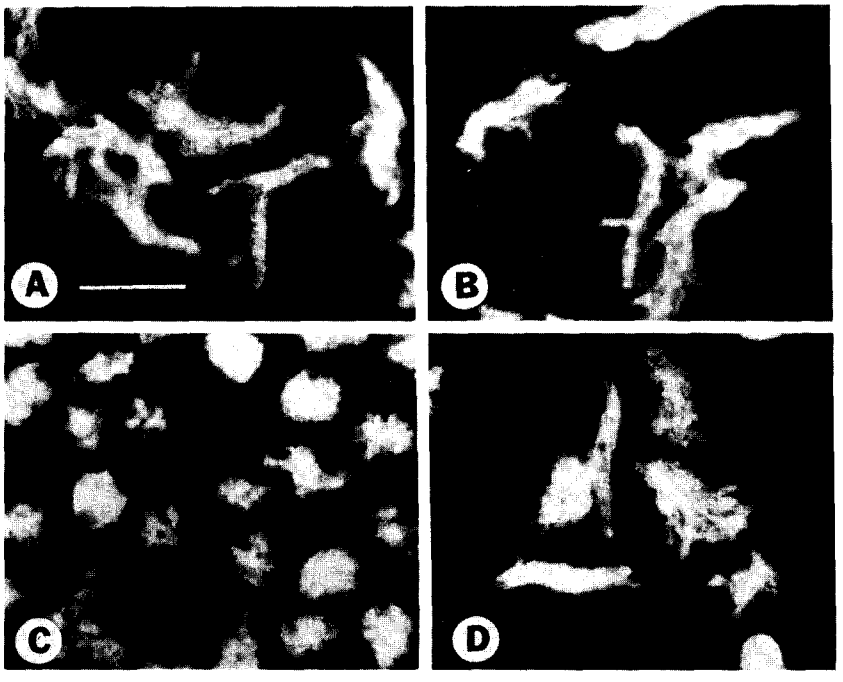

Fig. 3. Adherence of amoebae to substratum of tissue culture dish (Falcon plastic) after binding with BSA (A; for control) or the monoclonal antibodies (B; AMP, C; AMS, D; AMG). AMP or AMG bound cells showed normal attachment and celluler movement. AMS bound cells did not attach and could not form lobopods (C). Pictures were taken under dissecting scope. The bar in A represents 100 μm for all pictures.

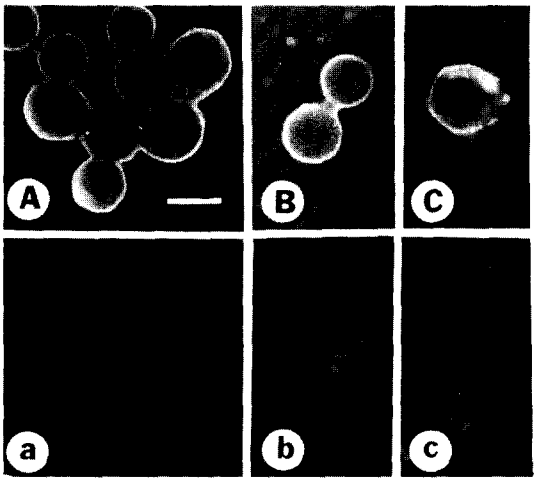

Fig. 4. AMS (A, a), AMP (B, b) and AMG (C, c) Immunoflourescence of 1 h (A, B, C.) and 7 h (a, b, c) old phagosomes containing ingested latex particies. Note phagosomes (A-C) are fluorescent for all 3 antibodies. The fluorescence is not readily visible in 7 h phagosomes (a-c).

mucopolysaccharides resolved in spacer gel or the 220 KD proteins resolved in the separating gel. As none of the AMS bound cells at various concentrations of antibody formed aggregates even after centrifugal packing, inability of the cell to substratum attachment could not be due to the masking of the sites by cross-linked antibody with the antigens. Since the binding of AMS was dramatically reduced by proteases (Fig. 2), the antigenic determinant could be either the protein or the sugar moieties linked onto the protein. PAS stained bands of the spacer gel (Fig. 1) was not stained any by BCB. This was the same with previous results (Ahn and Jeon, 1982; 1985). But these are not enough to say the determinant is the sugar moiety. Firstly the 220 KD band was not stained any by PAS reagent. Secondly, the stained intensity of the 220 KD band in transfer blot is so narrow compared with its BCB band. It should be further confirmed either by peptide mapping or by pro-

ducing the MAbs with unique specificity.

