Physiological and Biochemical Studies on Sexual Cell Fusion in *Dictyostelium discoideum*

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Contents

General Introduction

Complex carbohydrates are major constituents of the outer surface of plasma membranes and extend out some distance from the lipid bilayer. They can form polar bonds of various sorts and, by virtue of their structure, can confer a large degree of stereospecificity to glycoproteins and glycolipids. Because of these properties, it has been suggested that complex carbohydrates play a major role in cellular interactions. Although the molecular mechanisms are still a matter of conjecture, evidence has accumulated implicating carbohydrates in several processes of cell interactions, including fertilization, mating type cell interaction in micro organisms, cell-cell and cell-substratum adhesion and phagocytosis. (Bozzaro, 1985).

The cellular slime mold, Dictyostelium discoideum has been used as a suitable model system to study cell-cell interactions, and evidence indicates that in this organism carbohydrates, especially as prosthetic groups of glycoproteins, also play an important role in process of cell-cell interactions.

D. discoideum has two different developmental pathways, fruiting-body formation and macrocyst formation. These are considered to be the asexual and the sexual cycles in this organism, respectively (Clark et al., 1973; Erdos et al., 1973; MacInnes and Francis, 1974; Okada et al., 1986). During the growth phase, haploid myxamoeboid cells of D. discoideum feed on

bacteria and grow by fission. When bacteria are no longer present, the cells on a solid medium aggregate to form a fruiting-body consisting of spores and stalk cells (Bonner, 1959). On the other hand, under appropriate culture conditions, such as excessive water and certain period of darkness, the cells acquire fusion competence (Nickerson and Raper, 1973; Erdos et al., 1976; Saga and Yanagisawa, 1982; Saga et al., 1983) to fuse sexually between two opposite mating types, and produce a zygotic giant cell (O'Day, 1979; Robson and Williams, 1980; Szabo et al., 1982; Okada et al., 1986). Subsequently the giant cell develops into a macrocyst enclosed in a thick fibrillar wall. (Filosa and Dengler, 1972; Wallace and Raper, 1979; O'Day, 1979; O'Day and Durston, 1979; Abe et al., 1984; Okada et al., 1986) (Fig.1).

Recently, D. discoideum has been realized as a quite attractive system for studying cell interactions in the sexual event. Because, this organism has sexually heterothallic strains such as described above and homothallic strains. In addition, that has asexual and bisexual strains. (Erdos et al., 1973; Robson and williams, 1980) (Table 1). Therefore, the studies on sexual interactions of D. discoideum might give some clue to understand evolution of the sexuality.

Although D. discoideum had such many interesting properties, it had been impossible to analyze their sexual interactions on molecular level. One of the main reasons was the lack of

synchronous developmental system. However, in 1983, Saga *et al.* succeeded to develop the synchronous system and divided macrocyst formation process into 3 stages : 1) acquisition of fusion competence, 2) fusion of cells, and 3) zygotic giant cell development into macrocyst.

In the present experiments, attempts were made to investigate the mechanism of sexual cell fusion on cellular and molecular levels using the synchronous system. In part I, the effects of environmental factors at the each stage of the macrocyst development were investigated. A cell surface molecule involved in sexual cell fusion was identified in part II. Purification and characterization of this molecule were carried out in part III.

PART I

Environmental factors inducing sexual development

Introduction

Multicellular higher organisms possess two functionally different types of cells: germ and somatic cells, the former are capable of sexual fusion but not the latter. In some unicellular organisms such as cellular slime molds, a single cell should perform the role of a somatic cell at one time and that of a germ cell at another for sexual genetic exchange. Such a change in the function of a cell is often regulated by the particular surrounding environmental conditions.

In heterothallic strains of Dictyostelium discoideum, when cells of two opposite mating types, HM1 and NC4, are cultured with bacteria in the dark on a wet nutrient agar plate, fusion occurs to produce zygotic diploid giant cells, followed by development into macrocysts, the sexual structures of this organism (Clark et al.,1973; Erdos et al., 1973; MacInnes and Francis, 1974; O'Day, 1979; Saga et al., 1983; Okada et al.,1986). However, when the cells are cultured in the light on a dried agar plate, asexual fruiting-bodies are slightly produced.

Several researchers (Blaskovics and Raper, 1957; Nickerson and Raper, 1973; Erdos et al., 1976) studied environmental conditions responsible for macrocyst development in cells of many species and strains of cellular slime molds and found that darkness, excessive water, relatively high temperature (20^{o}C -

25°C) and the absence of phosphate ions in culture medium caused cells to develop into macrocysts in most species and strains, while the opposite conditions led to the development of fruiting-bodies without any giant cell formation. Furthermore, O'Day et al. reported calcium ions also are essential for the production of zygotic giant cells in D. discoideum (Chagla et al., 1980; McConachie and O'Day, 1986).

Saga and Yanagisawa (1982) developed a synchronous cell-fusion system for D. discoideum and found through its use that macrocyst formation occurred in at least two steps, giant cell production, and their subsequent development into macrocysts. They also found that in liquid culture, a certain period of darkness was necessary for the first step, but not for the second. In addition, HM1 cells became fusion competent only in darkness, but NC4 cells were able to become fusion competent in either darkness or light. In the present study, the process of macrocyst formation was divided into 3 stages: 1) acquisition of fusion competence, 2) fusion of cells, and 3) zygotic giant cell development into macrocysts. The effects of excess water, temperature, phosphate ions and calcium ions at the each stage were examined.

Materials and Methods

Strains

D. discoideum, strains NC4 and HM1, were used. These were maintained as a stock fruiting-body culture on nutrient SM-agar (Sussman, 1966) with *Klebsiella aerogenes*.

Tests for environmental effects

1) To examine the effects of excessive water on the acquisition of fusion competence, cells of the each strain were cultured in liquid medium, BSS (Bonner's Salt Solution) (Bonner, 1947) containing *K. aerogenes*, in the dark for 15 hrs at 22°C on a reciprocal shaker (120 strokes/ min). Growth-phase cells were used for the cell fusion assay. Cells grown on SM agar plates (22°C in the dark) were used as a control.

2) The effects of temperature on (a) the acquisition of fusion competence,(b) cell fusion and (c) giant cell development into macrocysts were examined as follows. For (a), NC4 and HM1 cells suspended separately in BSS containing bacteria were incubated at 11, 14, 18, 22, 25, or 28°C in the dark on a shaker (120 strokes/min). Small samples were taken at successive intervals during incubation and used for the cell fusion assay. For (b), fusion competent NC4 and HM1 cell suspensions were mixed in equal volumes and shaken at the following temperatures: 0, 5.5, 8.5, 11, 14, 16, 18, 22, 25, 28, or 30°C in the light.

The percentage of fused cells was determined after 30 min. For (c), NC4 and HM1 mixed cell suspension containing giant cells was placed on wet BSS agar plates, incubated at the above temperatures in the dark and observed for macrocyst production after 7 days.

3) The effects of phosphate ions on (a) the acquisition of fusion competence, (b) cell fusion and (c) giant cell development were studied as followed. (a), NC4 and HM1 cells separately suspended in different concentrations of bacteria containing phosphate buffers, 0, 2.5, 5, 10, 25, or 50 mM (pH6.5) were shaken in the dark at 22°C for 18 hrs and used for cell fusion assay. (b), The fusion-competent NC4 and HM1 cells mixed in equal numbers were suspended in different concentrations of the above phosphate buffers (containing 1mM $CaCl_2$), and examined for cell fusion after shaking in the light at 22 °C for 30 min. (c), The mixed cell suspensions containing giant cells in phosphate buffers (containing 1 mM $CaCl_2$) at the different concentrations were plated on plain agar plates, incubated at 22°C in the dark and examined for macrocyst production.

4) The effects of calcium ions on (a) fusion competence and (b) cell fusion were examined as follows. (a), NC4 and HM1 cells separately suspended with bacteria in either BSS or calcium free BSS (50 mM KCl or 50 mM NaCl) and shaken at 22°C in the dark. After 15 hrs incubation, the cell fusion assay was carried out. (b), $CaCl_2$ was introduced into the mixed fusion

competent cell suspensions (containing 50 mM NaCl) at different concentrations and the cell fusion assay was performed, after 60 min of shaking at 22°C.

Cell-fusion assay

The degree of fusion competence of cells was estimated from the percentage of fused cells according to the method of Saga et al. (1983).

Results

Effects of water

The fusion competence of NC4 and HM1 cells grown in liquid cultures was examined. As previously reported by Saga et al. (1983), HM1 cells cultured in the dark for 15 hrs possess fusion competence, but HM1 cells cultured in the light for 15 hrs do not. However, NC4 cells, cultured in either darkness or light possessed fusion competence. Figure 2 shows the acquisition of this competence in NC4 cells with time in liquid culture under either dark or light condition. It is evident that although NC4 cells cultured in both darkness and light are able to acquire fusion competence as previously reported by Saga et al. (1983), those cultured in the dark come to possess this competence to a slightly higher extent than those cultured in the light. It is of interest that in liquid, cells have no fusion competence at all at the beginning of cultivation. That is, a certain period of cultivation is required for the acquisition, which raises the question why these cells have no fusion competence at the beginning of liquid cultivation. A possible explanation for this is that cells grown on agar plates have no fusion competence, cells in liquid being taken from agar plates. To examine this possibility, the fusion competence of cells grown on agar plates was examined. Plate-cultured cells, both NC4 and HM1 in either darkness or light, were found to fail

to have any fusion competence. However, introducing excess water to the plates makes these cells fusion competent.

To determine whether or not fusion competent cells lose their competence with the removal of water, NC4 cells cultured in liquid for 15 hrs in the dark were placed on agar plate and cultured in the dark. As shown in Fig.3, their fusion competence was found to be lost within 1 hr.

It is also evident from Fig.2 that fusion competent NC4 cells lose their competence during continuous cultivation even in liquid. This has also been reported for HM1 cells (Saga et al., 1983). We investigated why this competence is lost during continuous liquid cultivation, and found that starvation of the cells was the reason for this loss. During liquid cultivation in the dark, NC4 cells were able to maintain fusion competence as long as bacteria were available as a food source. After thorough washing to remove bacteria and resuspension in BSS (5 X 10^6 Cells/ml), NC4 cells were noted to start losing their fusion competence immediately as shown in Fig.4, but when the cell suspension was replenished with bacteria, the acquisition of fusion competence was resumed.

Effects of temperatures

In most cellular slime molds, relatively higher temperatures (20°C - 25°C) are favored for macrocyst formation (Nickerson and Raper, 1973). According to Saga et al. (1983), HM1

cells cultured in the dark fail to acquire fusion competence at 25°C, but are able to fuse at temperatures between 15°C and 28°C. On the other hand, it is evident that NC4 and HM1 cells incubated as a mixture on wet agar plates in the dark actually develop into macrocysts even at 25°C. To reconcile these contradictory results, the effects of temperatures on macrocyst formation was again examined. The effect on fusion competence acquisition is shown in Fig.5. In both strains, cells cultured in the dark at 14°C were found to acquire fusion competence, although the time required for this was much longer. Both strains acquired this competence at 25°C, but in HM1 it was quickly lost by further incubation, before 15 hr (Fig.5). Since Saga et al. (1983) examined fusion competence after 15 hr of incubation, the HM1 cells had lost their competence before that time at 25°C. However, when in the presence of NC4, HM1 cells will fuse with the NC4 cells before this loss occurs. This should explain the variance of results mentioned above. Below 11°C or above 28°C, no acquisition of fusion competence was possible at all.

Next, the effects of temperatures on cell fusion were examined. The results are shown in Fig.6. Fusion competent NC4 and HM1 cells fused within 30 min between 16°C - 28°C, and with a longer period of incubation such as 120 min, the cells become fusible even at temperatures as low as 5.5°C, although not at 0°C.

Effects of phosphate ions

The effects of phosphate ions on each stage of macrocyst development were studied. As far as we have examined, phosphate ions were found to have no effects on any stage for the acquisition of fusion competence, cell fusion, or giant cell development.

Effects of calcium ions

Chagla et al. (1980), and McConachie and O'Day (1986) have reported that calcium ions are required for production of zygotic giant cells. In the present study, it was found that there was no indication at all of its requirement for the acquisition of fusion competence by NC4 and HM1 cells. NC4 cells grown on bacteria in either 50 mM KCl or 50 mM NaCl solution instead of BSS containing $CaCl_2$ acquired a high degree of competence after 15 hrs dark incubation at 22°C. However, calcium ions were found to be necessary during the cell fusion stage. As shown in Fig.7, when fusion competent NC4 and HM1 cells suspended in 50 mM NaCl solution were mixed at a 1 : 1 ratio, and incubated at 22°C for 60 min, fusion failed to occur, but with the introduction of 0.1 mM $CaCl_2$ into the cell suspension, cell fusion was initiated, and 0.5 mM $CaCl_2$ caused it to reach a maximum level. However, with the introduction of 25 mM $MgCl_2$ into the suspension, no cell fusion was initiated.

Discussion

In cellular slime molds, alternate modes of development either sexual or asexual are known to result from responses to various environmental factors (Blaskovics and Raper, 1957; Nickerson and Raper, 1973; Erdos *et al.*, 1976; Chagla *et al.*, 1980; McConachie and O'Day, 1986). The present findings with the *D. discoideum*, strains NC4 and HM1, generally agree with most previous reports except in regard to phosphate ions.

The present study demonstrates that excessive water and bacteria as food source are the conditions indispensable for the acquisition of fusion competence in cells. Even in liquid culture if the supply of bacteria is not replenished, fusion competence is quickly lost. Phosphate buffers at concentrations from 1 mM to 50 mM failed to affect any stage of macrocyst development of *D. discoideum* in the present experiment. In contrast, Nickerson and Raper (1973) have indicated previously in the same species that the production of macrocysts is greatly suppressed depending on the concentration of phosphate ion and that virtually complete inhibition occurs at 50 mM. One possible explanation for this conflict seems to be the difference in culture methods. I grew cells in BSS with *K. aerogenes*, while they used 0.1% lactose-peptone agar with *E. coli*. However, *D. discoideum* cells can produce macrocysts even on agar plate provided water is present in excess, and cells

cultured with either K. aerogenes or E. coli. can produce macrocysts to the same degree (Blaskovics and Raper, 1957). Another explanation is the difference in the strains used. Nickerson and Raper used a homothallic strain AC4, while I used the heterothallic strains HM1 and NC4. They have mentioned that the effects of the environmental conditions on macrocyst formation are quite varied depending on species and strain. Therefore, this would explain the variance in the results.

