Title: Factors Promoting Cell Aggregation in Conditioned Medium from CHL 36 cells.

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SUMMARY

From serum-free conditioned medium from the culture of CHL 36 cells, five factors which promote cell aggregation were found and characterized. Four of them, i.e., FGI, FQI, FQII and FQIII were purified by gel-filtration of Sephadex G-200 and ion-exchange chromatography of OAE-sephadex (A-50). The molecular weights of these were estimated by SDS-polyacrylamide gel electrophoresis, 13.3×10^4, 5.6×10^4, 8.1×10^4 and 6.3×10^4 daltons, respectively. Three factors, FQI, FQII and FQIII, could be inactivated by heat-treatment for 15 min at 100°C or treatment with trypsin. The factor FGI was stable during the heat-treatment. Treatment with trypsin cleaved the FGI factor into small fragments without losing activity to promote cell aggregation. The FGI factor can be inactivated by treatment with pronase. The fifth factor was found in the void fraction by Sephadex G-200 column chromatography. The activity promoting cell aggregation of this factor was lost after treatment with hyaluronidase, but not with trypsin or pronase. This factor was heat-stable. These suggest that this factor is reasonably assumed to be composed of acid mucopolysaccharide(s). Each factor promoted cell aggregation to different extent from another with several cell types, such as GMK, HeLa S3, L5178Y, 3Y1l0, liver cells and neural retina cells from chick embryos. Possible mechanism of these findings are discussed.

INTRODUCTION

The surfaces of animal cells are thought to play a dominant role in various complex phenomena, including cell recognition and adhesion (Moscona, A. A. and Moscona, M. H., 1952), contact inhibition of motility (Yamada, K. M. et al., 1976) and growth (Igarashi, Y. and Yaoi, Y., 1975), morphogenesis (Towne, P. L. and Holtfreter, J., 1955) and metastasis (Nicolson, G. L., 1975), though the molecular details underlying these phenomena are unknown. Above all, the mechanism of cell-to-cell adhesion and sorting-out has received a great deal of attention. In spite of many extensive studies, the basic mechanism of cell-to-cell adhesion has not been understood and it has not been determined whether the specificity of cell adhesion in higher animal cells is due to specific macromolecules (Moscona, A. A., 1963) or quantitative differences of some non-specific macromolecules (Steinberg, M. S., 1964). Studies aimed at isolation or identification of the surface macromolecules involved in the cell-to-cell adhesion are thought to be requisite to clarification of these points.

To study the problems with a more simplified and reproducible system, we employed an established cell line, CHL 36 derived from Chinese hamster lung cells. Using cell lines instead of embryonic cells, we can work with extremely homogeneous cell population, obtain a good cell viability and readily manipulate culture conditions. The conditioned medium of CHL 36 cells showed high level of activity promoting cell aggregation. In addition, we could observe a sorting-out phenomenon between a glucosamine-requiring mutant of CHL 36 (Onoda, T. and Matsushiro, A. 1972) and the non-requiring parent CHL 36. Thus, we tried to purify the factors promoting cell-to-cell adhesion from medium

conditioned with CHL 36.

Experiments in this report show that five factors promoting cell aggregation were identified in serum-free conditioned medium of CHL 36.

MATERIALS AND METHODS

Cells

Established line cells were passaged with 0.25% (w/v) trypsin and cultured in Eagle's minimal essential medium (MEM) containing 10% (v/v) calf serum.

Cells used were as follows: CHL 36 established from Chinese hamster lung cells, 3Y110 from rat whole embryo cells, GMK from green monkey kidney cells, L5178Y from mouse lymphoma, HeLa S3 from human cervix carcinoma, primary neural retina and liver cells from 10-day chick embryos.

Preparation of Conditioned Medium

Cells were cultured in glass bottles to confluent stage. Following the medium change at confluent stage and incubation for 24 hr in fresh medium, cells were washed four times with Dulbecco's phosphate-buffered saline (Ca^{++}, Mg^{++} free) (PBS(-)) to remove the remained calf serum and cultured in serum-free MEM at 37°C for 3 days. The media were collected, filtrated through glass fiber filter (Whatman GF/F) and concentrated in cellulose tubings (Union Carbide Co.) by negative pressure dialysis.

Purification of Cell-aggregation Promoting Factors

Concentrated conditioned medium (crude factor) prepared from CHL 36 was used for purification studies. Crude factor was applied on Sephadex G-200 column (3 x 100 cm) (Pharmacia Fine Chemicals) and eluted with PBS(-). Active fractions on Sephadex G-200 column were further purified by gel-filtration of Sephadex G-200 (3 x 100 cm or 2 x 65 cm) and ion-exchange chromatography of QAE Sephadex (A-50) (Pharmacia Fine Chemicals) (2 x 12 cm) or Sepharose 4B (2 x 65 cm) chromatography. Each sample for gel-filtration was concentrated in cellulose tubing by negative pressure dialysis,

centrifuged at 3,000 rpm for 30 min and the supernatant was applied. Each sample for QAE Sephadex column chromatography was diluted by 10 mM phosphate buffer (pH 7.5) for adjusting the ionic-strength to that of initial buffer, applied to QAE Sephadex column and eluted with linear gradients of NaCl in 10 mM phosphate buffer (pH 7.5). The ionic-strength of each fraction was measured by a conductive meter and was expressed by mMHO. The concentration of macromolecules in each fraction was measured by optical absorption at 225 nm (Murphy, J. B. and Kies, M. W., 1960).

