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Differentiation in *Acanthamoeba castellanii* Is Induced by Specific Monoclonal Antibodies

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Monoclonal antibodies that bind a large molecular weight plasma membrane protein of *Acanthamoeba castellanii* cause the cells to differentiate. A different monoclonal antibody that binds specifically to the major plasma membrane protein has no effect upon cell division or differentiation. The induction of differentiation by the monoclonal antibodies requires a bivalent attachment, more than a single binding cycle of the antibody to the plasma membrane protein, does not require cell-cell contact, and appears to be mediated by an inhibition of pinocytosis. These results suggest one of two alternatives: either (1) this free living amoeba possesses a cell surface receptor that serves to initiate the differentiation process when stimulated, or (2) the specific plasma membrane antigen for the differentiation-inducing monoclonal antibodies is an essential component of the pinocytotic mechanism. While it seems more likely on the basis of available evidence that we are observing the biological effects of a cell surface receptor, either of the two alternative circumstances open up investigative areas of large significance.

Key words: encystment induction, *Acanthamoeba castellanii*, pinocytotic inhibition

Vertebrate peptide hormones have been reported in simple organisms [1], and a steroid hormone receptor analogous to that found in mammalian cells has been reported in yeast [2]. Biological functions have not been demonstrated for these molecules. To our knowledge, no plasma membrane receptor that induces differentiation has been reported for any free living protozoan.

Acanthamoeba castellanii (Neff) is a small free living amoeba that has been studied for some time as a model of eukaryotic differentiation [3]. Trophozoites in axenic culture encyst as a response to lack of food, to the presence of DNA synthesis inhibitors, or following the stationary phase of growth. Very little is known about the mechanism by which encystment is induced. We report here that antibody binding to a specific plasma membrane antigen causes *A castellanii* cells to encyst.

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METHODS

Monoclonal Antibodies

The monoclonal antibodies used in this study, designated C8, F1, A9, and C5, were secreted by hybridomas prepared by polyethylene glycol mediated fusion of *A castellanii* plasma membrane-immunized BALB/C mouse splenocytes [4] with SP2/0-Ag14 myeloma cells [5]. C8, F1, and A9 belong to the IgG1 subclass and were purified by protein A affinity chromatography [6]. C5 is an IgM and was used as present in BALB/C mouse ascites fluid, or as a partially purified preparation (the 40% saturated ammonium sulfate-precipitable fraction from ascites fluid) in which the major contaminant was murine albumin. The $f(ab)'_2$ and f(ab)′ fragments of A9 was prepared as previously described [7].

Cell Culture

Acanthamoeba castellanii (Neff) was originally obtained from the American Type Culture Collection and has been maintained in this laboratory for a number of years. The organisms were cultured as described previously [8, 9]. Cell number was determined, in duplicate upon replicate samples, by counting aliquots with an improved Neubauer haemocytometer (average deviation was 17%). Cysts were differentiated from trophozoites by the presence of the thick cyst wall under phase contrast microscopy.

RESULTS

Binding competition studies indicate that C8, F1, and A9 all compete for the same antigen on the *A castellanii* plasma membrane, whereas C5 does not compete with any of the former three monoclonal antibodies. Western blot analysis [10] indicates that C8, F1, and A9 bind specifically to the same two bands, separated only slightly by SDS-polyacrylamide gel electrophoresis using an 8% gel, which had a mobility equivalent to polypeptides in the 100-kd range (data not shown). C5 binds exclusively [11] to the *A castellanii* major plasma membrane protein [12], which has a molecular weight of approximately 15 kd.

C5 did not inhibit cell division at any concentration tested, which included several above and below that required to saturate the cell surface antigens. In contrast, C8, A9 (data not shown) and F1 inhibited cell division when included individually in the growth media (Fig. 1). By employing several concentrations of antibody and correlating the extent of binding with the saturation binding, it is possible to estimate the percentage of binding sites occupied at any concentration of antibody. Complete inhibition of cell division took place with 23 μg/ml F1, with 90% of the available cell surface binding sites occupied (Fig. 1). Significant inhibition is also noted at 3 μg/ml F1, a concentration of antibody at which approximately 43% of the available cell surface binding sites are occupied.