In D. discoideum cells, the switching from sexually fusion incompetent to competent and vice versa occurs according to what environmental factors are present, such as darkness or light, the presence or absence of excessive water and bacteria as a nutrient source. These factors are believed to change directly or indirectly the properties of the cell surface, and surface proteins would be involved in this change. Attempts are now being made to find a cell surface protein(s) involved in the sexual cell fusion of D. discoideum.

PART II

Identification of the cell surface molecule involved in sexual cell fusion

Introduction

Sexuality plays an important role for evolution by exchanging genes. In an unicellular organism cellular slime molds Dictyostelium discoideum, haploid amoeboid cells acquire fusion competence under the condition of the presence of excess water, nutrient and certain period of darkness and produce diploid zygotic giant cells by fusion (Nikerson and Raper, 1973; Robson and Williams, 1980; Saga and Yanagisawa, 1982; Szabo et al., 1982 ; Saga et al., 1983; Suzuki and Yanagisawa, in press). In heterothallic strains such as NC4 and HM1, sexual fusion occurs only between cells belonging to opposite mating types (Blaskovics and Raper, 1957; Erdos et al., 1973; Saga and Yanagisawa, 1982; Szabo et al., 1982). This fusion process seems to be essentially the same as fertilization in multicellular higher organisms. In this study, D. discoideum was used as a model system for elucidating the molecular mechanisms of sexual cell fusion.

A zygotic giant cell excretes cyclic AMP (cAMP) to attract surrounding cells to engulf them, and subsequently grow into a mature macrocyst (Filosa and Dengler, 1972; O'Day, 1979; O'Day and Durston, 1979; Abe et al., 1984; Lewis and O'Day, 1985; Lewis and O'Day, 1986). Giant cells are often multinucleated, being the result of the fusion of more than two cells, but only two nuclei actually make fusion to produce a diploid nucleus, and

all others disappear (Okada et al., 1986). During the maturation of a macrocyst, meiosis takes place and subsequent mitosis produces dozens of haploid cells (MacInnes and Francis, 1974; Okada et al., 1986).

Saga et al. (1983) and Suzuki and Yanagisawa (in press) reported that both NC4 and HM1 cells required excess water and bacteria as food source to acquire fusion competence, but that darkness was only required by HM1 cells. NC4 cells are able to acquire fusion competence even under the condition of light. Recently, Urushihara et al. (1988) found a protein related to sexual cell fusion on cell surface of HM1. This protein is specifically present on the surface of fusion competent HM1 cells, but not on the surface of fusion incompetent HM1 cells.

The present paper will report another such protein involved in their sexual cell fusion. This protein is 138K dalton in molecular weight, and appears specifically only on the surface of fusion competent NC4 cells.

Materials and Methods

Organisms and culture conditions

Two heterothallic strains of D. discoideum, NC4 and HM1, were used. HM1 was derived from V12 (Kay et al., 1978), a strain of the opposite mating type to NC4. Stock fruiting-body cultures of each strain were maintained separately on nutrient SM agar with Klebsiella aerogenes (Sussman, 1966).

Fusion competent cells

Growth-phase cells harvested from agar plates were suspended in Bonner's salt solution (BSS) (Bonner, 1947)(3 - 5 x 10^5 cells / ml) with K. aerogenes and cultivated at 22^{o} C in the dark on a reciprocal shaker (120 strokes / min), and 15hr cultivated cells were used as fusion competent cells. Growth-phase cells from SM agar plates were used as fusion incompetent cells (Suzuki and Yanagisawa, in press).

Assay of cell fusion

The degree of fusion competence of cells was determined and expressed as the percentage of fused cells according to the procedure of Saga et al. (1983).

Enzyme treatments

Cells were treated either with trypsin or papain at various concentrations (10-1000 μg / ml at final), and incubated at

22°C on a shaker (120 strokes / min). Thirty min later, the cells were washed, resuspended in BSS (5 x 10^6Cells / ml) and mixed with an equal number of fusion-competent opposite mating-type cells. The percentage of fused cells was estimated after 30 min of continuous incubation.

Antibody preparation

Rabbits were immunized at weekly intervals with fusion competent NC4 cells (3 x 10^7 cells). One week following the 6th injection, antiserum was collected and stored at -80°C. Anti-fusion competent NC4 activity was assayed by titrating the ability for agglutination of fusion competent NC4 cells after heat inactivation. Antiserum with maximum cell agglutinating activity at a 2^{-8} dilution was used for the experiments.

The crude immunoglobulin G (IgG) fraction of antisera was obtained by 50% ammonium sulfate precipitation. Fab fragments of IgG were prepared according to the procedure of Utsumi (1969).

Inhibition assay of cell fusion by Fab

Fusion competent NC4 cell suspensions (1 x 10^7 cells/ml in BSS) were preincubated with various concentrations of Fab at 0°C for 15 min. Equal number of fusion competent HM1 cells were introduced into the NC4 cell suspensions and incubated at 22°C on a shaker (120 strokes / min). The inhibitory activity at Fab concentration C (I A(C)) was calculated as follows:

$$IA(C) = \frac{FA(0) - FA(C)}{FA(0)} \times 100$$

FA(0) and FA(C) are fusion activities at Fab concentrations of 0 mg/ml and C mg/ml, respectively.

Inhibition assay of cell adhesion by Fab

Fusion competent NC4 cells (1 x 10^7 cells/ml in BSS) preincubated with Fab at 0^oC for 15 min, were mixed with an equal number of competent HM1 cells and the percentage of cell adhesion was determined after 30 min of incubation. The adhesion was expressed as the percentage of number of the initial cells and cell clumps. Thus, 100% correspond to the complete inhibition of cell adhesion (Chadwick and Garrod, 1983).

Absorption of fusion inhibitory activity of Fab by cells

An NC4 cell suspension (1 x 10^7 cells/ml in BSS) was mixed with an equal volume of a Fab suspension (4 mg/ml in BSS) in a siliconized microfuge tube and kept on ice with occasional shaking. After 1hr incubation, the supernatant was collected as absorbed Fab and assayed for fusion inhibition.

Absorption of Fab fusion inhibitory activity by polyacrylamide gel

The absorption was carried out by a modification of the method of Steinemann et al. (1979). Fusion competent NC4

cells (5 X 10^8) extracted in incubation medium (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, in 125mM Tris/HCl buffer, pH6.8) were boiled for 5 min and centrifuged (10,000xg for 10 min). The supernatant was separated by SDS polyacrylamide gel electrophoresis. After fixation with isopropanol / acetic acid / water (5/2/13 by vol.), the gel was thoroughly washed to remove SDS, sliced from top to bottom, and washed again with BSS. Each washed gel slice was finely cut, suspended in 3ml BSS and mixed with 1ml Fab (1.5 mg). After incubation for 24hr at 4°C on a rolling-mixer, Fab that did not bind to proteins in the gel (supernatant) was collected, and assayed for fusion inhibitory activity.

Lactoperoxidase-catalyzed iodination

Lactoperoxidase-catalyzed iodination was conducted according to the procedure of Hynes (1973). Well washed fusion competent NC4 cells (1 x 10^7) were suspended in 17 mM sodium phosphate buffer (pH 7.2). Five mM glucose and 200 µCi/ml of carrier-free $Na^{125}I$ were introduced into the cell suspension. The reaction was initiated by the addition of lactoperoxidase and glucose oxidase at final concentrations of 20µg/ml and 0.1units/ml, respectively, then allowed to remain on ice with occasional swirling. Labeling was stopped after 20min by addition of 5µl phosphate buffer containing 1 mg/ml KI. The cells (1 x 10^7) were washed with the above buffer, dissolved in

120ul RIPA buffer (Collett and Erikson, 1978) and centrifuged (10,000xg for 10min). The supernatant (RIPA extract) thus obtained was used for immuno-precipitation and electrophoresis.

Immuno-precipitation

Immuno-precipitation was performed according to the procedure of Richert et al. (1979). For preparation of boiled Staphylococcus aureus, formalin-fixed S. aureus suspended in 20mM Tris buffer saline (pH7.2) containing 10% 2-mercaptoethanol and 3% sodium dodecyl sulfate (SDS) was heated at 95°C for 30min. After centrifugation, the bacteria were resuspended in the fresh buffer and heated again. The bacterial pellet was washed and suspended in RIPA buffer and stocked for immuno-precipitation.

Six μl antiserum was adsorbed to the boiled 100μl S. aureus at 0°C for 30min, and antisera-S. aureus complexes were suspended in 100 μl RIPA buffer. Varying amounts of RIPA extracts of cells were incubated with the antisera-S.aureus complex at 0°C for 30min. The immuno-precipitates were washed with RIPA buffer, and centrifuged. For gel electrophoresis of the immuno-precipitated proteins, the precipitates were boiled for 5min in incubation medium, centrifuged (10,000xg for 10min), and the supernatant was subjected to the gel electrophoresis.

SDS-polyacrylamide gel electrophoresis

RIPA extracts of cells and immuno-precipitated proteins were boiled for 5min in incubation medium containing 0.02% Bromo

phenol Blue (BPB), and centrifuged (10,000xg for 10 min). Supernatant was analyzed on 8 - 16% gradient poly-acrylamide/SDS gels using the discontinuous buffer system of Laemmli (1979). Electrophoresis was started by application of a constant current of 10mA for the first half hour and then of 30mA current until the BPB dye reached the lower end of the separation gel. The gel was fixed and stained in methanol/acetic acid /water (10/3/7 by vol.) containing 0.1% Coomassie Brilliant Blue R for 2hr, and destained in ethanol/acetic acid/water (2/1/7 by vol.) for 4hr. For autoradiography, the gels were dried on filter paper and placed against Fuji-RX X-ray film for 6 - 24hr.

Results

Inhibition of cell fusion by Fab-fragment

Cell fusion is generally mediated by cell surface molecules, and that is also suggested in Dictyostelium discoideum (O'Day and Rivera, 1987). To determine which molecule(s) on the surface of NC4 cells is involved in the sexual cell fusion of D. discoideum, first, Fab fragment of antiserum for fusion competent NC4 cells was prepared, and its effect on cell fusion was examined (Fig.8A - 8D).

Mixing fusion competent NC4 cells with fusion competent HM1 cells produced many cell clumps (Fig.8A). However, when 5mM EDTA was introduced into the cell suspension the agglutinated cells were separated each other and only fused cell remained as clumps (Fig.8B). On the contrary, the fusion competent NC4 cells pre-treated with Fab produced cell clumps after mixing them with fusion competent HM1 cells (Fig.8C), but no fused cell clumps remained after introduction of EDTA. Addition of 5mM EDTA caused dissociation of all cell clumps (Fig.8D).

Fusion competent NC4 cells pre-treated with Fab at various concentrations were mixed with fusion competent HM1 cells and the percentage of cell-fusion inhibition was determined. It was found that the degree of cell-fusion inhibition was dependent on the concentration of Fab. Cell fusion was completely inhibited at concentrations exceeding 0.5mg/ml (Fig.9B). However, cell

adhesion was not inhibited even at a concentration of 2mg/ml (Fig.9A).

For assay of the antiserum specificity, the cell fusion inhibitory activity of Fab absorbed by fusion competent NC4 cells was examined, and compared with that of Fab absorbed by fusion incompetent NC4 cells. While there was a loss of inhibitory activity in the former, which is dependent on the number of cells absorbing Fab, in the latter, no loss of inhibitory activity was observed (Fig.10). It is evident from these findings that a specific antigen(s) is present on the cell surface of only fusion competent NC4 as the target(s) of fusion-blocking Fab, but not on the cell surface of fusion incompetent NC4.

Identification of a protein involved in cell fusion

For the identification of a specific antigen(s) on NC4 cell surface involved in sexual cell fusion, immuno-precipitation analysis was carried out. First, attempts were made to detect all of the antigenic proteins of fusion competent- and incompetent- NC4 cell surfaces. At least 27 and 26 bands were reproducibly detected on fusion competent cells and incompetent cells, respectively. There were two proteins, 39K and 138K dalton in molecular weight, detected specifically on fusion competent cells (Fig.11).

Next, fusion competent cells were treated with proteolytic enzymes, and examined for the loss of fusion competence.

Treatment with trypsin had no effect even at a concentration of 1,000μg/ml (Fig.12A), while cells treated with 300μg/ml papain lost their fusion competence almost completely (Fig.12B). Study of the immuno-precipitate patterns of fusion competent NC4 cells treated either with 500μg/ml of trypsin or papain showed that bands for the 39K and 138K proteins could still be seen following trypsin treatment, but after papain treatment, the 138K band disappeared completely (Fig.13). Therefore, the specific protein related to sexual cell fusion appears to be the 138K protein.

For confirmation, blot-absorption experiments were performed. Solubilized fusion competent NC4 cells were separated by SDS-polyacrylamide gel electrophoresis. A gel was sliced into 7 fractions (#1-#7). Each of which was incubated in Fab at 4°C for 24hr, and assay was made of the fusion inhibitory effect of Fab. It was found that Fab absorbed by the #1 gel fraction containing the 138K protein lost its inhibitory activity to a remarkable degree, but there was no loss at all in Fab absorbed by the #4 fraction containing the 39K protein or the other gel fractions (Table 2). From these findings, it was concluded that the 138K surface protein on NC4 cells was involved in their sexual cell fusion.

Relation of 138K protein to acquisition of fusion competence by cells

NC4 cells cultured on a agar plate containing minimum water are not fusion competent (Suzuki and Yanagisawa, in press), but start to become fusion competent 6 hrs after being transferred to a liquid culture. This competence reaches a maximum by 12hr (Fig.14A). We thus made an attempt to determine the time required for the 138K protein to be expressed in liquid cultivation. To our surprise, the protein was expressed after 3hrs of liquid cultivation and was continuously present thereafter (Fig.14B). This is 3-6 hrs earlier than the time at which cells start acquiring fusion competence.

In order to confirm this result, an experiment was conducted from a different approach. NC4 cells from agar plates were transferred to a liquid medium, and after 4hr of cultivation, prior to the acquisition of fusion competence, they were harvested, washed and used for Fab absorption. The inhibitory activity of the absorbed Fab on cell fusion was then examined. As a control, 15 hr cultivated cells with sufficient fusion competence were employed to absorb Fab. Fab absorbed by both 4 and 15 hrs liquid-cultivated cells lost their inhibitory activity, and the extent of loss being dependent on the cell numbers used for absorption (Fig.15).