Cell-aggregation promoting activities were assayed by using CHL 36 cells. The activity in each fraction through a gel-filtration was tested directly. However, in the case of QAE Sephadex column, the activity was examined after dialysis of each fraction against PBS(-) for more than 30 hr for eliminating the effects of ionic-strength on cell aggregation.

Cell-to-cell Binding Assay

After established line cells were passaged with 0.25% (w/v) trypsin and cultured at 37°C for 40 hr, cells at growing stage were treated with 0.12% (w/v) trypsin at 37°C for 30 min and washed four times with PBS(-) containing 1% (v/v) calf serum. After these treatments, cells were suspended in Ca^{++}-free MEM bufferized with 10 mM Hydroxy-ethylpiperazine ethanesulfonic acid [HEPES] (pH 7.5) and the medium finally contained 1 mM $CaCl_2 \cdot 2H_2O$, 1% (w/v) glutamine, 5% (v/v) dialyzed calf serum and an appropriate volume of sample solution. Each test was performed in duplicate and the cell concentration was adjusted at 30×10^4 cells per 1.5 ml medium in each 10-ml Erlenmeyer flask. All flasks were shaked on a gyratory shaker (70 rpm) at 37°C for 24 hr.

Tissues from chick embryos were treated with 0.25% (w/v) trypsin and prepared as described above.

The level of cell aggregation was determined as follows. All flasks were arranged in order of the size of cell aggregate by microscopical and macroscopical observations, and numbered. These numbers show the level of cell aggregation. Specific activity of each factor was estimated as follows. When serial-diluted factor could not promote the level of cell aggregation, its protein concentration (μg/ml) (Lowry, O. H., et al., 1951) at the end point of activity was determined. The protein concentration of the purified factor at the end point was divided by that of crude factor at the end point. And the specific activity was represented by the reciprocal of the quotient, i.e., the specific activity could be shown as follows.

$$\text{specific activity} = \frac{\text{protein concentration of crude factor at the end point}}{\text{protein concentration of purified factor at the end point}}$$

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE (12% gel) was performed on slab gel (Ostrove, S. and Maizel, J., 1973). Samples were precipitated with 5% (w/v) cold trichloroacetic acid followed by precipitation with cold aceton. The pellets were dried up and solubilized at 30°C for 12 hr with sample buffer containing 1% (w/v) sodium dodecyl sulfate (SDS), 10% (w/v) glycerol, 0.01% (w/v) phenol red and 0.15 M 2-mercaptoethanol in 0.05 M Tris-HCl buffer (pH 8.2), and boiled for 1.5 min. Slab gels were run at 100 V until phenol red marker reached the end of the gels (about 5 hr). Gels were fixed and stained for proteins with 0.2% (w/v) Coomasie Brilliant Blue, 50% methanol and 7% acetic acid in distilled water and destained by 50% methanol and 7% acetic acid in distilled water.

Estimation of Molecular Weight

On Sephadex G-200 column (2 x 65 cm), molecular weight was estimated (P. Andrews, 1964)by using standard markers as follows: 2 x crystallized trypsin (2.3 x 10^4 daltons) (T'sin), monomer (6.8 x 10^4 daltons) (HSA) and dimer (13.6 x 10^4 daltons) [$(HSA)_2$] of human serum albumin. Void fraction was estimated by Blue Dextran (BD). Phenol red (PR) was used as an internal marker. These markers were eluted with PBS(-).

In the case of SDS-polyacrylamide gel electrophoresis (12% gel), following standard markers were used (Weber, K. and Osborn, M., 1969): myosin (20 x 10^4 daltons), β-galactosidase (13 x 10^4 daltons), human serum albumin (6.8 x 10^4 daltons), ovalbumin (4.3 x 10^4 daltons) and lysozyme (1.43 x 10^4 daltons). Each factor was run with these markers on slab gels at the same time.

RESULTS

Purification of Cell-aggregation Promoting Factors

In the case that most of cells in suspension are single cells, there are methods appropriate to quantitative measurement of cell aggregation level (Curtis, A. S. G., 1969). However, in the case of most cells being cell aggregates, there is only a less quantitative method for measurement of aggregate size (Moscona, A. A., 1963). The cell-aggregation promoting activity of conditioned medium showed the latter property. Therefore, all flasks were arranged in order of aggregate size and numbered from the flask showing the lowest cell-aggregation level. Since this number corresponded to the size of cell aggregate, the level of cell aggregation could be measured semi-quantitatively. Using this method, we could readily find the chromatographic fractions having maximal promotive activities of cell aggregation.

