Journal of Cellular Biochemistry 56:592–596 (1994)

Cell Surface Control of Differentiation in *Acanthamoeba*

Shiguang Yang and Clarence Villemez

Department of Molecular Biology, University of Wyoming, Laramie, Wyoming 82071-3944

Abstract *Acanthamoeba castellanii (Neff)* is a free-living soil amoeba with close relatives that are opportunistic pathogens. Trophozoites differentiate into cysts when deprived of nutrients; cysts convert into trophozoites, leaving the wall behind, in the presence of nutrients. The data presented here, which includes immunoaffinity purification of the receptor, indicate that cell surface molecular signals also control *Acanthamoeba* differentiation in both directions. Monoclonal antibodies that bind specifically to a 40 kD trophozoite protein initiate the encystment of trophozoites. When bound to cysts the same monoclonal antibodies prevent excystment. Washing away the antibody allows both trophozoites and cysts to resume normal activity. One of these monoclonal antibodies inhibits pinocytosis, while another has no effect on pinocytosis. © 1994 Wiley-Liss, Inc.

Key words: amoeba, encystment, excystment, receptors, pathology, therapy

Acanthamoeba castellanii and closely related species are free-living amoebae that are widely distributed in soil and water worldwide. They are responsible for *Acanthamoebic* keratitis [Visvesvara and Stehr-Green, 1990] which can still prove impossible to treat [Holz et al., 1993] although recent improvements have been made [Bacon et al., 1993]. *Acanthamoeba* keratitis is notable for being the only disease caused by this organism that afflicts individuals with unimpaired immune systems. *Acanthamoeba* diseases are rare and usually affect relatively immune deficient organs such as the cornea and the brain [Martinez, 1991]. Disseminated amebiasis has only been observed in immune deficient individuals of which a recent example is an AIDS patient [Friedland et al., 1992]. Although rare, *Acanthamoebic* diseases are not trivial. Amoebic encephalitis is invariably fatal, and amoebic keratitis still calls forth extensive treatment which can include multiple corneal transplants [Wiens and Jackson, 1988; Bacon et al., 1993; Holz et al., 1993]. The results of the work presented here suggest that *Acanthamoeba's* communication with the extracellular world through the cell surface may be medically exploitable.

Acanthamoebae differentiate into a metabolically inactive, walled form (termed a "cyst") under what are called "unfavorable conditions" [Byers, 1979]. The latter term is presently somewhat ambiguous, since very few naturally occurring conditions have been clearly shown to cause encystment. The huge majority of previous studies of *Acanthamoeba* encystment include food deprivation in a salt media in the induction of encystment. DNA, RNA, and protein synthesis inhibitors have been reported to induce encystment in nutrient media [see Byers et al., 1991, for a review]. There are two troublesome aspects to the latter information. First, it gives few hints regarding the natural mechanism since (1) complete information regarding the specific molecular effects of these compounds on *Acanthamoeba* is unavailable, and (2) the effects are not completely reproducible over long periods of time, perhaps due to mutation or adaptation of the organism. Secondly, changes in DNA, RNA, and protein metabolism are expected consequences of any differentiation process.

Cysts differentiate into free-living trophozoites in the presence of nutrients and the wall is left behind. However, *Acanthamoeba* cysts are universally present in infected corneas [Ferrante, 1991] and brain tissue [Ma et al., 1990]. No satisfactory explanation has heretofore been available for the presence of cysts in what should be, in terms of nutrition, pH, oxygen levels,

Received March 16, 1994; accepted June 2, 1994.

Address reprint requests to C.L. Villemez, Department of Molecular Biology, University of Wyoming, Laramie, WY 82071-3944.

Shiguang Yang's present address is Department of Animal, Dairy and Veterinary Sciences, Utah State University, Logan, UT.

temperature control, the highly "favorable" environment of human tissue. Interactions of the human immune system seem likely to be involved. Antibodies binding the cell surface protein described here provide a reasonable explanation for the presence of cysts in human tissues.