This result suggests a possibility such as that there is the presence of another factor(s) that affects the action of the 138K protein and its modification during the course of cell fusion.

Discussion

The 138K protein present on heterothallic strain NC4 cell surface was found to be involved in their sexual cell fusion. This protein can be detected on the surface of fusion competent NC4 cells, but not on the surface of fusion incompetent cells. Furthermore, as additional data, it was found that the 138K protein was also present on the surface of fusion competent HM1 cells (Fig.16). Since cells fuse only with cells of the opposite mating type, there must be some mechanisms operative within them for recognition and adhesion prior to fusion. In other words, at least two steps are required for complete cell fusion to occur: 1) mutual recognition and adhesion between cells and 2) membrane fusion. Whether the 138K protein is actually involved in either one or both of these steps remains to be determined. However, an evidence that this protein is present on the surface of both fusion competent NC4 and HM1 cells suggests that there is greater possibility of its involvement in membrane fusion rather than cell recognition.

I previously reported that there is another protein which is also related to cell fusion, 70K dalton in molecular weight, on the surface of HM1 cells (Urushihara et al., 1988). This protein is found to be only on fusion competent HM1 cells, but not on fusion incompetent HM1 cells or both fusion-competent and -incompetent NC4 cells. Therefore, I assume that the 70K

protein has involvement in mutual cell recognition.

As described above, if the 138k protein is actually involved in cell fusion, there are the problems of explaining the conflicting results that this protein is present even on the surface of fusion incompetent NC4 cells cultivated for only 3hr in liquid culture. Furthermore, Fab absorbed by 4hr liquid-cultured fusion incompetent NC4 cells, decreases markedly in inhibitory activity of cell fusion much like Fab absorbed by 15hr liquid cultured fusion competent NC4 cells. These conflicting findings are resolved allowing the following presumptions, 1) In addition to the 138K protein, some other molecule(s) which could not be detected by the present experimental methods, must also be present on the cell surface for cell fusion to occur. 2) reorganization or/and modification of the 138K protein is necessary for its function. 3) Intracellular structures, such as cytoskeleton, must undergo certain changes for function of this protein.

PART III

Purification and Characterization of gp138 : A Surface Glycoprotein Involved in Sexual Cell Fusion

Introduction

Dictyostelium discoideum cells fuse sexually between two opposite mating type strains, NC4 and HM1 to produce macrocysts. O'Day and Rivera (1987) reported that certain kinds of lectins, such as Con-A and WGA, inhibited macrocyst formation, and suggested involvement of cell surface sugar in the early event of macrocyst development including cell fusion. Cell fusion should consist at least of two steps, mutual recognition between cells and membrane fusion. Urushihara and Yanagisawa (1987) found that the mutual recognition and membrane fusion were taken even between intact cells and cell ghosts. Recently, Urushihara et al. (1988) discovered a protein, 70K dalton in molecular weight, appeared specifically in fusion competent HM1 cells. More recently, Suzuki and Yanagisawa detected the presence of another protein on fusion competent cells of both NC4 and HM1. Molecular weight of this protein was 138K dalton. Out of these two proteins, 70K might be involved in mutual recognition between cells. Because, it exists only on HM1 cell surface. Therefore, fusion competent HM1 cells having 70K protein could be recognized from fusion competent NC4 cells which do not have the molecule. After completion of mutual recognition, 138K protein, which appears on both fusion competent NC4 and HM1 cell surface, would work for membrane fusion.

In the present experiment, purification and some

characterization of 138K protein were attempted. It was found that 138K protein was a glycoprotein. Antiserum against 138K protein was also prepared to perform further experiments.

Materials and Methods

Organisms and culture conditions

Two heterothallic strains of *D. discoideum*, NC4 and HM1 were used. HM1 derived from V12 (Kay *et al.*, 1978) is a strain of the opposite mating type to NC4. Stock fruiting-body cultures of each strain were maintained separately on nutrient SM agar with *Klebsiella aerogenes* (Sussman, 1966).

Preparation of fusion competent cells

Growth-phase cells suspended in Bonner's salt solution (BSS)(Bonner, 1947)(3 - 5 x 10^5 cells / ml) with *K. aerogenes* were cultured in the dark at 22^{o} C on a reciprocal shaker (120 strokes / min). The 15hr cultivated cells were used as fusion competent cells, while, growth-phase cells from SM agar plate were used as fusion incompetent cells.

Assay for cell fusion

The degree of fusion competence of cells was indicated as the percentage of fused cells (Saga *et al.*, 1983).

Inhibition assay of cell fusion

Fusion competent NC4 cell suspension (1 x 10^7 cells/ml in BSS) were preincubated with equal volume of various concentrations of Fab suspension (in BSS) at 0^{o}C for 15 min. Equal number of fusion competent HM1 cells were then introduced

into the cell suspension and further incubated at 22°C on a shaker (120 strokes / min). The inhibition activity at Fab concentration C (IA(C)) was calculated as follows:

$$IA(C) = \frac{FA(0) - FA(C)}{FA(0)} \times 100$$

FA(0) and FA(C) are fusion activity at Fab concentrations of 0 mg/ml and C mg/ml, respectively.

Preparation of cell ghosts

Cell ghosts were prepared according to the method of Sussman and Boschwitz (1975). Cells were suspended in a ghost solution (20mM Na-phosphate buffer, pH6.5; 50mM NaCl; 2% glycerol) and stored at -20°C. The frozen cell suspensions were thawed before use, washed twice by centrifugation, and resuspended in cold BSS.

Preparation of antiserum

Antiserum FRA-4 was prepared against fusion competent NC4 cells as follows. Rabbits were immunized at weekly intervals with 3×10^7 cells, and one week after the 6th injection, antiserum was collected and stored frozen at -80°C.

Antiserum FRA-6 was prepared against purified gp138 according to the method of Hayashi et al. (1977). After SDS-PAGE as the final step of purification, the gp138 band which could be detected by a 10 min stain with Coomassie Brilliant Blue R solution and 20 min destain with destain solution (see SDS-

PAGE), was cut out. The gels containing the protein were dialyzed against buffered physiological saline (10mM Na-phosphate buffer, pH6.8 containing 0.15M NaCl). This gel was mashed, mixed with an equal volume of Freund's complete adjuvant (Difco) and injected subcutaneously into the backs of rabbit at monthly intervals. The reactivity of antisera with the antigen was checked by immuno-blotting analysis using a small amount of blood from the ear vein. After 2 injections, antiserum was collected and stored frozen at -80°C. Antiserum FRA-5 was prepared against the 120K dalton protein of D. discoideum following the description above.

Preparation of Fab fragments

The crude immunoglobulin G (IgG) fraction of antisera was obtained by 50% ammonium sulfate precipitation. Fab fragment of IgG were prepared according to the procedure of Utsumi (1969).

Absorption of fusion inhibitory activity of Fab by cells or cell ghosts

Cells or cell-ghosts suspended in BSS were mixed with equal volume of Fab suspensions (4 mg/ml in BSS) in a siliconized microfuge tube and placed on ice with occasional shaking. After 1hr incubation, the suspensions were centrifuged and the supernatants were collected for fusion inhibition assay.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Cells, cell-ghosts and immuno-precipitated proteins were boiled 5 min in incubation medium (2% SDS, 10% glycerol, 5% 2-mercapto-ethanol in 125mM Tris/Hcl buffer (pH 6.8) containing 0.02% Bromophenol Blue (BPB)) and centrifuged (10,000 x g for 10 min). Supernatant were analyzed on 8 - 16% gradient or 5.1% non-gradient polyacrylamide/SDS gels using the discontinuous buffer system (Laemmli, 1970). The electrophoresis was started with a constant current of 10 mA for the first half an hour and thereupon continued at 30 mA until the BPB dye reached the lower end of the separation gel. Some gels were used for immuno- or lectin- blotting analysis, and the other ones were fixed and stained in methanol/acetic acid/water (10/3/7 by vol.) containing 0.1% Coomassie Brilliant Blue R for 2hr, and destained in ethanol/acetic acid/water (2/1/7 by vol.) for 4hr. For autoradiography, the gels were dried onto filter paper (3MM, Whatman) and placed against Kodak-XAR-5 X-ray film for 6 - 24hr.

Lectin affinity column chromatography

Fusion competent NC4 cells ($2x10^7$) were surface labeled with ^{125}I and suspended in 150µl Tris/HCl buffer (10mM, pH7.5) containing 1% deoxycholic acid, sodium salt (DOC), 0,2M NaCl. The suspension was vortexed for 1 min and allowed to stand on ice for 30min. Insoluble material was then removed by centrifugation at 10,000xg for 10 min. A 850µl 10 mM Tris/HCl buffer (pH7.5) containing 0.2M NaCl, was added to the

supernatant (final 0.15% DOC), and used for lectin affinity chromatography (DOC-extract).

Columns (1 ml syringe) of WGA-agarose and Con-A-agarose (HOHNEN, Tokyo) were washed successively with 10ml of 10 mM Tris/HCl buffer (pH7.5) containing 0.15% DOC,0.2M NaCl (DOC-Tris buffer). The DOC-extract was then applied to the column and unbound components were eluted with 5ml of DOC-Tris buffer. Lectin binding proteins were then specifically eluted with 5ml of DOC-Tris buffer containing 0.2M N-acetyl-D-glucosamine (SIGMA) (for WGA-agarose), or 0.2M methyl-α-D-mannopyranoside (SIGMA) (for Con-A-agarose). Each fraction was concentrated to a small volume by ultrafiltration with Centricon-10 (Amicon), dialyzed against 100 volumes of DOC-Tris buffer for 24hr, and applied to Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE).

Purification of gp138

Ghost pellets of fusion competent NC4 cells ($5x10^9$) were solubilized in 5ml 50mM Tris/HCl buffer (pH6.8) containing 1% deoxycholic acid (DOC), 1% cholic acid. The suspension was vortexed for 3min and stand on ice for 30min. Insoluble material was then removed by centrifugation at 10,000 x g for 20min. Tris/HCl buffer (45ml)(50mM, pH6.8) was added to the supernatant (final 0.1% DOC, 0.1% cholic acid), and applied to phenyl-sepharose CL-4B chromatography (Pharmacia) in a bed

volume of 15ml. After washing with 100ml 50mM Tris/HCl buffer (pH6.8) containing 0.1% DOC, 0.1% cholic acid, the column was eluted by rising the concentration of cholic acid in the buffer from 0.1% to 1%. The elution (50ml) was concentrated to a small volume with collodion bags (12K-cut, Sartorius), dialyzed against 100 volumes of 10mM Tris/HCl buffer (pH7.4) containing 0.15% DOC, 0.2M NaCl, and subjected to Con-A agarose column (2.5ml) according to described in "Lectin affinity column chromatography". Con-A binding fraction was concentrated with collodion Bags, dialyzed against 10mM NH_4HCO_3 for 48hr and lyophilized. Dry powder contained 80μg protein.

To prepare large amounts of protein, this purification series was done 16 cycles, and applied to preparative SDS-PAGE (5.1% acrylamide). Gels containing gp138 were cut out, and used for immunization of rabbit as described in "Preparation of antiserum".

Lactoperoxidase(LPO)-catalyzed iodination

Iodination was performed by the method of Hynes (1973).

Immuno-blotting analysis

SDS-PAGE was performed as described above. Proteins in the gels were transferred to nitrocellulose membranes (BA85, Schleicher and Schnell, Dassel) at 10 V/cm for 1-2hr in 5mM sodium borate (Urushihara and Yamada, 1986). The nitrocellulose membranes were then incubated in 1%

polyvinylpyrrolydone K90 (PVP) for 1-16hr to block further binding of proteins.

For immuno-blotting analysis, the membrane was washed with 20mM Na-phosphate buffer, pH7.6 containing 0.15M NaCl (PBS) and incubated with 1/100 diluted antiserum in PBS containing 5% bovine serum albumin (BSA-PBS) for 1hr. After washing with PBS containing 0.1% Tween 20 (Tween-PBS), the membrane was incubated with 1 μCi/ml ^{125}I-protein A (New England Nuclear, Immunology grade) in BSA-PBS for 1hr, washed with Tween-PBS, dried and then exposed to X-ray film (XAR-5, Kodak).

Immuno-precipitation

Immuno-precipitation with Staphylococcus aureus was performed according to the procedure of Kessler (1975), and Richert et al. (1979).

Two dimension polyacrylamide gel electrophoresis (2D-PAGE)

The immuno-precipitates were treated with 0.1M Tris/HCl buffer containing 8M guanidine hydrochloride and 10% 2-mercaptoethanol, and boiled in a water bath for 5 min. The solution was then dialyzed against a 7M urea solution containing 2% Noident P-40 (NP-40) at 4^{o}C for 3 hr.

2D-PAGE was carried out according to the method of Hirabayashi (1981). Cylindrical 1% agarose gels containing 2% NP-40 were used for the first dimension isoelectric focusing.

Samples were loaded on the anode side of the gel and isoelectric focusing was conducted at 500V for 24hr in a cold room (4°C). The pH range covered pH4 to pH9. After being fixed with 10% trichloroacetic acid and 5% sulfosalicylic acid solution (at room temp. for 1hr) and washed with distilled water, the agarose gels were placed on SDS-polyacrylamide gel. SDS-PAGE was carried out as described in SDS-PAGE.

Lectin-blotting analysis

Proteins in the gels of SDS-PAGE were transferred to nitrocellulose membrane as described in "Immuno-blotting analysis", but membranes were not treated with PVP after transfer. The membranes were washed with 10mM Tris/HCl buffer (pH7.4) containing 0.9% NaCl, 0.05% Tween 20 (Buffer-A) and incubated with horse radish peroxidase (HRP) conjugated lectin solution (5μg/ml, in Buffer-A) at room temp., for 1hr. Seven HRP-conjugated lectins, HRP-WGA, HRP-Con-A, HRP-LCA, HRP-PNA, HRP-E_4PHA, HRP-RCA60, HRP-RCA120 (Lectin stain kit, HOHNEN, Tokyo) were used. After washing with Buffer-A, the membranes were treated with 15mM Na-phosphate buffer (pH6.8) for 20sec, and detected the bound lectin with 15mM Na-phosphate buffer (pH6.8) containing 0.02% Diaminobenzidine, Tetrahydrochloride (DAB), 0.006% H_2O_2 at room temp. for 15min.

Results

Since 138K protein is located on cell surface, to isolate the molecule more efficiently, cell-ghosts were used instead of intact cells in most of the present experiments. Cell-ghosts can be easily stocked in a large amounts and prepared anytime for experiments.