Cell-aggregation promoting factors in serum-free conditioned medium of CHL 36 were purified as shown in Fig. 1. The conditioned medium, i.e., crude factor (85 mg protein from 8 litre of conditioned medium obtained from the culture of ca. 5×10^{10} cells) was fractionated on Sephadex G-200 column (Fig. 2). There exist three peaks of the cell-aggregation promoting activity; G 0, G I and G II fractions. These three fractions were pursued by further chromatography through the process shown in Fig. 1.

CI fraction: The first activity peak (G I fraction) in included fraction on Sephadex G-200 column was further purified by re-chromatography on Sephadex G-200 column, and fractionated three times by QAE Sephadex (A-50) chromatography. There was obtained a single absorption peak on the third QAE Sephadex column (Fig. 3a). This purified substance was once applied

to Sephadex G-200 column in order to check the deviation in the eluted position and in cell-aggregating activity (Fig. 3b). One sharp absorption peak was seen at the region corresponding to molecular weight of $12.0 \pm 1.0 \times 10^4$ daltons and a small shoulder was observed at the region corresponding to the void volume. Cell-aggregating activity was seen at both regions. This shoulder was thought not to be some contaminated other materials, but the aggregated one resulted from aggregation of the macromolecules at the main peak region, since this small shoulder could be observed in the same proportion even when the main peak substance was re-chromatographed on Sephadex G-200 column and the SDS-PAGE of the small shoulder substance at the void volume showed a single band, which corresponded to the single band of the main peak substance (Fig. 6a). This single-banded region corresponded to $13.3 \pm 0.5 \times 10^4$ daltons when calculated from the electropherogram (Fig. 6 b and Table 1). This purified factor from G I fraction was named FG I.

G II fraction; The second activity peak in the included volume of Sephadex G-200 column yielded three active fractions designated as QAE I, II and III fraction by QAE Sephadex (A-50) chromatography (Fig. 4). From the fraction of QAE I, single absorption peak was obtained by rechromatography of QAE Sephadex (A-50) (Fig. 5a) and further its active fraction was applied to Sephadex G-200 column in order to examine the molecular weight of active macromolecule and whether the elution pattern at 225 nm corresponds to the cell-aggregating activity (Fig. 5b). Fig. 5b shows that the single activity peak closely corresponding to the absorption pattern in the included volume of Sephadex G-200 column is accompanied with the active substance at the void fraction. This substance eluted at the void fraction could be

considered as the aggregated macromolecules of the substance at the single activity peak in the included volume. This purified factor from QAE I fraction was named FQ I and estimated to correspond to a molecular weight of $7.0 \pm 0.5 \times 10^4$ daltons on Sephadex G-200 column. SDS-PAGE of FQ I showed a single band corresponding to the molecular weight of $5.6 \pm 0.4 \times 10^4$ daltons (Fig. 6b).

From active fractions of QAE II and III, macromolecules having cell-aggregating activity were further purified through similar procedures as described in the case of FQ I (Fig. 1) and these factors were designated as FQ II and FQ III, respectively. Molecular weight of FQ II and FQ III were estimated to correspond to 6.7 ± 0.5 and $5.5 \pm 0.6 \times 10^4$ daltons, respectively, on Sephadex G-200 column. By SDS-PAGE, FQ I and FQ II were estimated to correspond to the molecular weight 8.1 ± 0.3 and $6.3 \pm 0.2 \times 10^4$ daltons, respectively (Fig. 6b). On Sephadex G-200 column of FQ II or FQ III, cell-aggregation promoting activity was observed similarly at both the excluded and the included fractions as in the case of FG I and FQ I.

G 0 fraction; The activity peak named G 0 fraction existed in the excluded fraction on Sephadex G-200 column (Fig. 2). The G 0 fraction was further fractionated by Sepharose 4B chromatography. The major activity of cell aggregation was observed in the slightly included fractions (Fig. 7a).

It was reported that some cell-aggregation promoting substance, which had molecular size from 10^5 to 5×10^6 daltons, might be acid mucopolysaccharide resistant to pronase treatment (Pessac, B. and Defendi, V., 1972). In order to examine whether active substances in G 0 fraction consists of some pronase-resistant substance or only aggregates derived from FG I, FQ I, FQ II and FQ III, G 0 fraction was treated with pronase

(200 μg/ml, 37°C for 24 hr) followed by heat treatment (100°C, 30 min) and fractionated on Sepharose 4B column (Fig. 7b). The absorption profile of treated G 0 fraction was greatly different from that of intact G 0 fraction (Fig. 7a, b). However, the activity profile of treated G 0 fraction was similar to that of untreated G 0 fraction (Fig. 7a, b). These data suggest that most proteins in the included fraction on Sepharose 4B column were digested by pronase while the absorption in the excluded fraction is due to other substances than proteins. Therefore, the activity in G 0 fraction is considered to depend mainly on the activity of pronase-resistant substance(s) and partly on that of aggregated FG I, FQ I, FQ II and FQ III, which are sensitive to pronase-treatment.