Modification of the plasmalemma by trypsin or proteinase K exerted damage on the plasmalemma (Ahn and Jeon, 1982; 1985). These two enzymes modified the apparent morphology of the amorphous layer of the plasmalemma (Ahh and Kwak, 1986). Ultrastructural localization of the MAb binding antigens by immunocytology using gold particle-conjugated second antibody is in prograess. Then the structure and composition of the antigenic components of the plasmalemma could be further confirmed.

However the adherence in amoeba is located on the outer surface and distribute evenly all over the plasmalemma as evidenced by AMS fluorescence microscopy using either acetic acid fixed cells (Fig. 2) or the live cells (result is not shown). Distribution of adherence in *Amoeba* has been suggested as diffuse around the cell surface (Ambrose, 1961). If the effective molecules are the antigens of the AMS antibody, the adherence in amoebae appears to distribute as highly dense manner. Such a distribution is in contrast comparing with the sporadic distribution of adhesiveness visualized in cultured vertebrate cells (Damsky *et al.*, 1985).

The antigenic determinants reacting with the three MAbs, internalized simultaneously into the phagosomal membranes. Internalization of membrane in phaocytosis is not like the one shown in receptor-mediated endocytosis where most of the protein components of the plasma membranes were excluded in endosome membrane (Pearse and Bretscher, 1981). The simultaneous internalization of membrane components was also noted in the comparisons of protein composition of the plasmalemma and phagolysosomes (Ahn and Jeon, 1982). At that time there were no discrete distinction between phagosomes and phagolysosomes as they were isolated 1-2 hr after phagocytosis.

In terms of membrane differentiation with cellular activities, the study of membrane composition and functional modification of the plasmalemma in phagocytosis and formation of symbiotic vacuoles may be of interest (Ahn and Jeon, 1982), if the plasmalemma of amoebae has both discrete compositional and functional markers. Thus far there was not any known analytical marker molecule on the plasmalemma of *Amoeba proteus*. The MAbs produced in this study can be used as probes in the study of membrane differentiation accompanying endocytosis and endosymbiosis.

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아메바 세포막에대한 단항체 생산 및 이를 이용한 막 조성 물질의 역할규명

안태인 · 최지영(서울대 생물교육과)

*Amoeba proteus*의 세포막과 반응하는 단세포군 항체를 생산하였다. SDS polyacrylamide gel을 transfer blotting하여 이들 항체의 반응 특이성을 조사해 본 결과 AMS 단항체는 PAS로 염색되는 spacer gel의 mucopolysaccharide 벤드, resolving gel의 220 KD 및 50 KD 단백질과 반응하였으며, 세포막의 주요 당단백질인 175 KD 및 165 KD 벤드와 50 KD 단백질은 AMG 단항체에 의해서 인지되었다. 그리고 AMP단항체는 공통인 50 KD 단백질과 특이하게 반응하였다. 효소처리한 아메바의 면역형광현미경적 조사에서 이들 항체에 대한 항원분자들은 모두 단백질분해효소에 민감하였으며 neuraminidase에 대해서는 변화가 없었다. 이들 항체를 결합시킨 아메바의 용기표면 부착 가능성을 분석한 결과 AMP 및 AMG 단항체는 아무런 영향을 미치지 못하였으며 AMS 단항체는 세포의 용기표면 부착 및 세포의 펴짐을 저해하였다. 따라서 아메바의 용기표면 부착은 mucopolysaccharide 및 220 KD 단백질에 의해서 매개되는 것으로 나타났다. 그리고 latex particle을 담고 있는 식포막은 면역형광형미경적 조사에서 세포막과 차이가 없었다. 따라서 식포막은 항원 조성에 있어서 비선택적으로 세포막에서 유도되는 것으로 나타났다.