Cells from the two samples containing the highest concentrations of antibody (100 and 76 μg/ml—Fig. 1) were kept in the growth media containing F1 for one week, during which time no cell division took place, and following which time all observable cells were cysts. The cysts were isolated by centrifugation, washed, and placed in fresh growth media. A normal exponentially growing culture resulted, indicating that the cells excysted and divided. Experiments similar to that in Figure 1

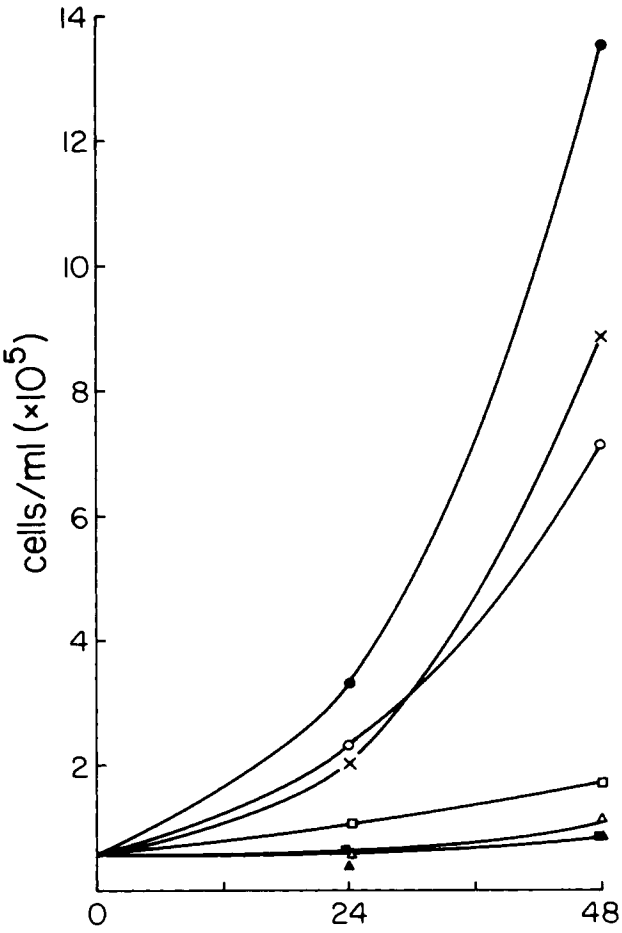

Fig. 1. Inhibition of mitosis by F1 monoclonal antibody. Log phase *A castellanii* cells were grown in 2.1 ml normal growth media to which was added 0.4 ml phosphate buffered saline (PBS) containing the additives indicated. PBS x−x, mouse IgG (100 μg/ml), (●−●); F1 (1 μg/ml), (○−○); F1 (3 μg/ml), (□−□); (23 μg/ml), (△−△); (76 μg/ml), (■−■); (100 μg/ml), (▲−▲). The saturating concentration of F1 was 50 μg/ml. The difference in growth rate in the PBS control and the mouse IgG control is due to mouse IgG serving as a source of protein nutrient, ie, the growth media is less diluted than with PBS.

resulted in A9 and f(ab)′$_2$-A9, causing cell division inhibition and subsequent encystment. However, in the presence of saturating concentrations of f(ab)′-A9, cell division continued and no cysts resulted, indicating that monovalent binding of the antigen was insufficient to cause encystment.

Saturating the cell surface with A9, followed by washing the cells and adding fresh media lacking A9, inhibited cell division completely for about 18 hr (Fig. 2). A normal rate of cell division followed the period of no cell division. This circumstance indicates that, by whatever mechanism encystment is induced, the plasma membrane antigen must be bound continuously for some latent period before encystment is induced. Lacking continous binding during the latent period, cells resume the normal cell cycle rather than enter the encystment phase of development.