Molecular weight estimated by SDS-PAGE and recovery of FG I, FQ I, FQ II or FQ III are shown in Table 1. Generally, through each condensation process in cellulose tubings by negative pressure dialysis for application to gel filtration, about 30% of total protein was found in precipitated fraction by centrifugation at 3,000 rpm for 30 min. While, in the case of column process, more than 90% of applied sample was recovered by using gel-filtration column and 80 to 90% was recovered by QAE column chromatography.

Specific activity of each purified factor was expressed by the reciprocal of ratio of the protein concentration of each factor to that of crude factor at the end point of cell-aggregation promoting activity in serial-diluted factor (Table 1) (Pessac, B. and Defendi, V., 1972). Thus, the specific activity of FG I, FQ I, FQ II and FQ III was determined as 71, 28, 14 and 33-fold, respectively, of the activity of crude factor, i.e., conditioned medium from CHL 36.

Characterization of the Factor Activity

In order to examine whether each factor was protein or acid mucopolysaccharide, effects of 2 x crystalized trypsin, pronase E, hyaluronidase and heat treatments on activity of each factor were tested (Table 2). Further, the effect of DNase I was examined because Steinberg, M. S. (1963) reported that exogeneous DNA promoted cell-aggregation level. Activity of G 0 fraction was sensitive to hyaluronidase treatment but little or not to trypsin, DNase I, pronase or heat treatment. Judging from these data, active substance(s) in the pronase-treated G 0 fraction appears to correspond to acid mucopolysaccharide as described by Pessac, B. and Defendi, B. (1972). Activity of FG I was sensitive to pronase treatment and insensitive to DNase I, trypsin and heat treatment. However, FG I treated with trypsin was cleaved into the fragments of ca. 9×10^4 daltons and smaller ones than 3×10^4 daltons estimated by SDS-PAGE (data not shown). Activity of FQ I, FQ II and FQ III were sensitive to trypsin and heat treatment.

Effects of the Factors on Heterotypic Cells

We tried to examine effects of the cell-aggregation promoting factors on heterotypic cells, i.e., the differences of responsibility to the factors between one type cell and another type cell. The differences could only be shown by comparison between the patterns of the responsibilities to the factors. In order to compare among the patterns of the responsibilities of several type cells (GMK, HeLa S3, L5178Y, 3Y110, liver and neural retina cells from chick embryos), pronase-treated G 0 fraction, G I fraction and G II fraction on Sephadex G-200 column (Fig. 2) and crude factor were tested. Crude factor promoted the aggregation level of CHL 36, 3Y110, HeLa S3 and liver cells, but did not in the case of GMK, L5178Y and neural retina cells (Table 3).

Though each sample promoted cell aggregation of CHL 36, 3Y110 and HeLa S3, the pattern of responsibility of 3Y110 to each sample was similar to that of CHL 36, but was different from that of HeLa S3. Crude factor remarkably promoted aggregation level of liver cells and G II sample also promoted it, but G I and G 0 sample were ineffective. GMK, L5178Y and neural retina cells did not respond to each sample. These data indicate that promoted levels of cell aggregation by each factor depend on cell types and the promotion effect is different from factor to factor on the same cells.

DISCUSSION

Most cell types employed for studying cell-to-cell adhesion can form cell aggregate by itself in suspension. Because of this character of spontaneous cell-aggregate formation, it is hard to measure quantitatively the level of cell aggregation promoted by the factor. Therefore, it is difficult to purify the cell-aggregation promoting factor by pursuing its activity. But such a difficulty was overcome by the method using in this paper. In spite of being less-quantitative, this method is very useful for determination of activity peaks promoting cell aggregation by column chromatography (Fig. 2, 3b, 4, 5b). By using this method, five factors were detected in the conditioned medium from CHL 36.

It was suggested that activity substance in pronase-treated G 0 fraction was acid mucopolysaccharide, judging from the fact that the activity of the factor was resistant to DNase I, pronase, trypsin and heat treatments, but sensitive to hyaluronidase (Fig. 7b and Table 2) and that macromolecules labeled with C^{14}-glucosamine existed at the region with cell-aggregation promoting activity on the Sepharose 4B column of pronase-treated G 0 fraction (data not shown). This factor may be similar to the substance reported by Pessac, B. and Defendi, V. (1972), though cell origin in their experiment was different from ours. They did not report the existence of other factors in conditioned medium of 3T3-SV40 cells. However, we detected the presence of four factors in conditioned medium of CHL 36. These factors are considered to contain protein by reason that they were sensitive to proteolytic digestion and stained with Coomasie Brilliant Blue.

The estimated molecular weights of the factors by Sephadex G-200 column chromatography were different from those by SDS-PAGE (see Results section). In the case of FG I, FQ II or FQ III, this discrepancy may depend on the factor being glycoprotein, whose molecular weight is apt to be over-estimated by SDS-PAGE (Okuyama, T. and Kondo, M., 1974). While, in the case of FQ I, the discrepancy may depend on highly negative-charged residues in FQ I.