In a preliminary experiment, it was examined that whether function of 138K protein on cell-ghosts is the same as that of intact cells or not. First, it was tested that whether cell-ghosts prepared from fusion competent NC4 cells are able to absorb the inhibitory activity of Fab of antiserum FRA-4 (anti-rabbit serum for fusion competent NC4 cells) as same as fusion competent intact NC4 cells do. As shown in Fig.17, the cell-ghosts absorbed the inhibitory activity of Fab as almost same as the intact cells did. This indicates that 138K protein on cell-ghosts is functionally same to that on intact cells.

Purification of 138K protein

Many cell surface proteins, especially the molecules involved in cell recognition and cell adhesion, are known to have a sugar chains. Therefore, it may be reasonable to assume that 138K protein has also (a) sugar chain(s), and that if 138K protein bind specifically to certain lectin, the molecule will be

purified easily. In order to test this, lectin-affinity column chromatography was performed. Results showed that 138K protein was found only in a binding fraction of both Con-A and WGA affinity chromatographies (Fig.18). Since the molecule is located on cell surface and has (a) sugar chain(s) which binds to Con-A, hereafter it was called gp138, phenylsepharose and Con-A affinity column chromatography were used for purification. Cell-ghosts were prepared from ^{125}I-labeled fusion competent NC4 cells, solubilized, centrifuged. The supernatant was then applied first to phenylsepharose column chromatography and second to Con-A affinity column chromatography. Fig.19A shows autoradiograms after application of SDS-PAGE electrophoresis, taken from each fraction of the purification steps described above. It is clear that gp138 is concentrated remarkably after application of the two affinity column chromatographies. There were approximately 30 bands detectable on autoradiogram of a phenylsepharose and Con-A binding fraction (Fig.19A-d) but no band located closely next to 138K band. This makes isolation of the molecule for purification easy.

Next, it was examined whether gp138 is detectable by Coomassie blue staining or not. It was not detectable in a ghost lysate fraction (Fig.19B-a), but detectable in a fraction after application of the two chromatographies (Fig.19B-b).

Function of gp138

In order to test function of gp138, furthermore, antiserum against gp138 was prepared. Cell-ghost lysate from cell fusion competent NC4 was applied first to phenylsepharose chromatography and then to Con-A affinity chromatography. Con-A binding fraction was eluted and used for SDS-PAGE electrophoresis. The gel was stained with Coomassie blue. The pieces of gel containing gp138 and the next band closest to gp138, which is approximately 120Kd in molecular weight, were cut out separately, mashed and injected each with adjuvant to rabbits. After the second injection, serums (FRA-5 for 120K protein and FRA-6 for gp138) were taken and Fab fragments of the each serum, were prepared. When fusion competent NC4 cells were pretreated with Fab of antiserum FRA-6, fusion activity of the cells was found to be inhibited. The inhibition was dependent on concentrations of the Fab, and fusion competence of NC4 cells was completely inhibited at a concentration of 2 mg/ml (Fig.20). On the contrary, the cells pretreated with Fab of FRA-5 did not lose their fusion competence.

Next, immuno-blotting analysis of cell-ghosts prepared from fusion-competent and -incompetent NC4 using FRA-6 were carried out. The band of gp138 was detectable in a fusion competent cell ghost lysate, but not in a fusion incompetent cell ghost lysate (Fig.21). In addition, it was found that there were approximately 15 bands, besides the gp138 band, detectable in the

both lysates. That is, antiserum FRA-6 cross-reacts with other molecules besides gp138. This could be happened, however, if antiserum against sugar chain of gp138 reacts with molecules which have the same kind of sugar chain.

Some characterizations of gp138

1). Isoelectric point : Isoelectric point of gp138 was determined by two dimension SDS-polyacrylamide gel electrophoresis (2D-PAGE). Solubilized fusion competent NC4 cells were immunoprecipitated with FRA-6, and applied for 2D-PAGE. Result showed that the isoelectric point of gp138 was pH 4.5-4.9 (Fig.22)

2). Lectin binding : Binding of Con-A and WGA to gp138 were previously shown. Here, binding activity of these lectins and the other lectins, LCA, E4-PHA, PNA, RCA60 and RCA120 were further investigated. Results indicated that only three lectins, Con-A, WGA and LCA bound to the molecule, but the other ones did not (Fig.23).

Discussion

Purification and some characterization of 138K protein which is involved in sexual cell fusion of D. discoideum were carried out in the present study. The 138K protein was found to be a glycoprotein, and its sugar chain bound to lectins, Con-A, WGA or LCA.

In D. discoideum, several cell surface proteins have been isolated and characterized. Most of them are glycoprotein. For example, a cell surface protein called gp80 which is playing an important role for cell-cell adhesion during aggregation stage of fruiting-body formation, is glycoprotein (Muller and Gerisch, 1978). Cell surface proteins which appear during migration stage, 95Kd and 150Kd in molecular weight, are also glycoproteins (Steinemann and Parish, 1980; Geltosky et al.,1979). Furthermore, proteins which appear in growth- and pre-aggregation-stage, 126Kd and 24Kd, respectively, are glycoproteins (Chadwick and Garrod, 1983; Knecht et al., 1987). In glycoproteins, sugar chains are known to be playing important roles for stability (Hori et al., 1988) or functions such as intercellular reactions of the molecule. For example, a mutant strain cells in D. discoideum having 80K protein without sugar chain which includes N-acetyl-glucosamine, show very weak adhesion (Gerisch et al., 1985; Bertholdt et al., 1985; Loomis et al., 1985).

From the above evidence, it was quite expectable that 138K protein had (a) sugar chain(s), and the sugar chain of 138K protein would play an important role in the sexual cell fusion. O'Day and Rivera (1987) found that formation of binucleate cells in a mixture of NC4 and V12, a parental strain of HM1, were inhibited remarkably by addition of Con-A or WGA, but not PNA, RCA60 (RCA-II) or Gorse. This result is agree with our present data that 138K protein bind to Con-A, WGA or LCA, but not to PNA, RCA60, RCA120 or E-$_4$PHA, and supports strongly our conclusion that gp138 is involved in the sexual cell fusion.

General Conclusion

In cellular slime molds alternate modes of development, either sexual or asexual, are known to result from responses to various environmental factors, such as excessive water, certain period of darkness and absence of phosphate ions in the medium (Blaskovics and Raper, 1957; Nickerson and Raper, 1973; Erdos et al., 1976; Chagla et al., 1980; McConachie and O'Day, 1986). The effects of these environmental factors in the stage of macrocyst development, 1) acquisition of fusion competence, 2) cells fusion, and 3) zygotic giant cell development into macrocyst, were examined using the synchronous development system, in Part I. In the present work, it was found that excessive water and bacteria were the conditions indispensable for the acquisition of fusion competence in the cells. Even in liquid culture if the supply of bacteria was not replenished, fusion competent cells quickly lost their fusion competence. However, on the contrary to the previous reports, phosphate ions had no effect to the any stage of macrocyst development. In addition, it was detected that calcium ions were essentially required for cell fusion.

During macrocyst development, switching from sexually fusion incompetent to competent in the cells and vice versa occurred depending on the factors described. These factors could change directly or indirectly the properties of cell surface, and

surface proteins of the cells might be involved in this change. When the fusion competent cells were treated with papain, cells found to lose their fusion activity. In part II, attempts were made to detect the papain-sensitive, surface proteins which involved in the sexual cell fusion. First, monovalent antibodies (Fab) were prepared from rabbit antiserum against fusion competent NC4 cells (FRA-4). It was shown that Fab of FRA-4 completely inhibited sexual cell fusion. On the cell surface of fusion competent NC4 cells, there were two specific antigenic proteins, 39k and 138k daltons in molecular weight, and only 138K protein found to be papain-sensitive. This molecule was capable of neutralizing the fusion inhibitory activity of the monovalent antibody. Thus, we concluded that 138K protein was the protein involved in the sexual cell fusion.

To study the function of 138K protein in the sexual cell fusion, purification and characterization of that molecule were carried out, in Part III. The protein was found to be a glycoprotein (gp138), and its isoelectric point was pH 4.5-4.9. The gp138 was partially purified using phenylsepharose chromatography and Con-A agarose chromatography, and gp138 band obtained by SDS-polyacrylamide gel electrophoresis, was cut out to inject into rabbits. Fab fragments of rabbit antiserum against gp138 (FRA-6) inhibited completely the sexual cell fusion. In addition, it was shown that gp138 bound to the

lectins such as WGA, Con-A and LCA, but didn't bind to E_4-PHA, PNA, RCA60 and RCA120.

Urushihara et al. (1988) have isolated another protein, called 70K protein, which involved in the sexual cell fusion. This protein might be involved in mutual recognition between cells, because it exists only on HM1 cell surface. It is reasonable to assume that fusion competent HM1 cells having 70K protein could be recognized from fusion competent NC4 cells which do not have this molecule. Since gp138 appears on both fusion competent NC4 and HM1 cell surface, it might participate in membrane fusion after mutual recognition. For future study of sexual cell interactions, author is now attempting to prepare monoclonal antibodies against gp138, and determine its N-terminal amino acids sequence. Furthermore, author would like to isolate the gene coding gp138 by using synthesized DNA probe. The mechanisms regulating the gp138 gene expression by the environmental factors and the precise role of its gene product in the sexual cell interactions are also intended to be investigated.

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Table 1. Macrocyst formation in sexual, asexual, bisexual and seifing (homothallic) wild isolate of *D. discoideum*

Haploid strains	mat A taster	mat a taster	self	reference
Sexual				
V12	+++	-	-	1
HM1	+++	-	-	(1)
WS567	++	-	-	1
NC4	-	+++	-	1
WS472	-	+++	-	1
Asexual				
WS576	-	-	-	2
WS269A	-	-	-	2
WS380B	-	-	-	2
Bisexual				
WS2162	++	+	-	2
WS112B	++	+	-	2
Homothallic				
AC4	+++	+++	+++	2
ZA3A	++	+++	+	2

Table 1 Macrocyst formation in sexual, asexual, bisexual and selfing (homothallic) wild isolates of D discoideum.

Macrocyst formation was assayed using strains WS583, HU1 and HU235 as mat A testers, and strains WS582 and HU89 as mat a testers.

+++ ; Strong mating reaction

++ ; Moderately strong mating reaction

\+ ; Weak mating reaction

\- ; No mating reaction

Reference

1 : Erdos, Raper and Vogen (1973)

2 : Robson and Williams (1980)

Table 2. Absorption of fusion-inhibitory activity of Fab by sliced polyacrylamide gels

Range of molecular weight (K dalton)	Fusion inhibitory activity		
	Exp.1	Exp.2	Exp.3
Gel #1 : (300 - 97)	11%	52%	38%
#2 : (97 - 65)	72%	100%	-
#3 : (65 - 42)	92%	96%	-
#4 : (42 - 33)	89%	85%	71%
#5 : (33 - 24)	92%	85%	-
#6 : (24 - 18)	84%	87%	-
#7 : (18 - Front)	96%	79%	-
Control	96%	100%	97%

Control gel slice contains no protein.

Figure legends

Figure 1. The sexual life cycle of D. discoideum. The cellular slime molds feed on bacteria and grow by fission. When bacteria are no longer present, amoeboid cells on a solid medium aggregate to form a fruiting-body consisting of spores and stalk cells. However, the haploid myxamoeboid cells of heterothallic strains in D. discoideum acquire fusion competence during growth under appropriate culture conditions and two opposite mating type cells fuse to produce a giant cell containing two nuclei or sometimes more. The giant cell subsequently develops into a macrocyst, enclosed in a thick fibrillar wall, the sexual structure of this organism. When two strains NC4 and HM1, are cultured together in the dark in liquid medium, a considerable number of macrocysts are produced within a few days.

Figure 2. Acquisition of fusion competence by NC4 cells cultured in liquid in the dark and light. They were taken from a stock SM agar plate and suspended in BSS containing bacteria. The cell suspension was shaken at 22°C in either light (○) or darkness (●). During cultivation, aliquots were examined at successive intervals for the percentage of fused cells.

Figure 3. Loss of fusion competence by NC4 cells on agar

plates. Fusion competent NC4 cells cultured in liquid in darkness for 15 hrs were transferred to SM agar plates, incubated at 22°C with bacteria in the dark and examined for fusion competence. Arrows indicate times of the transfer.

Figure 4. Effects of nutrition on acquisition and loss of NC4 cell fusion competence. Fusion competent NC4 cells suspended in BSS containing bacteria (●) maintained their fusion competence, while those suspended in BSS without bacteria (■) lost it gradually after 6 hrs incubation. Bacteria replenishment in BSS (↑) resulted in resumption of fusion competence (□).

Figure 5. Temperature effects on acquisition of fusion competence. Growth-phase NC4 and HM1 cells from SM agar plates were suspended separately in BSS containing bacteria and incubated at different temperatures (11°C - 28°C) in the dark. During incubation small sample amounts were assayed for cell fusion.

Figure 6. Temperature effects on cells fusion. Fusion competent NC4 and HM1 cells suspended at a 1 : 1 ratio in BSS were incubated at different temperatures (0°C - 30°C) in the light. Aliquots at certain intervals were examined for the

percentage of fused cells.

Figure 7. Effects of calcium ions on cell fusion. Fusion competent NC4 and HM1 cells were mixed and suspended in 50 mM KCl solutions containing various concentrations of $CaCl_2$ (0.025 mM - 5 mM) and assayed for the percentage of fused cells after 60 min of shaking at 22°C in the light.

Figure 8. Inhibition of cell fusion by Fab fragments of antiserum for fusion competent NC4 cells. A) Agglutinated cell masses produced after mixing fusion competent NC4 and HM1 cells suspended in BSS in equal volumes, and followed by incubation at 22°C for 30min on a shaker (120 rpm). B) Agglutinated cell masses were dissociated by introducing a final concentration 5mM EDTA into the suspension. Only fused cell masses (indicated by arrows) remained. C) Agglutinated cell masses of HM1 and NC4 cells treated with a final concentration 1mg/ml Fab for 15min at 22°C. D) Cells dissociated by the addition of 5mM EDTA to the cell suspension pre-treated with Fab (C). No fused cell masses could be found. Each bar indicates 40um.