Cell surface receptors allow cells to react to external signals. The general process by which the receptors cause cellular change is similar in all known systems. All are either transmembrane macromolecules themselves, or are closely associated with transmembrane molecules. The conformational change in the receptor produced by the binding of an extracellular ligand is thought to be the actual extra- to intracellular signal transmission device. Consequently, anything that causes the appropriate conformational change in the receptor should produce the biological effect of the natural ligand. Some receptor-binding antibodies satisfy these conditions and can thereby substitute for the natural ligand [see for example, Kahn et al., 1978].

We have reported that the addition of any of three monoclonal antibodies (C8, F1, and A9) to exponentially growing cultures of *Acanthamoeba castellanii (Neff)* causes the cells to stop dividing and encyst in full growth media [Villemez et al., 1985]. Another monoclonal antibody (C5) which bound a major plasma membrane protein had no apparent effect on the organism, indicating that the cause of encystment was due to relatively specific binding. C8, F1, and A9 competed for cell surface binding sites, suggesting that they bound plasma membrane moieties that are in close proximity. Further, the $F(ab)'_2$ form of A9, but not the F(ab)', was as effective as the intact antibody, suggesting that antibody-caused association of plasma membrane moieties was responsible for causing encystment. Also, C8 caused cells in isolation to encyst, eliminating cell-cell contact as a requirement for the antibody-induced encystment. All three encystment-inducing antibodies inhibited pinocytosis within a few minutes of application, producing the possibility that lack of food was the stimulus for encystment. Our new data, presented below, indicates that another monoclonal antibody (2D4) binds a similar cell surface molecule as A9, induces encystment in full growth media, but does not inhibit pinocytosis. Further, both A9 and 2D4 bind to the cyst cell surface and inhibit excystment in full growth media. Taken with the previous data, these experiments suggest strongly that the amoebae possess cell surface receptors that induce encystment and prevent excystment when stimulated.

METHODS

Replication

All experiments were repeated. Results similar to those presented were obtained from all experiments. *Acanthamoeba castellanii (Neff)* was obtained from the American Type Culture Collection (Rockville, MD). Culture conditions were those described previously [Villemez et al., 1985].

Preparation and Purification of Monoclonal Antibodies

The preparation of the hybridoma that secretes monoclonal antibody A9 was described previously [Villemez et al., 1985]. The hybridoma that secretes the monoclonal antibody 2D4 was prepared similarly except that electrofusion [Ohnishi et al., 1987] was used to fuse the cell partners. A9 and 2D4 were purified from ascites fluid by Protein G chromatography. The antibodies were pure as indicated by SDS gel electrophoresis, as was the irrelevant mouse IgG referred to below.

Immunoaffinity Purification

Ten milligrams of each of the monoclonal antibodies A9, 2D4, and irrelevant mouse IgG (Pierce Chemicals, Rockford, IL), was coupled to 1 ml of Affi-Gel 10 (Bio-Rad, Richmond, CA) according to the manufacturer's instructions. Trophozoites (10 ml for each column at 10^8 cells/ml) were lysed in a buffered saline solution containing 1% NP40, the lysates allowed to interact with 1 ml of the immunoaffinity gels, and the gels eluted as described previously [Chang and Chang, 1986]. The eluate from each column was subjected to SDS-polyacrylamide gel electrophoresis (4–15% gradient gel), and silver staining (sensitivity = 0.3–0.5 ng/band) using a Phast-Gel system (Pharmacia, Piscataway, NJ) according to the manufacturer's instructions. Molecular weight indicated was estimated using a lane of low molecular weight standards (Bio-Rad, Richmond, CA).

Measurement of Pinocytosis

Log phase *A. castellanii* cells, at a concentration of 3.5×10^6/ml in growth media, were assayed for pinocytotic activity by the method of

Bowers and Olszewski [1972] measuring the uptake of ^{14}C-leucine.