Concerning the characters of the factors, these promoted the aggregation of cells fixed with glutaraldehyde. In addition, cell-aggregation promoting factor could be solubilized from the fixed cells treated with NaSCN and it corresponded to FQ I by SDS-PAGE. These facts suggested that the factors may play a role in cell-to-cell adhesion on cell surfaces. Purified factors, i.e., FG I, FQ I, FQ II and FQ III were apt to form self-aggregates of each macromolecule at condensation process or in the case of the factors being stocked with high concentration in cold room. A part of these aggregates was observed as the large aggregated molecule at the void fraction on Sephadex G-200 column (Fig. 3b and 5b). Specific activity of aggregated factor was observed to be comparatively higher than that of purified factor in included fraction on Sephadex G-200 column. This fact was assumed to be due to the formation of more stable cell aggregation by multivalent ligands. The other part of the aggregates was precipitated by centrifugation at 3,000 rpm for 30 min but some portion of precipitated substances treated with 7 M urea, 1 M NaSCN or 5 M guanidine hydrochloride (37°C, 24 hr) was found in the supernatant by the centrifugation and had cell-aggregation promoting activity. Such aggregation of purified factor appears to be a common feature of cell-aggregation promoting substances isolated here and was also

observed in the case of crude factor or partially purified factors, though such phenomena were not observed in the case of the purified factor from conditioned medium of chick neural retina cells (Hausman, R. E. and Moscona, A. A., 1975).

It has not been determined whether the specificity of cell adhesion in higher animal cells is due to specific macromolecules (Moscona, A. A., 1963) or quantitative differences of some non-specific macromolecules involved in cell adhesion (Steinberg, M. S., 1964; Rutshauser, U. et al., 1976). In our case, there are five factors promoting cell aggregation in serum-free conditioned medium from the established cell line (CHL 36), whose cell population is highly homogeneous compared to embryonic tissue cells. Such a variety of factors was suggested by Pousséque, J. M. and Pastan, I. (1976). They reported that the substances having molecular weight of 24×10^4, 13.7×10^4, 11.5×10^4 or 9×10^4 daltons estimated by SDS-PAGE might be involved in the regulation of cell adhesion in 3T3 cells by analysis of mutant of 3T3 cells defective in cell-to-substratum adhesion. These suggest that cell has several own factors.

Several workers purified cell-adhesion promoting factors: collagen-dependent cell attachment factor (more than 20×10^4 daltons) in calf serum (Kleba, R. J., 1974), glycoprotein (5×10^4 dalton estimated by SDS-PAGE) in conditioned medium of chick embryonic retina cells (Hausman, R. E. and Moscona, A. A., 1975) and glycoprotein (22×10^4 daltons on SDS-PAGE) from chick embryo fibroblasts (Yamada, K. M. et al., 1975). Concerning molecular weight and some of its character, we could not find any macromolecules in conditioned medium of CHL 36 corresponding to these by our assay system. This discrepancy may come from the

different cell types used for these experiments, and we have some results suggesting this. The band corresponding to FG I could not be detected by SDS-PAGE of conditioned media from GMK and chick embryonic neural retina cells. But, in the case of HeLa, 3Y110 or liver cells from chick embryo, the band corresponding to FG I was detected (unpublished data). These facts suggest that certain factors from one cell type are different from factors of the others. Judging from the existence of both a variety of factors and their different effects on cell adhesion (Table 3), it can be considered that there are quantitative and qualitative differences of macromolecules involved in cell adhesion and that specific phenomena like a sorting-out phenomenon (Moscona, A. A., 1963) might be due to both quantitative and qualitative differences of these macromolecules.

Though there are few known macromolecules involved in cell adhesion how to reflect on physiological phenomena, we can expect that studies of cell aggregation, by using purified cell-aggregation promoting factors, play an important role in understanding the basic mechanism of cell adhesion and complicated phenomena, i.e., cell-to-cell interaction, cell transformation, differentiation and development.