Figure 9. Inhibition of cell-adhesion and cell-fusion by Fab fragments. A) Fusion competent NC4 cells pre-treated

with Fab at various concentrations, of 0.06, 0.13, 0.25, 0.5, 1 and 2 mg/ml (final concentration) for 15min at $0^{o}C$ were mixed with an equal number of fusion competent HM1 cells in BSS. After incubation for 30min at $22^{o}C$ on a shaker (120rpm), assay was made of inhibition of cell adhesion. B) Fab treated fusion competent NC4 cells were mixed with the same number of fusion competent HM1 cells in BSS. Five mM EDTA was added (a final concentration) and the percentages of fused cells and of inhibition of cell fusion were determined.

Figure 10. Inhibitory activity of Fab absorbed by intact cells. Small samples of Fab varying in concentration were absorbed by either fusion competent or incompetent NC4 cells at $0^{o}C$ for 1hr. Inhibitory activities of the Fab on cell fusion were then assayed. a) Fab absorbed by fusion incompetent NC4 cells. b) Fab absorbed by fusion competent NC4 cells.

Figure 11. Immuno-precipitate patterns of NC4 cells. Fusion competent- and incompetent- NC4 cells were surface labeled with ^{125}I, solubilized and incubated with antiserum for fusion competent NC4 cells. The Immuno-precipitates were washed and subjected to SDS-polyacrylamide gel electrophoresis. The gels thus obtained were exposed to X-ray film. a) Fusion incompetent NC4 cells incubated with the antiserum. b) Fusion competent NC4 cells incubated with serum. The numbers indicate

molecular weight standards in K daltons. 38K and 139K protein bands are indicated by arrows.

Figure 12. Fusion competence of NC4 cells treated with proteolytic enzymes. Fusion competent NC4 cells pre-treated with either trypsin or papain at various concentrations (10,30, 100,300 and 1000μg/ml) at 22°C for 30min, were assayed for fusion competence. A) and B) show changes in fusion competence following trypsin- and papain-treatment, respectively.

Figure 13. Immuno-precipitate patterns after proteolytic enzyme treatment. Fusion competent NC4 cells pre-treated with 500μg/ml of trypsin or papain for 30min at 22°C were washed and labeled with ^{125}I, solubilized and incubated with antiserum for fusion competent NC4 cells at 0°C for 30min. The Immuno-precipitates were washed and subjected to SDS-polyacrylamide gel electrophoresis. The gels were exposed to X-ray film. a) and b) indicate immuno-precipitate patterns following trypsin- and papain-treatment, respectively. c) shows immuno-precipitate pattern for no enzyme treatment, as a control.

Figure 14. Relation between the period required for acquisition of fusion competence and the time at which the 138K protein was first detected. A) Fusion incompetent NC4

cells, transferred to a liquid medium from an agar plate, were incubated at 22°C on a shaker (120 rpm). Small samples during the course of incubation were taken and assayed for fusion competence. B) Cells at certain intervals during the above liquid incubation (3, 6, 9, 12 and 15hr) were labeled with ^{125}I and their immuno-precipitate patterns were examined.

Figure 15. Inhibitory activity on cell fusion of Fab absorbed by either 4hr- or 15hr-liquid cultivated NC4 cells. Fab for fusion competent NC4 cells was incubated with 4hr cultivated fusion incompetent NC4 cells (a) or 15hr cultivated fusion competent NC4 cells (b) at 0°C for 30min. Their inhibitory activity on cell fusion were then assayed.

Figure 16. Immuno-precipitation patterns of NC4 and HM1 cells. Fusion competent NC4 and HM1 cells were surface labeled with ^{125}I, solubilized and incubated with antiserum for fusion competent NC4 cells. The immuno-precipitates were washed and subjected to SDS-polyacrylamide gel electrophoresis. The gels were exposed to X-ray film. a) Fusion competent NC4 cells, b) fusion competent HM1 cells. 138K protein bands are indicated by arrows.

Figure 17. Absorption of inhibitory activity of antiserum FRA-4 with intact cells and cell ghosts. Fab of FRA-4,

antiserum for fusion competent NC4 cells, were prepared, mixed with either intact cell (●) or cell-ghosts (O) of fusion competent NC4, and placed on ice for 1hr. Each Fab was isolated by centrifugation and their inhibitory activities on sexual cell fusion were estimated by inhibition assay of cell fusion.

Figure 18. Fusion competent NC4 cells labeled with ^{125}I were solubilized, and applied to WGA (b, c) or Con-A (d, e) affinity chromatography. After washing off the non-binding fractions (b, d), the binding fractions were eluted with 0.2M N-acetyl-D-glucosamine (c), or 0.2M metyl-α-D-mannopyranoside (e). Each fraction was subjected to SDS-PAGE and the gel was exposed to X-ray Film. Arrow indicates 138K protein band.

Figure 19. Purification of gp138. A) ^{125}I labeled cell-ghosts were solubilized and applied first to phenylsepharose column chromatography. Phenylsepharose binding fraction was concentrated and applied next to Con-A affinity column chromatography. Each fractions of cell ghosts (a), phenylsepharose binding (b), Con-A non-binding (c) and Con-A binding (d) were applied separately to SDS-PAGE electrophoresis, and the gel was exposed to x-ray films. Arrow indicates gp138 band.

B) Fractions of solubilized cell-ghosts (a) and phenylsepharose and Con-A binding (b) were applied SDS-PAGE. The gel was then stained with Coomassie Brilliant blue.

Figure 20. Effect of antiserum FRA-5 and FRA-6 on sexual cell fusion. Fusion competent NC4 cells were pretreated with different concentrations of Fab of FRA-5 (O) and FRA-6 (●), antiserum for 120K protein and gp138, and mixed with fusion competent HM1 cells. Inhibitory effects of the serums on sexual cell fusion were estimated by inhibition assay of cell fusion.

Figure 21. Immuno-blotting of fusion incompetent and competent cell-ghosts by antiserum FRA-6. Cell-ghosts from fusion incompetent (a) and competent (b) NC4 cells were lysed and applied to SDS-PAGE. Separated proteins were transferred to nitrocellulose sheet and immuno-blotted with antiserum for gp138.

Figure 22. Isoelectric point of gp138. Fusion competent NC4 cells labeled with ^{125}I, were solubilized and incubated with FRA-6. The immuno-precipitate was washed and applied for 2D-PAGE. The gel was then exposed to X-ray film. Arrow indicates spot of gp138.

Figure 23. Lectin binding of gp138. Fusion competent NC4 cell-ghosts were solubilized, immunoprecipitated with FRA-6, and applied to SDS-PAGE electrophoresis. Separated proteins were transferred to a nitrocellulose sheet and stained with HRP conjugated lectins, HRP-Con-A (a), HRP-WGA (b), HRP-LCA (c), HRP-E_4PHA (d), HRP-PNA (e), HRP-RCA60 (f), HRP-RCA120 (g). Arrow indicates gp138 band.