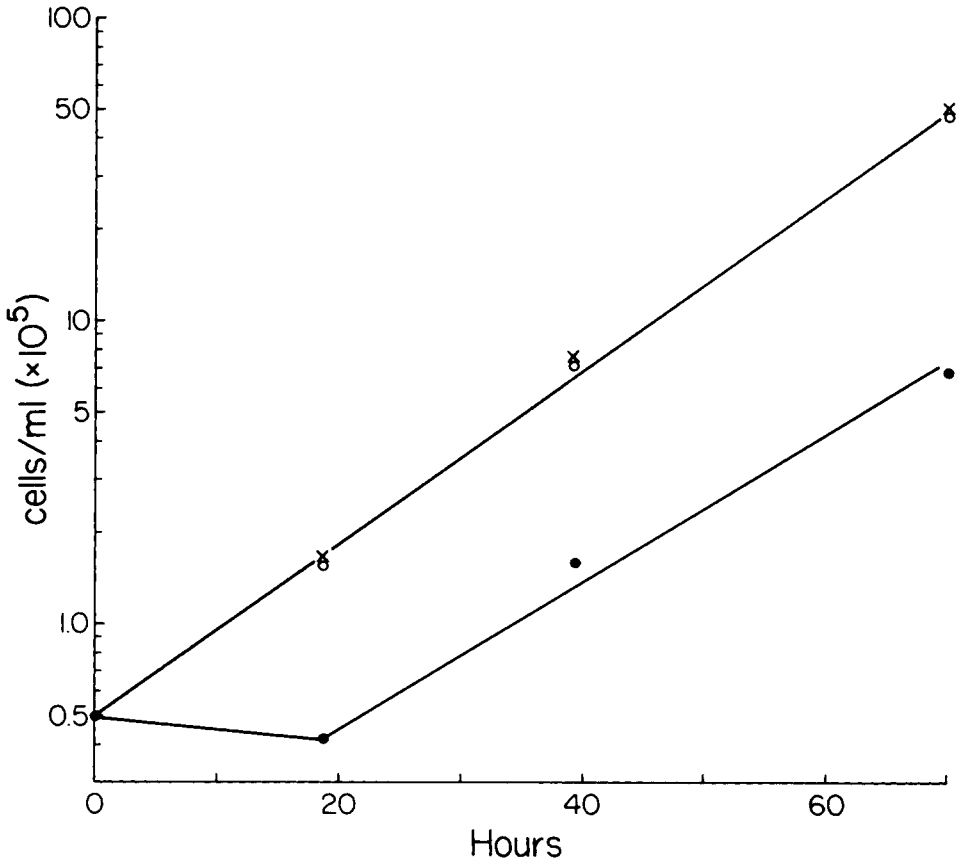

Fig. 2. Inhibition of mitosis by short-term exposure to A9 monoclonal antibody. *A castellanii* cells were exposed to 27 μg/ml A9 or to 45 μg/ml f(ab)'-A9 for 15 min at 0°C. The cells were isolated by centrifugation, washed with growth media, and distributed into 2.5-ml samples. Control, (○−○); A9, (●−●); f(ab)-A9,(x−x). Saturating concentrations were 15 μg/ml A9 and 33 μg/ml f(ab)'-A9.

Exponential phase trophozoites were dispersed at very low densities (50 and 500 cells per well) into the 0.28-cm^2 wells of Linbro microtiter plates with 0.2 ml growth media, in replicates of 10, and incubated at 30°C. The initial monocellular distribution was confirmed microscopically. C5-treated control cells divided rapidly, eventually packing the bottom of the well. No aggregated cells and only an occasional encysted cell were ever observed in these samples, even after 9 days. In contrast, C8-treated cells did not divide, and were encysted within 5 days. This observation is especially noteworthy, since, in our experience, unagitated cultures of this strain of *A castellanii* in growth media do not encyst. No cell-cell contact was observed in the C8-treated samples prior to encystment, demonstrating that antibody-induced encystment is a primary effect as opposed to the secondary effect of bringing about an artificial cell crowding.

All of the binding molecules that stimulate encystment, ie, A9, C8, F1, and $f(ab)'_2$-A9, inhibit pinocytosis. Typical results are shown in Figure 3, in which a saturating concentration of A9 resulted in an 87% inhibition of pinocytotic rate. In