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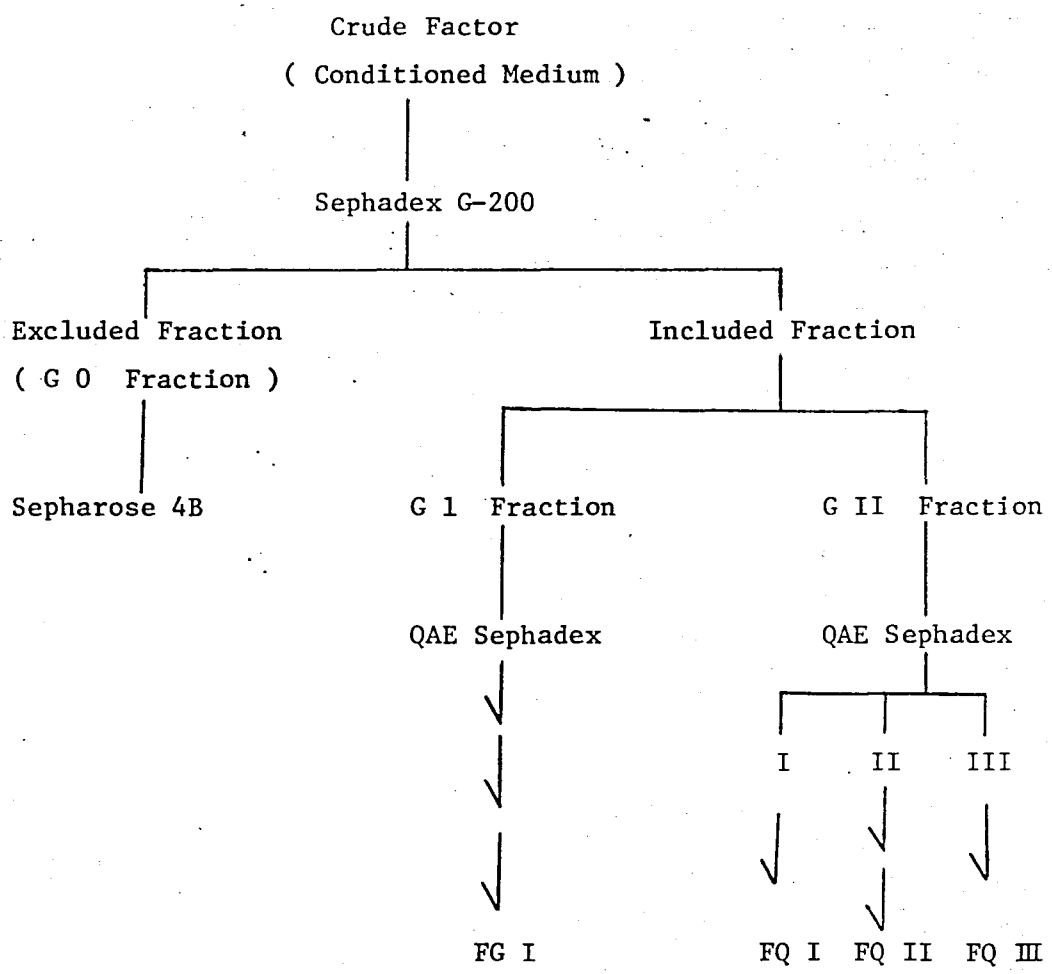


Figure 1. Purification procedures.

(↓): This symbol means that the sample of the activity peak region was re-chromatographed on the same size column of QAE Sephadex (A-50). Purified materials, i.e., FG I, FQ I, FQ II and FQ III were applied to Sephadex G-200 column (2 x 65 cm) and SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

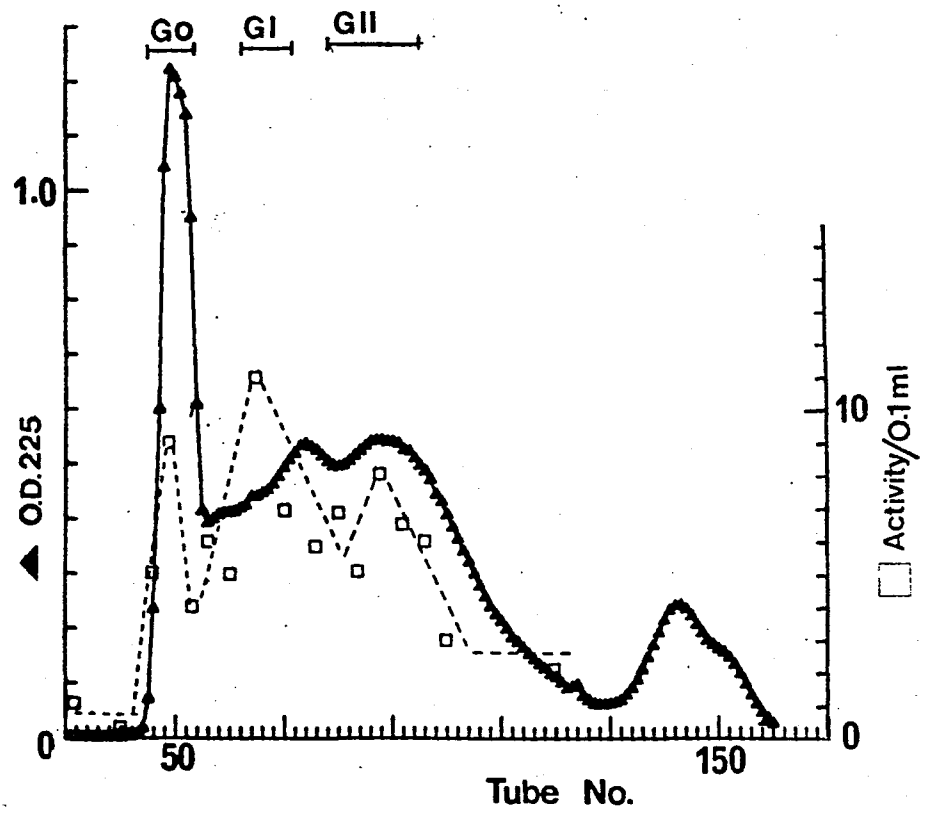
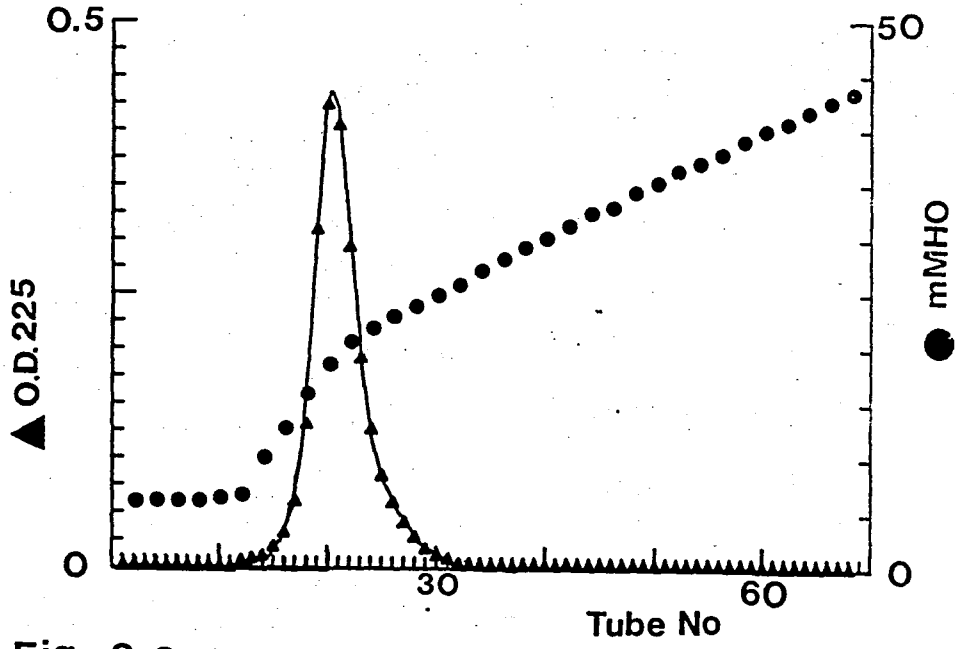