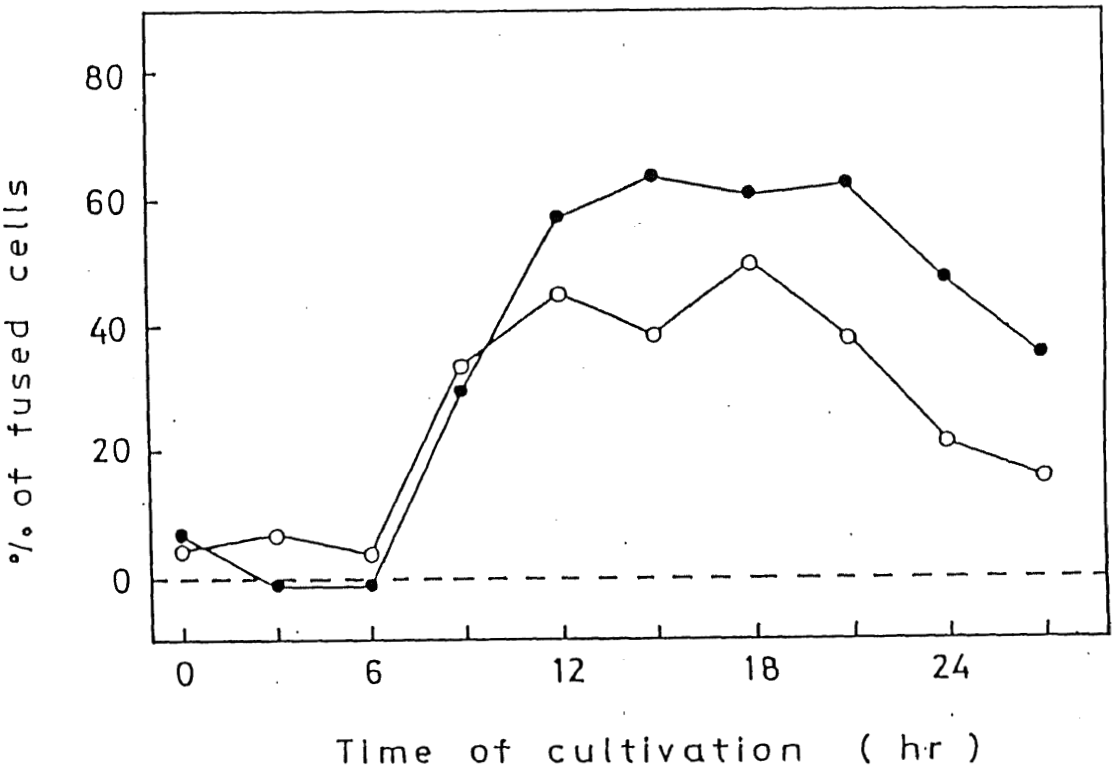

Fig. 2

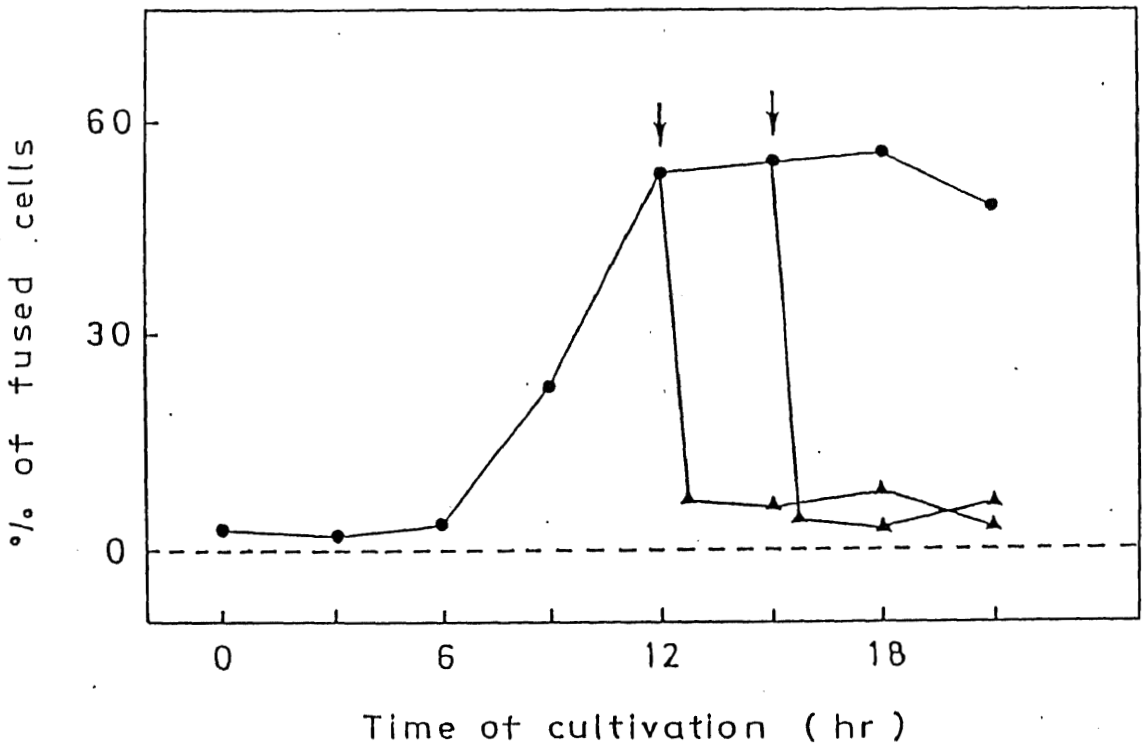

Fig. 3

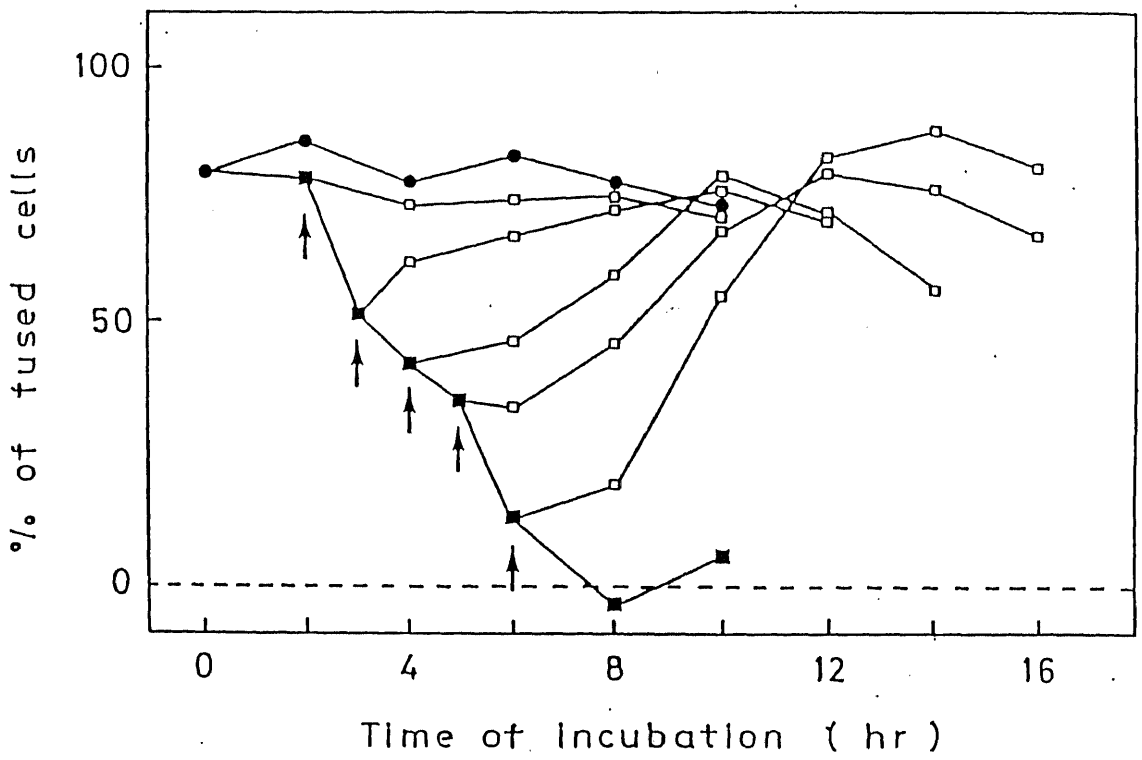

Fig. 4

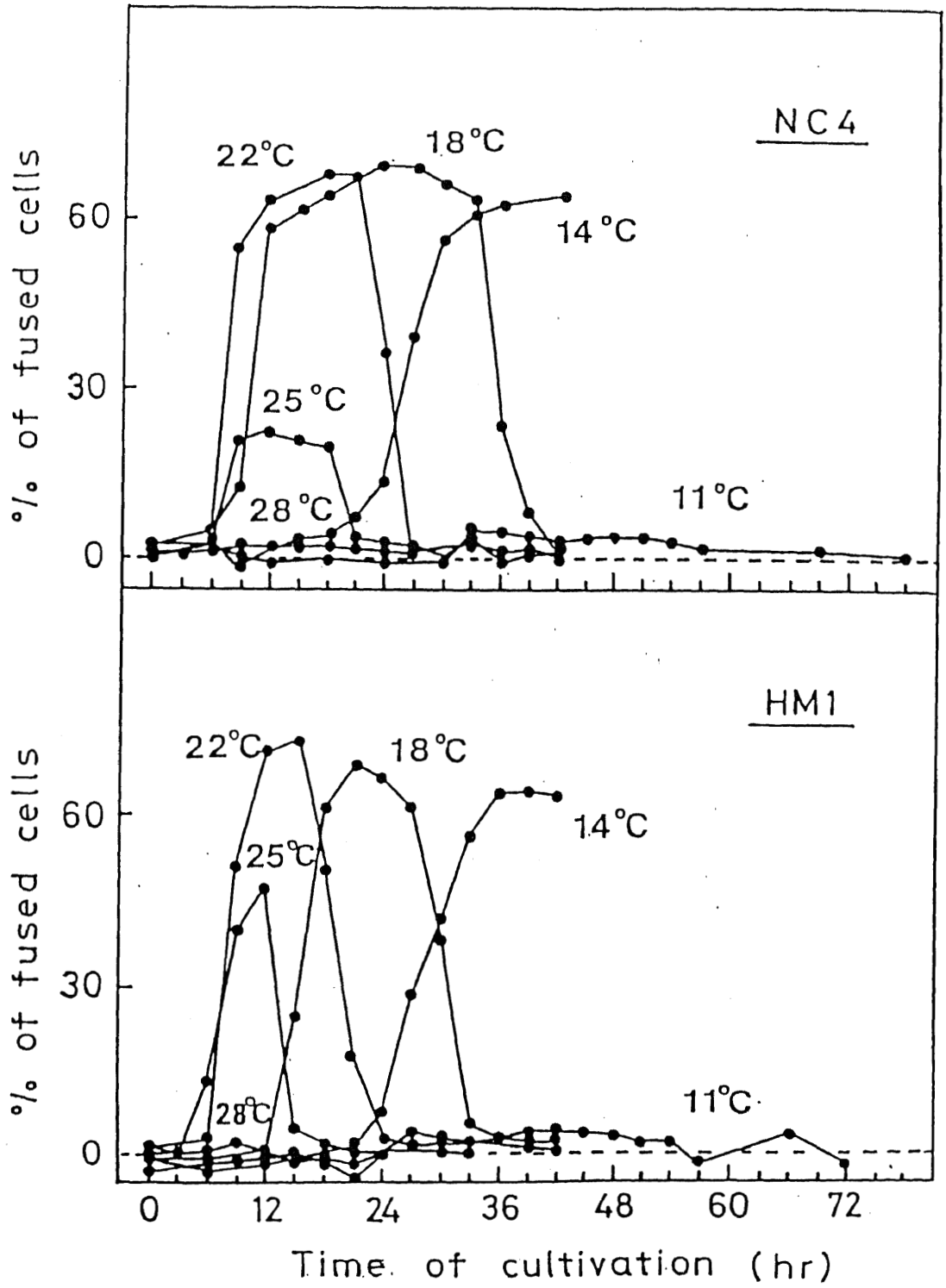
NC4
22°C
18°C
14°C
25°C
28°C
11°C
% of fused cells
60
30
0
HM1
22°C
18°C
14°C
25°C
28°C
11°C
% of fused cells
60
30
0
0
12
24
36
48
60
72
Time of cultivation (hr)

Fig. 5

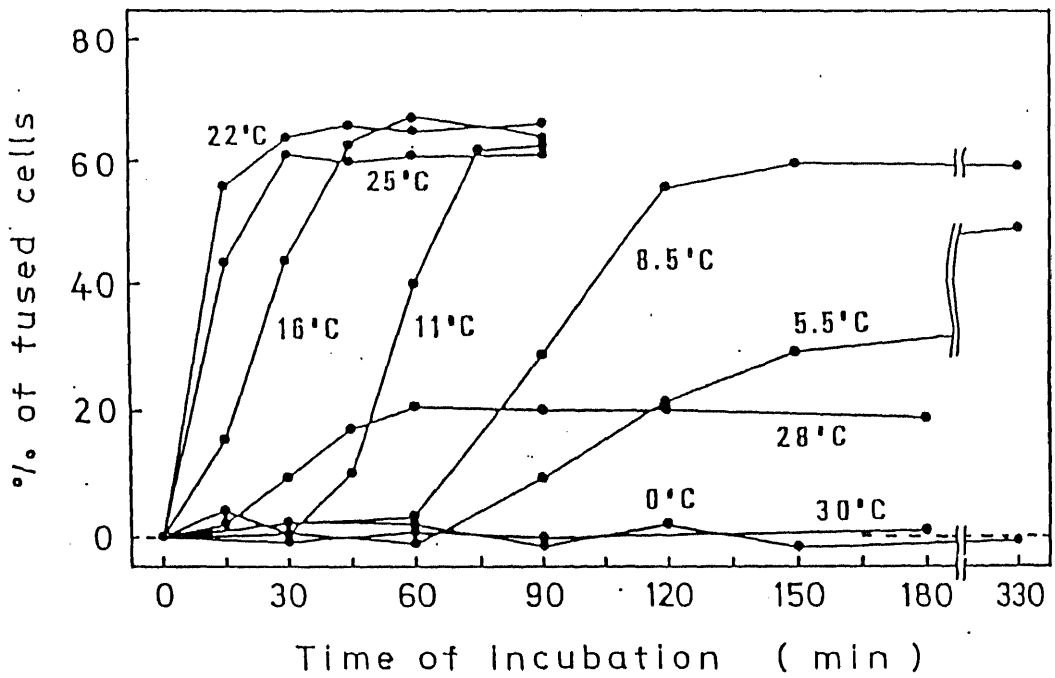
80
60
40
20
0
% of fused cells
22°C
25°C
16°C
11°C
8.5°C
5.5°C
28°C
0°C
30°C
0
30
60
90
120
150
180
330
Time of Incubation (min)

Fig. 6

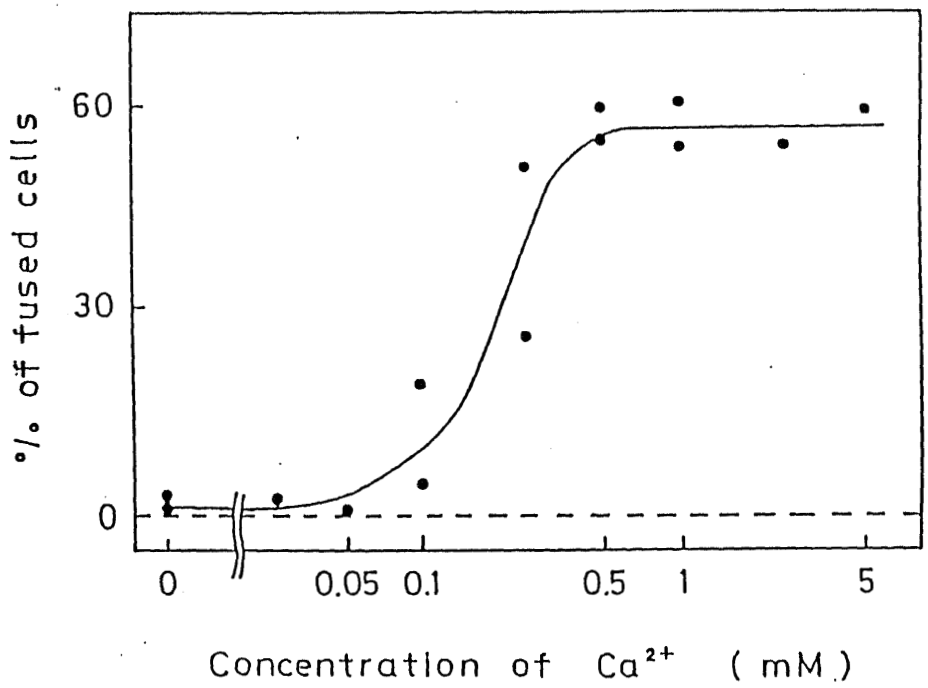

Fig. 7

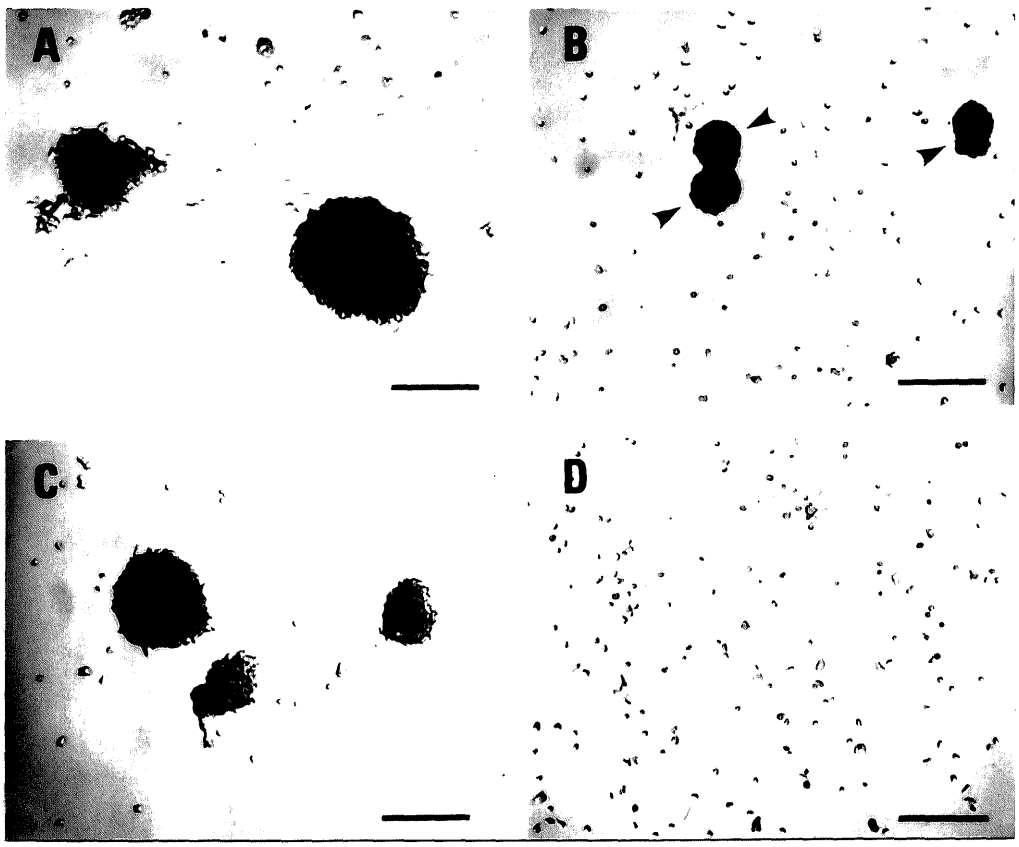

Fig. 8

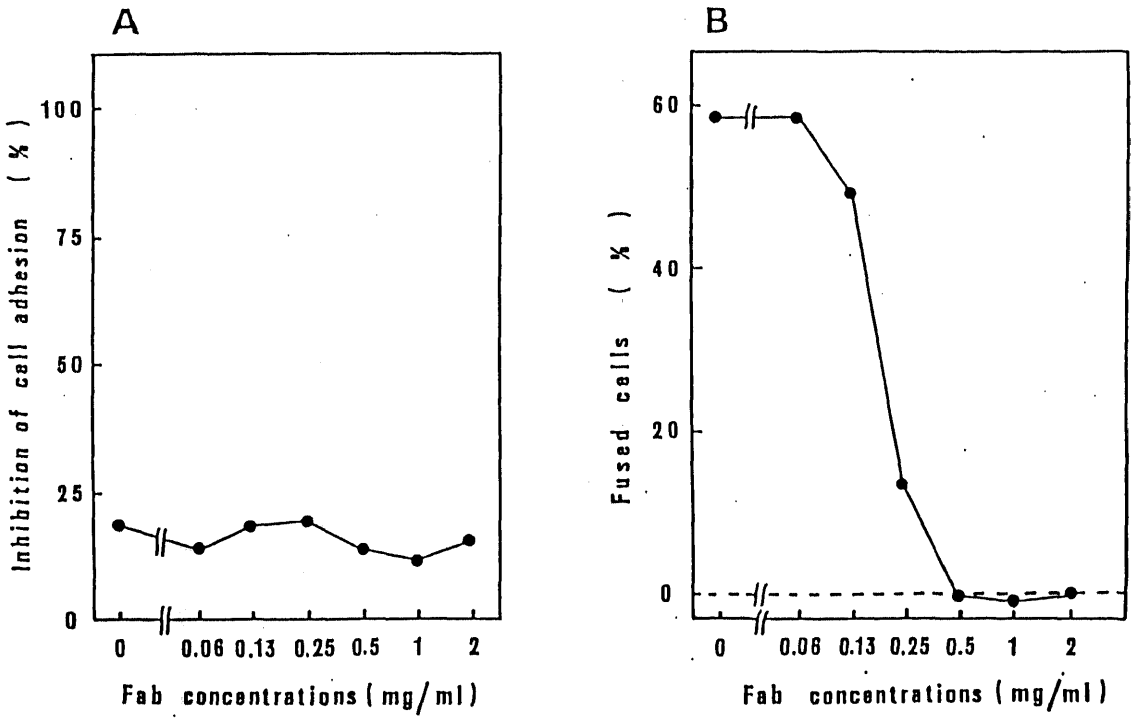
A
Inhibition of cell adhesion (%)
100
75
50
25
0
0 0.06 0.13 0.25 0.5 1 2
Fab concentrations (mg/ml)
B
Fused cells (%)
60
40
20
0
0 0.06 0.13 0.25 0.5 1 2
Fab concentrations (mg/ml)