Measurement of Excystment

Excystment was measured by the increase in trophozoite number. Experimental points are the average of duplicate samples. Cells were counted with a haemocytometer. Standard errors decreased with increasing cell density ranging from 71% at 10,000 cells per ml to 25% at 80,000 cells per ml. Daily microscopic examination determined that all cells in the A9 and 2D4 samples remained as cysts, no cell division or excystment occurred during the experiment. After 15 days the cysts in the A9 and 2D4 samples were collected, washed, and placed in fresh growth media. Normal excystment occurred following removal of the antibodies.

RESULTS AND DISCUSSION

Monoclonal Antibodies A9 and 2D4 Bind Specifically to Similar Proteins

Fixed cell ELISA and immunofluorescence microscopy show that 2D4 and A9 bind to the cell surface of both trophozoites and cysts (data not shown). Further, both antibodies appear to bind to similar trophozoite proteins (Fig. 1), in that the proteins isolated with the two monoclonal antibodies have identical mobilities by SDS gel electrophoresis and indistinguishable amino acid composition (data not shown). We have named these proteins of around 40 K molecular weight Encystment Stimulating Protein (ESP).

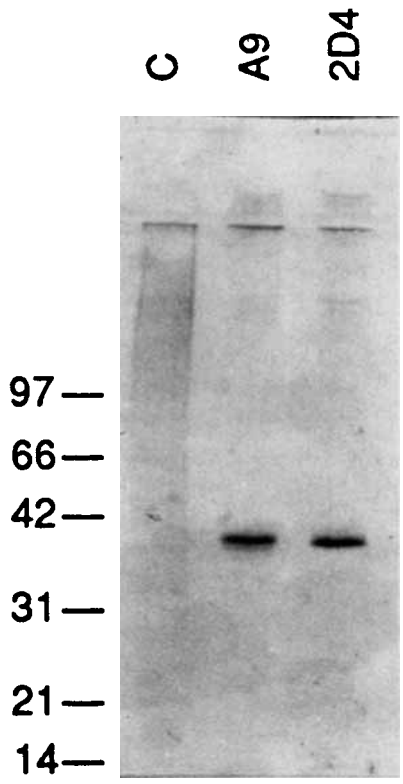

Fig. 1. Purification of ESP by immunoaffinity chromatography. Monoclonal antibodies A9, 2D4, and irrelevant mouse IgG were each covalently linked to AffiGel 10 (see Methods). The total lysate from *Acanthamoeba* trophozoites was passed through each column, the unbound material removed, the bound material eluted at high pH, and the fractions containing the bound material subjected to SDS gradient gel electrophoresis. Lane markers: C = irrelevant IgG, A9 = monoclonal antibody A9, 2D4 = monoclonal antibody 2D4.

Different Epitopes Must Be Recognized by the Monoclonal Antibodies

A9 and 2D4 must either bind different epitopes of ESP, or they bind different, closely related, ESPs since pinocytosis is inhibited by A9 binding, but not by 2D4 binding (Fig. 2). Further, that 2D4 binding induces encystment without inhibiting pinocytosis suggests that the main objective of the signaling pathway is differentiation control. But since C8, A9, and F1 cause the immediate inhibition of pinocytosis indicates that ESP can also associate with molecules controlling this physiological function.

ESP Is Also Present on the Cyst Surface and Stimulation Prevents Excystment

The addition of 2D4 or A9 to cysts in growth media prevents excystment, while control cysts in the same media differentiate and divide in the expected fashion (Fig. 3). It should be noted that energy metabolism in cysts is so low as to be undetectable by criteria such as oxygen uptake. Cysts also remain viable for years, indicating that stored energy supplies are used, if at all, at an extremely low rate. Consequently, it is very unlikely that the antibodies are artificially inhibiting an ongoing metabolic process required for excystment. While the antibodies could inhibit a process activated by excystment conditions, the inhibition would have to take place from the exterior surface of the cyst and not damage the cyst's future ability to excyst in the absence of antibody, i.e., it would probably fulfill the conditions of a signal mechanism designed to prevent excystment. The antibody-treated cysts, when placed in fresh growth media containing no antibody, excyst and divide identical to untreated cysts (data not shown), indicating that the antibodies do no permanent damage to the cell. The