Figure 2. Elution profile of conditioned medium from CHL 36 by Sephadex G-200 column chromatography.

Sample condensed by negative pressure dialysis was centrifuged at 3,000 rpm for 30 min to remove particulate substances. The supernatant (4 ml, 85 mg protein) was applied to Sephadex G-200 column (3 x 100 cm) and eluted with PBS(-). The fraction size was 6 ml/tube. Cell-aggregation activity was tested with 0.1 ml of each fraction as described in Materials and Methods. G 0 fraction was collected from tube No. 45 to 54. G I fraction was from No. 61 to 72. G II fraction was from No. 78 to 95. ▲: Absorbance at 225 mn. □: Activity of cell aggregation.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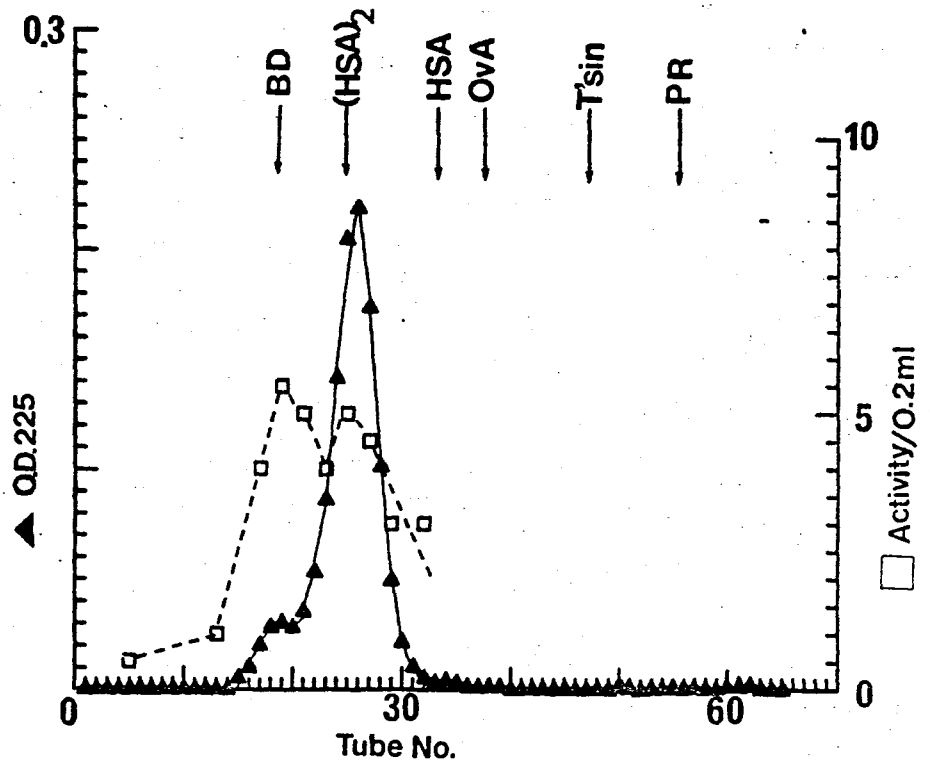
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BD
(HSA)2
HSA
OvA
T'sin
PR
▲ O.D.225
□ Activity/0.2ml