Fig. 9

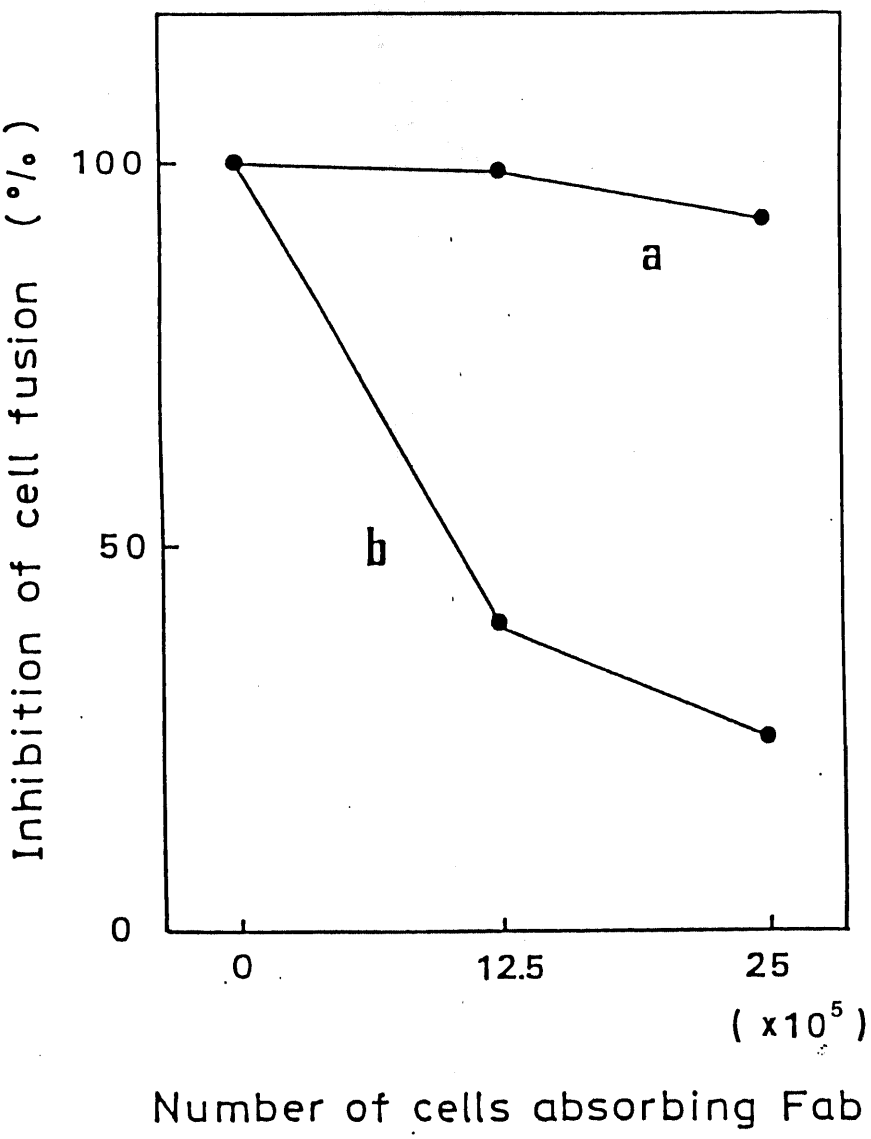
Inhibition of cell fusion (%)
100
50
0
a
b
0
12.5
25
(x10^5)
Number of cells absorbing Fab

Fig. 10

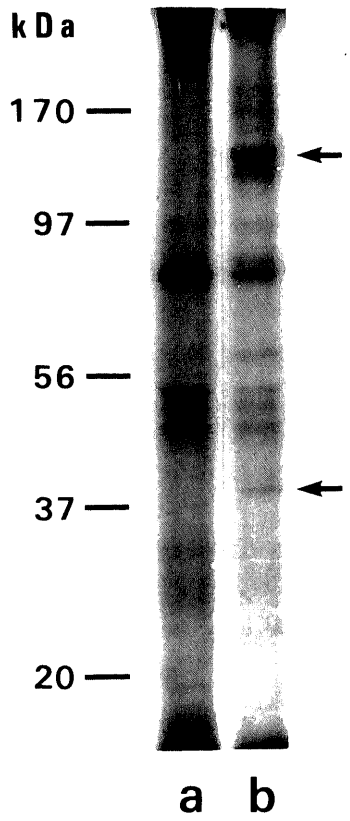

Fig. 11

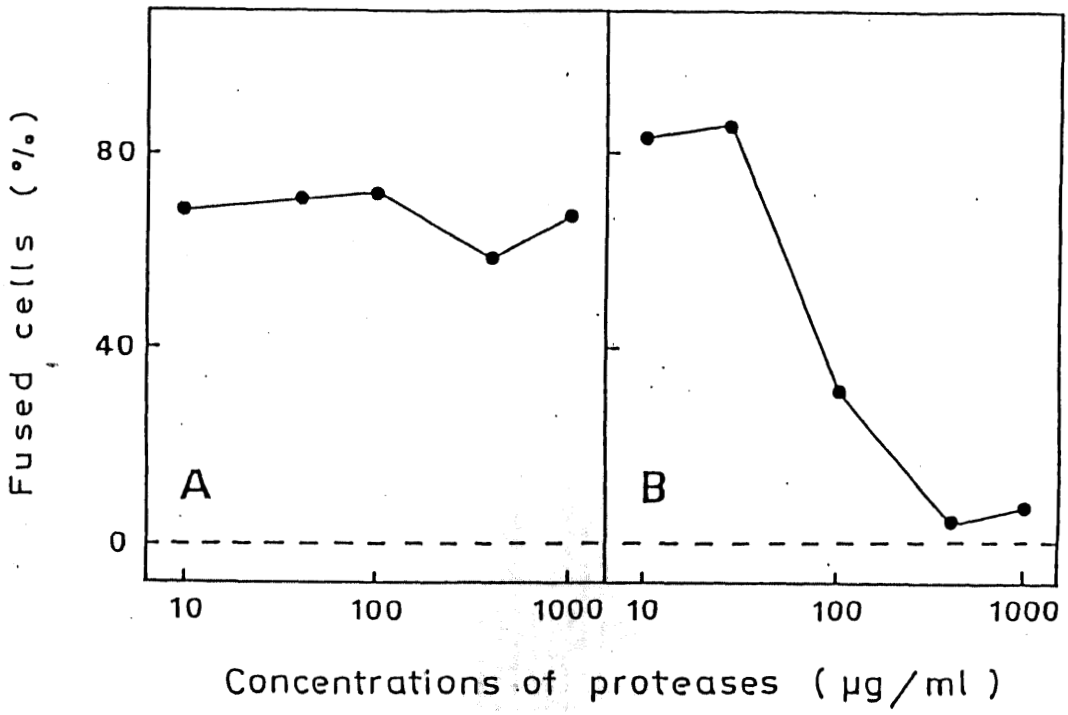
Fused cells (%)
80
40
0
A
B
10
100
1000
10
100
1000
Concentrations of proteases (μg/ml)

Fig. 12

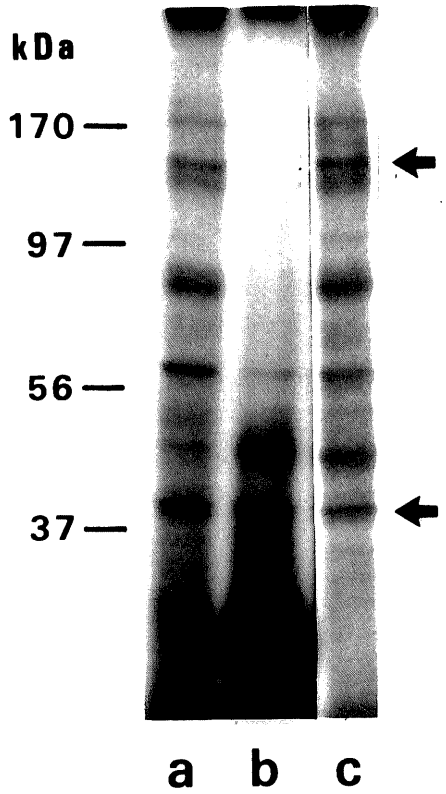

Fig. 13

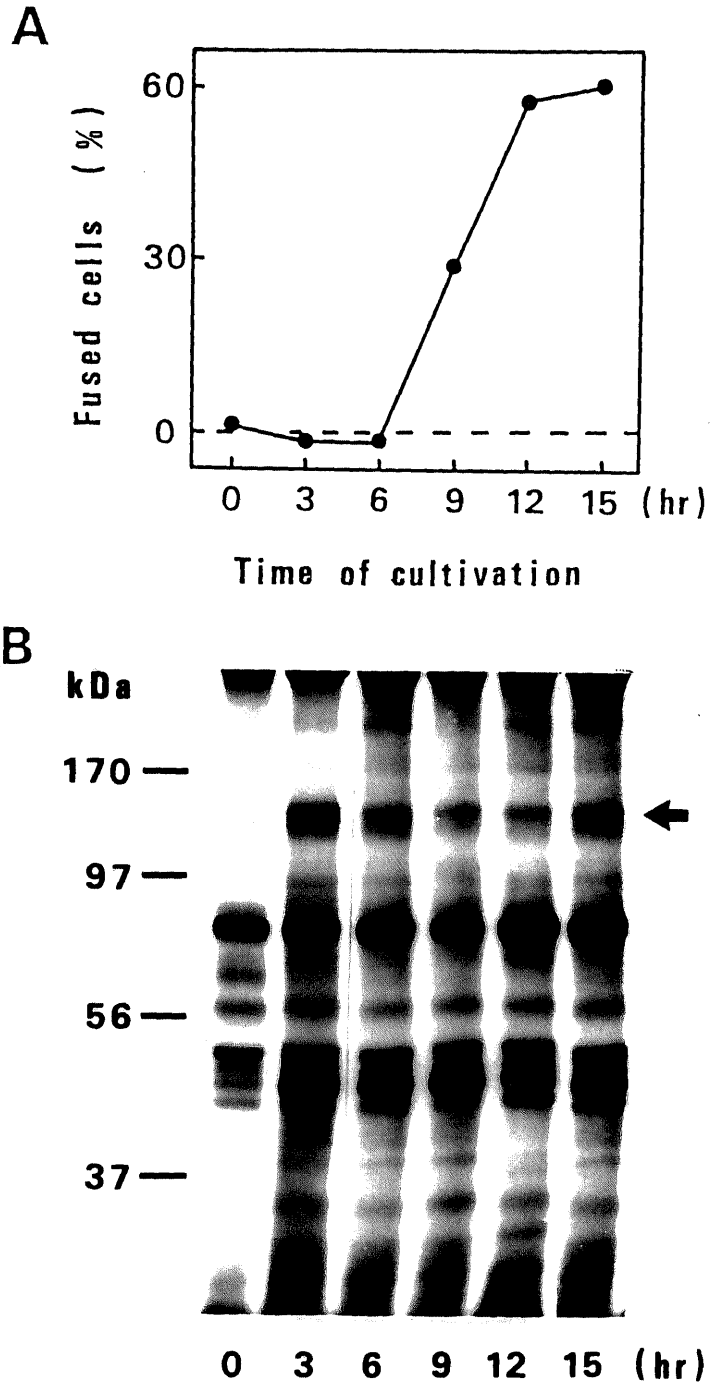
A
60
30
0
Fused cells (%)
0 3 6 9 12 15 (hr)
Time of cultivation
B
kDa
170
97
56
37
0 3 6 9 12 15 (hr)

Fig. 14

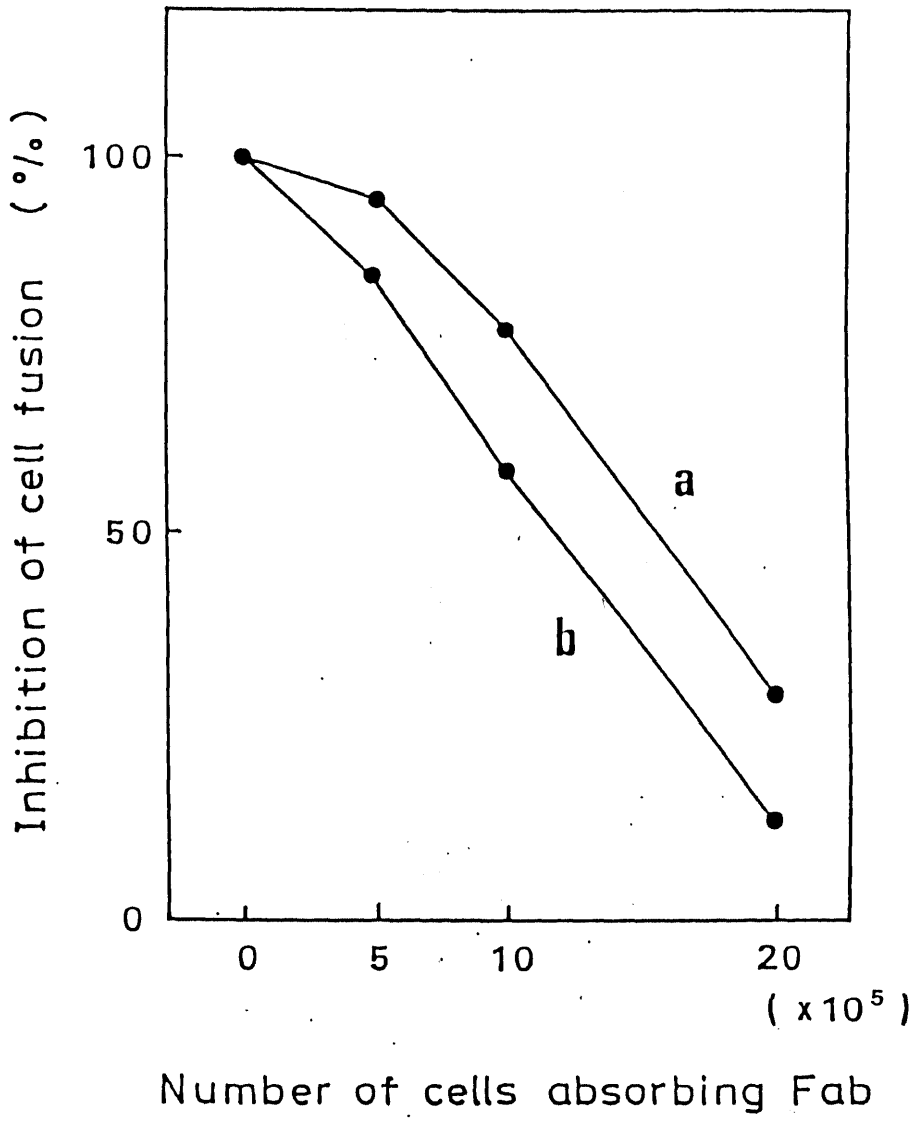
Inhibition of cell fusion (%)
100
50
0
a
b
0
5
10
20
(x10^5)
Number of cells absorbing Fab