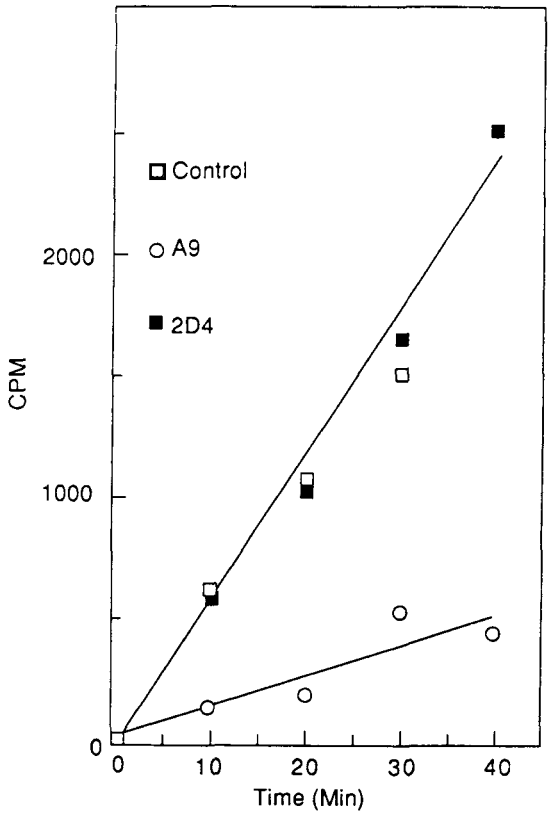

Fig. 2. Inhibition of pinocytosis by A9 antibody. Control = no additions, A9 and 2D4 = 25 μg/ml of the individual antibody added. The filled square symbol furthest to the right covers an open square. Radioactivity from intracellular ^{14}C-leucine was measured (see Methods).

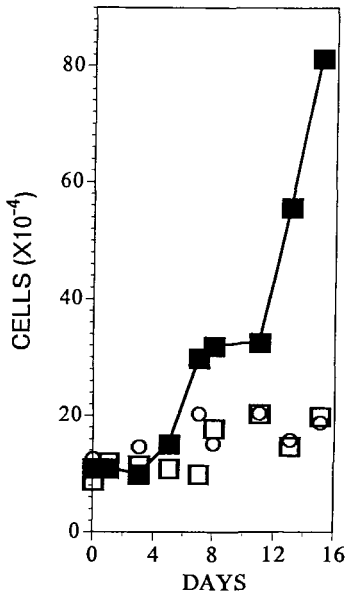

Fig. 3. Inhibition of excystment. *A. castellanii* cysts were placed in growth media containing either no additions (■) or 85 μg per ml A9 monoclonal antibody (○) or 2D4 monoclonal antibody (□).

encystment-inducing antibodies are apparently recognizing unique structures in the trophozoite: of the tens of thousands of macromolecules (the entire cell repetoire) passed through A9 and 2D4 affinity columns, only protein of similar electrophoretic mobility and amino acid content was retained by each column. This specificity makes it likely that the antibody-binding moiety on the cyst surface is ESP or a closely related macromolecule. The presence of ESP on the cyst surface, inhibiting excystment when stimulated, supports the suggestion of the encystment data: that ESP is part of a signalling system designed to maintain the organism in the encysted state as long as the natural ligand is present in the environment.

Signaling Through the Cyst Wall Suggests New Mechanisms

The detailed structure of the *Acanthamoeba* cyst wall is unknown. However, it is apparent from microscopic studies that it is very thick [Bowers and Korn, 1969] and contains cellulose fibrils similar to those found in the primary plant cell wall [Blanton and Villemez, 1978]. Signal transduction through a structure like the thick cyst wall suggests novel mechanisms, since it is not immediately obvious from existing information how the signal would be transmitted.