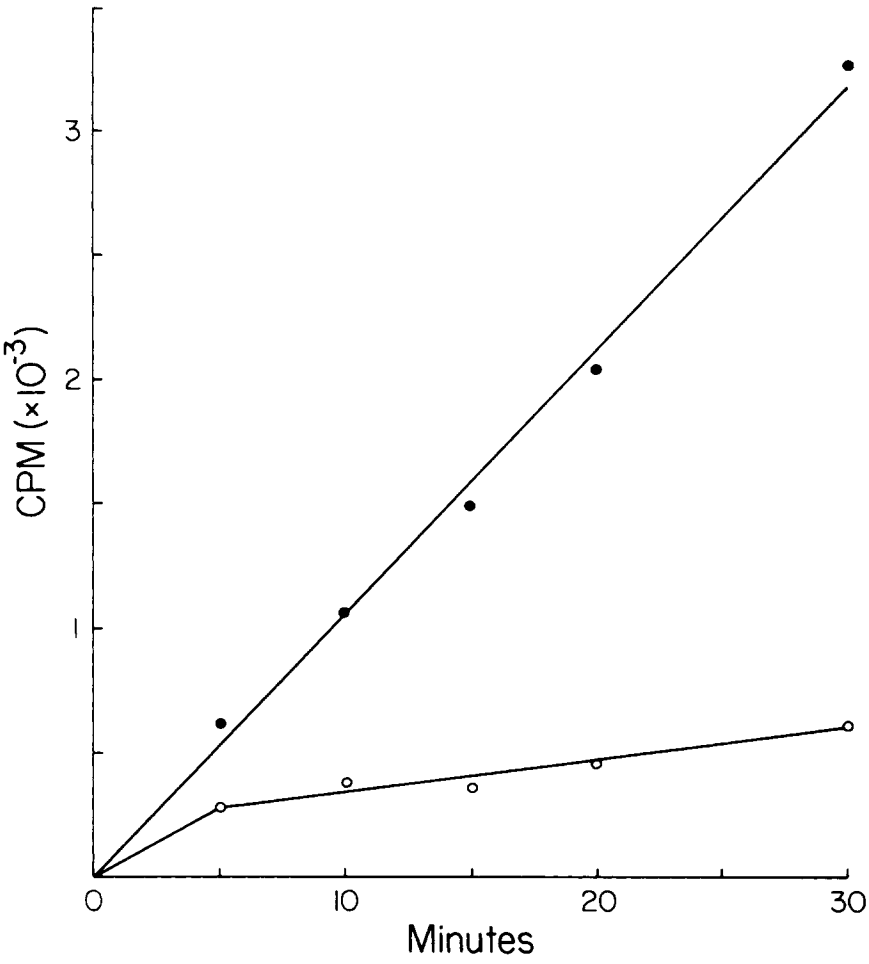

Fig. 3. Inhibition of pinocytosis by A9 monoclonal antibody. Log phase *A castellanii* cells, at a concentration of 10^6/ml, were assayed for pinocytotic activity by the method of Bowers and Olszewski [19]. Control, (●–●); 23 μg/ml A9, (○–○).

contrast, those binding molecules which do not stimulate encystment, ie, C5 and f(ab)'-A9, did not inhibit pinocytosis (several concentrations above and below saturating concentrations were used). A lag before full inhibition of pinocytotic rate indicates that several minutes are required for the antibody binding to exert the inhibitory effect. The lag suggests that the binding itself is not immediately responsible for the inhibition of pinocytosis. Rather, it appears that a relatively rapid secondary reaction triggered by binding to the plasma membrane antigen is responsible for the shutting off of pinocytosis. Since the binding takes place at 0°C and the pinocytosis reactions are started by warming to 30°C, the secondary reaction apparently cannot take place at 0°C. This data, taken with the fact that monovalent fragments of antibody do not cause encystment, suggest that one aspect of the secondary reaction may be the aggregation of specific membrane proteins.

DISCUSSION

Two pieces of experimental evidence indicate that C8, F1, and A9 monoclonal antibodies bind the same cell surface receptor. First, each of these antibodies competes for cell surface binding sites with the two other antibodies, whereas none of them compete with C5. Also, C5 does not compete for binding sites with these three antibodies. Secondly, C8, F1, and A9 bind the same two peptides (100-kd size range) separated by sodium dodecyl sulfate (SDS) gel electrophoresis, whereas C5 binds exclusively to the major plasma membrane protein (15-kd size range) [12].

C8, F1, and A9 all stimulate encystment and all inhibit pinocytosis. C5, a decavalent antibody against the most prevalent plasma membrane protein, neither inhibits cell division or pinocytosis nor stimulates encystment. These data argue that the plasma membrane binding site for C8, F1, and A9 has a special nature, and that the induction of encystment is a direct result of the binding of that particular protein. The physiological function of that plasma membrane protein is unknown. However, two explanations for this observation appear most likely. The differentiation-inducing antibodies could be binding a cell surface receptor, similar in mechanism to mammalian hormone receptors, whose physiological function is to induce encystment when stimulated. It has been known for some time that antibody binding to a cell surface hormone receptor can mimic the action of the hormone [13], and that antibody binding to the antigen receptors of B-lymphocytes can produce the same biological effects as natural antigen [14]. On the other hand, no differentiation-inducing receptors have ever been described for a free living amoeba, nor has a enhanced survival function for such receptors been postulated. As an alternative explanation, the antigen for the differentiation-inducing antibodies may be a molecular component of the pinocytosis mechanism. Should binding to this component interfere with its function, pinocytosis, the only means of feeding available in the defined media, would be shut off, and the organism would differentiate in response to lack of nutrient. No one has ever isolated a protein that was a part of the pinocytotic mechanism.

There are several analogies which argue in favor of the receptor hypothesis. The hormone interleukin-2 interacts with specific T-cell plasma membrane receptors to cause mitosis. Cantrell and Smith [15] have shown that interleukin-2 stimulation of cell division is dependent upon concentration of the hormone, receptor density, and the duration of hormone-receptor interaction. These critical factors for stimulation of T-cell cycle progression are remarkably similar to the conditions necessary for induction of *A castellanii* encystment with C8, F1, and A9 antibodies. Cell surface molecules that, upon being bound by monoclonal antibodies, prevent the differentiation of a trypanosome from the insect to the mammalian form have been described [16]. In that case as with antibody-replacing hormone [13], B-lymphocyte activation [14], and these results, f(ab)′ fragments were ineffective. Also, a correlation between decreasing pinocytotic activity and the onset of differentiation has been noted [17]. Finally, the presence of a cell surface receptor that regulates cell division and encystment in *A castellanii* would explain early observations on the deceleration and termination of growth [8,18].

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