Fig. 15

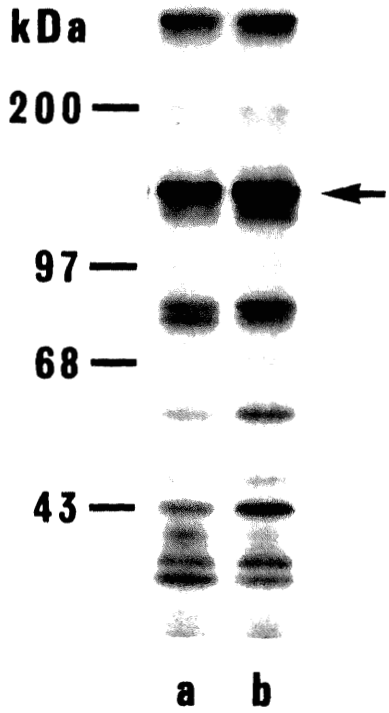

Fig. 16

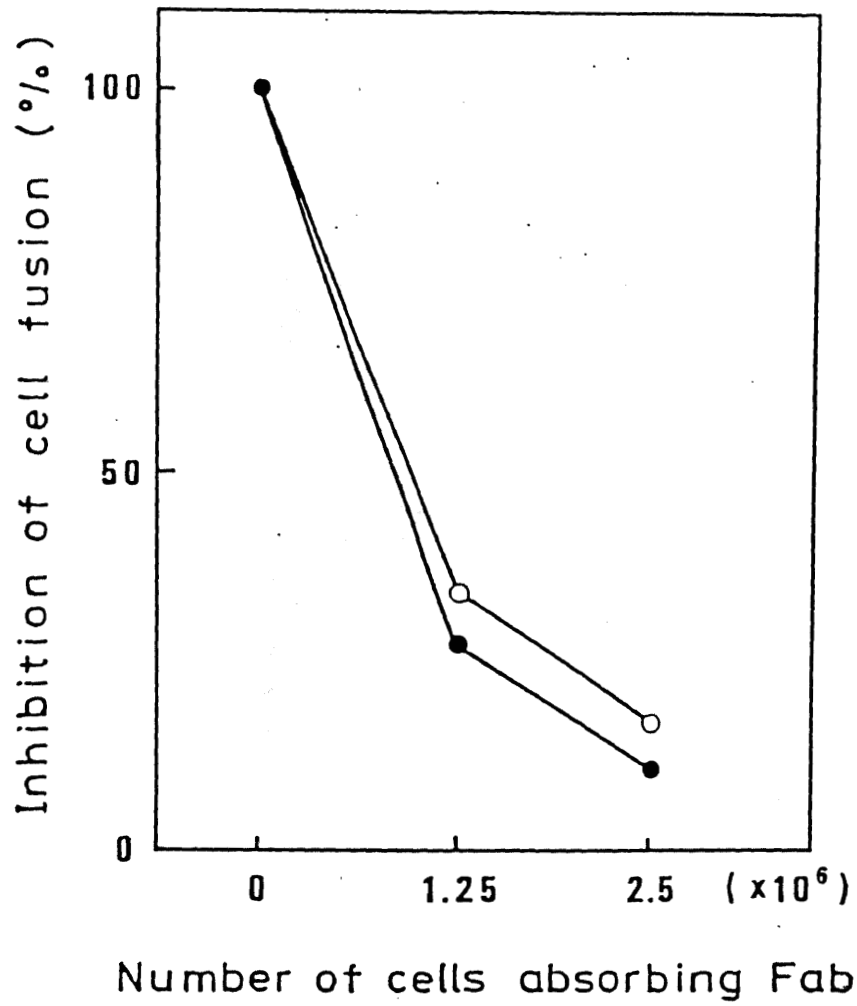
Inhibition of cell fusion (%)
100
50
0
0
1.25
2.5
(x10^6)
Number of cells absorbing Fab

Fig. 17

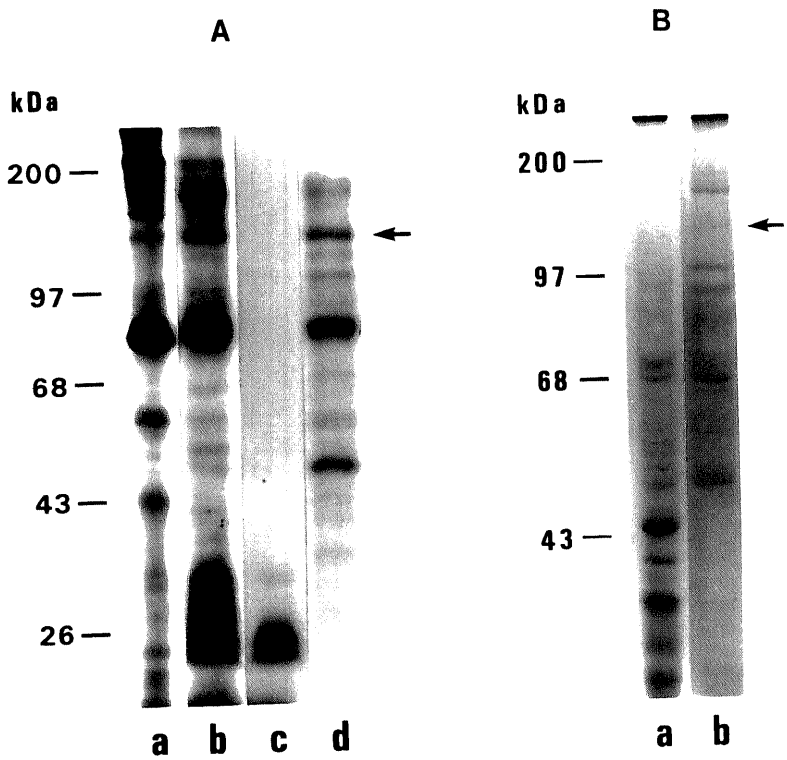

Fig. 19

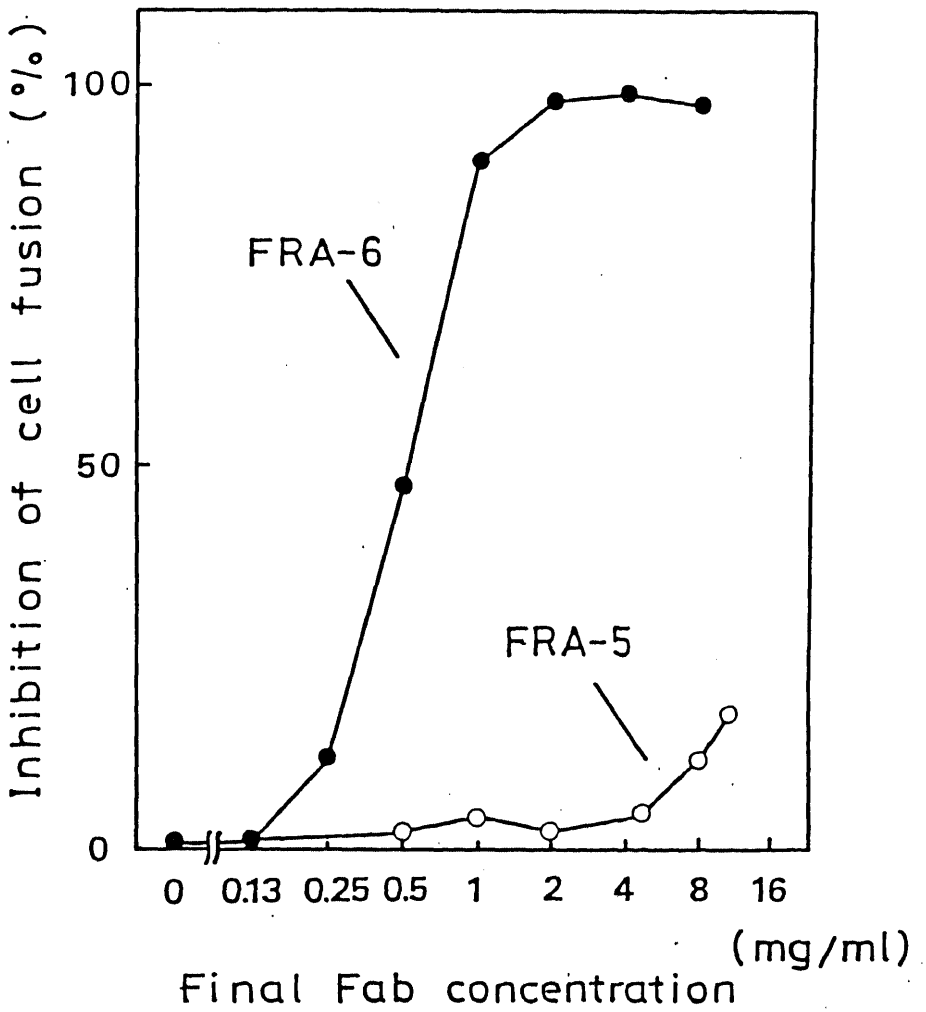
Inhibition of cell fusion (%)
100
50
0
FRA-6
FRA-5
0
0.13
0.25
0.5
1
2
4
8
16
(mg/ml)
Final Fab concentration

Fig. 20

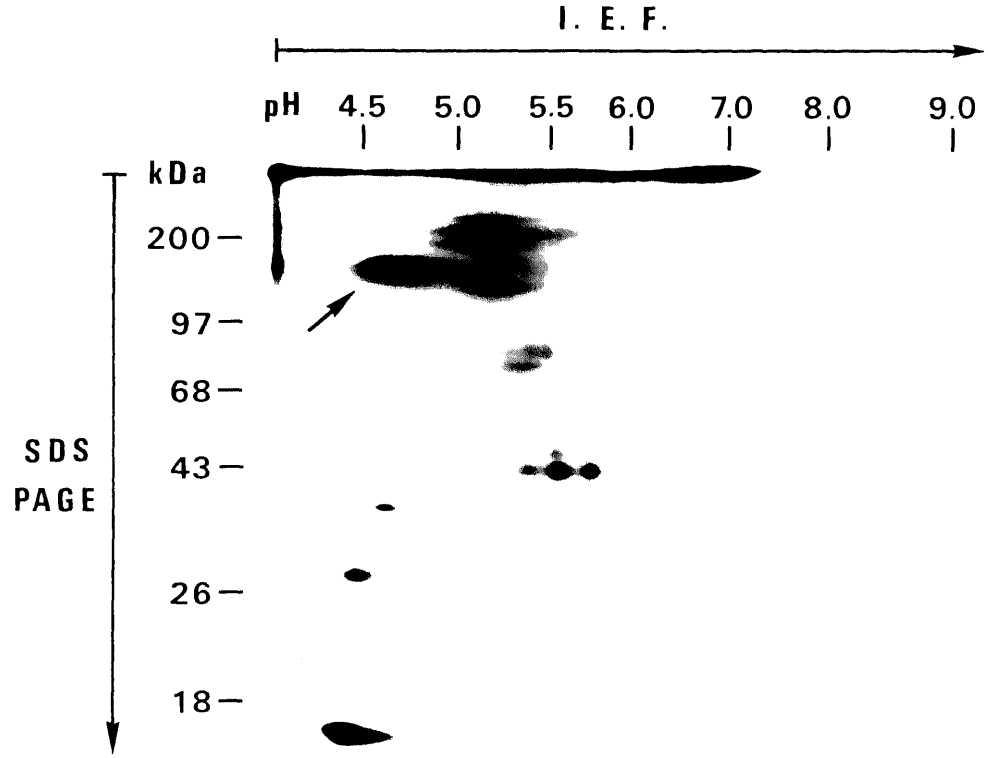

Fig. 22

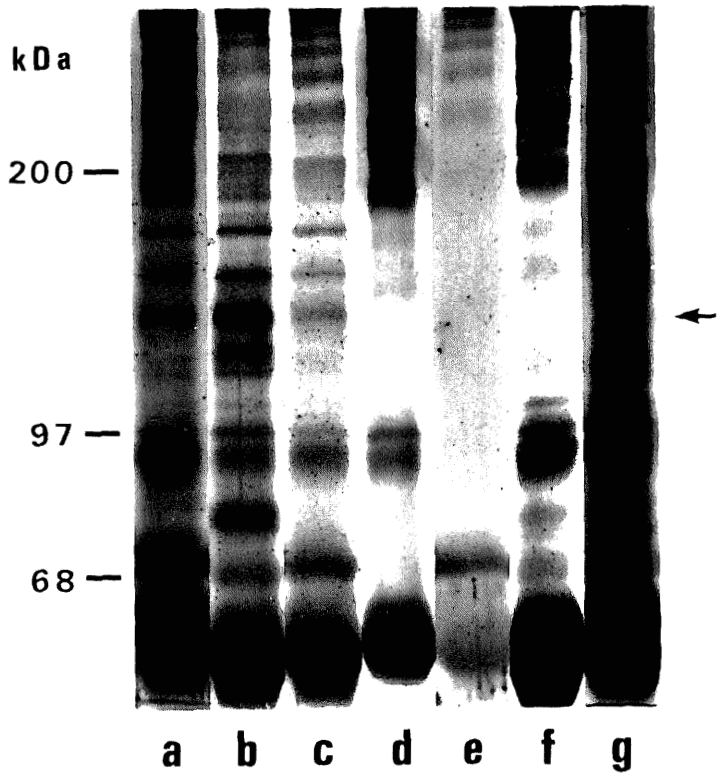

Fig. 23