Possible Nature of the Natural Ligand

We have no information concerning the natural ligand for this apparent signaling system. There are numerous possibilities for the biological purpose including inter-amoeba communication and protection from amoebacidal agents.

Pathological Trophozoites, Benign Cysts

Tissue destruction by the trophozoite is apparently responsible for the pathogenic effects of opportunistic infections by *Acanthamoeba* and its relatives. The cyst neither divides nor destroys tissue. Binding of ESP by antibodies causes the organism to differentiate into a cyst, and to remain encysted as long as the antibodies are present. This latter circumstance is a good explanation for why the infections are so rare, and further, why the infected organs are those, brain and cornea, known to be relatively immunodeficient when infections do occur. In addi-

tion, the presence of cysts in infected brain and corneal tissue is most likely caused by the limited immune response in those tissues. Further, ESP could be a potential target in the treatment of *Acanthamoeba* diseases. Boosting the titer of anti-ESP antibodies by vaccinating patients with ESP could ameliorate these diseases.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health Grant AI23996.

REFERENCES

Bacon AS, Frazer DG, Dart JKG, Matheson M, Ficker LA, Wright P (1993): A review of 72 consecutive cases of Acanthamoeba keratitis, 1984–1992. Eye 7:719–725.

Blanton WE, Villemez CL (1978): Molecular size and chain length distribution in *Acanthamoeba* cellulose. J Protozool 25:264–267.

Bowers B, Korn ED (1969): The fine structure of *Acanthamoeba castellanii* (*Neff* strain) II. Encystment. J Cell Biol 41:786–805.

Bowers B, Olszewski TE (1972): Pinocytosis in *Acanthamoeba castellanii*. J Cell Biol 53:681–694.

Byers TJ (1979): Growth, reproduction, and differentiation in *Acanthamoeba*. Int Rev Cytol 61:283–338.

Byers TJ, Kim BG, King LE, Hugo ER (1991): Molecular aspects of the cell cycle and encystment of *Acanthamoeba*. Rev Infect Dis 13(Suppl 5):S378–S384.

Chang CS, Chang K-P (1986): Monoclonal antibody affinity purification of a *Leishmania* membrane glycoprotein and its inhibition of Leishmania-macrophage binding. Proc Natl Acad Sci USA 83:100–104.

Ferrante A (1991): Free-living amoebae: Pathogenicity and immunity. Parasite Immunol 13:31–47.

Friedland LR, Raphael SA, Deutsch ES, Johal J, Martyn LJ, Visvesvara GS, Lischner HW (1992): Disseminated *Acanthamoeba* infection in a child with symptomatic human immunodeficiency virus infection. Pediatr Infect Dis J 11:404–407.

Holz FG, Burk ROW, Volcker HE (1993): Bilateral *Acanthamoeba* keratitis. Klin Monatsbl Augenheilkd 203:418–422.

Kahn CJR, Gair KL, Jarrett DB, Flier JS (1978): Direct demonstration that receptor crosslinking or aggregation is important in insulin action. Proc Natl Acad Sci USA 75:4209–4213.

Ma P, Visvesvara GS, Martinez AJ, Theodore FH, Daggett PM, Sawyer TK (1990): Naegleria and Acanthamoeba infections: Review. Rev Infect Dis 12:490–513.

Martinez AJ (1991): Infection of the central nervous system due to *Acanthamoeba*. Rev Infect Dis 13:S399–S402.

Ohnishi K, Chiba J, Goto Y, Tokunaga TJ (1987): Improvement in the basic technology of electrofusion for generation of antibody-producing hybridomas. J Immunol Methods 100:181–189.

Villemez CL, Carlo PL, Russell MA (1985): Differentiation in *Acanthamoeba castellanii* is induced by specific monoclonal antibodies. J Cell Biochem 29:373–379.

Visvesvara GS, Stehr-Green JK (1990): Epidemiology of free-living amoeba infections. J Protozool 37:25S–33S.

Wiens JJ, Jackson WB (1988): *Acanthamoeba* keratitis: An update. Can J Ophthalmol 23:107–110.