Fig. 3 b

Figure 3. Elution profiles of G I factor (FG I).

a) Elution profile of FG I on the third QAE Sephadex (A-50) column (2 x 12 cm). Sample was applied to QAE Sephadex column in 0.06 M NaCl in 10 mM phosphate buffer (pH 7.5) and eluted with a linear gradient from 0.06 M (100 ml) to 1.0 M NaCl (100 ml) in 10 mM phosphate buffer (pH 7.5). The fraction size was 3 ml/tube.

b) Elution profile of FG I on Sephadex G-200 column. FG I purified on QAE Sephadex column was condensed by negative pressure dialysis and 2.5 ml sample was applied to Sephadex G-200 column (2 x 65 cm) and eluted with PBS(-). The fraction size was 2.5 ml/tube. Activity check was carried out with 0.2 ml of each fraction as described in Materials and Methods.

Standard markers were as follows. BD: Blue Dextran. $(HSA)_2$: Dimer of human serum albumin. HSA: Human serum albumin. OvA: Ovalbumin. T'sin: Trypsin. PR: Phenol red. ▲: Absorbance at 225 nm. ●: Ionic strength (mMHO). □: Activity of cell aggregation.

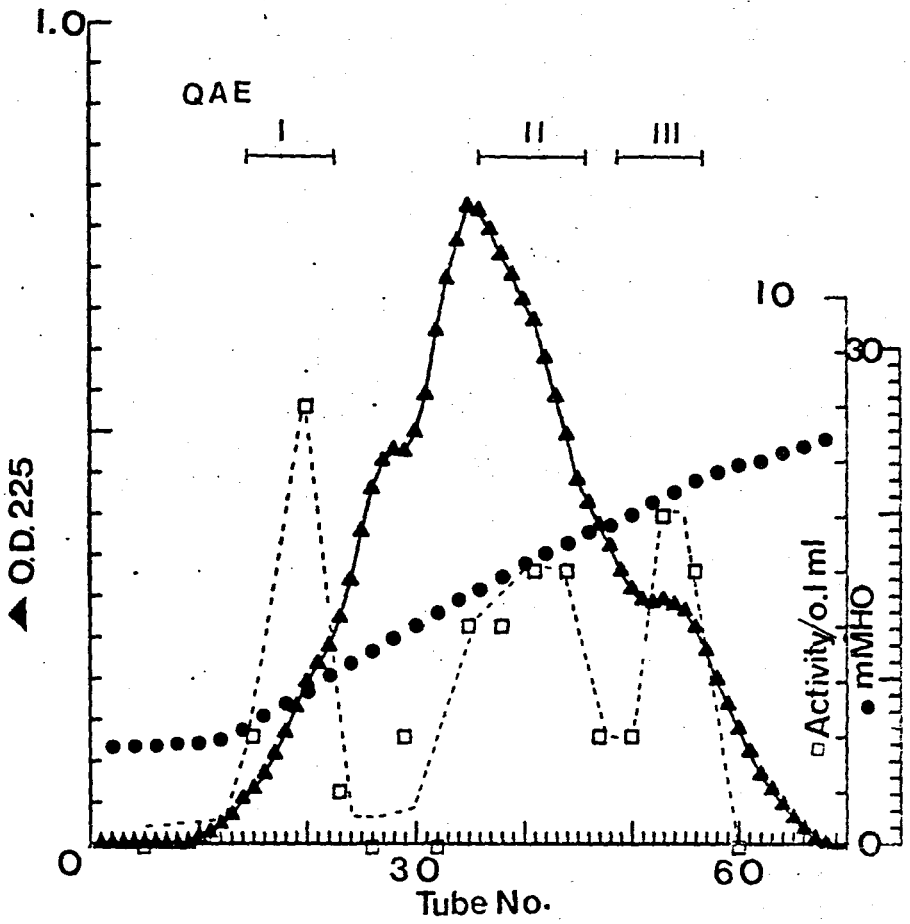

Figure 4. Separation of G II fraction by QAE Sephadex (A-50) column chromatography.

G II fraction on Sephadex G-200 column (Fig. 2) was adsorbed to QAE Sephadex (A-50) column (2 x 12 cm) and eluted with a linear gradient from 100 ml of 0.06 M to 100 ml of 0.5 M NaCl in 10 mM phosphate buffer (pH 7.5). The fraction size was 3 ml/tube. Aliquots of each fraction was dialized against PBS(-) for 40 hr to eliminate the effect of ionic strength on cell-aggregation and then cell-aggregation activity was tested with 0.1 ml of each fraction as described in Materials and Methods. QAE I fraction: Tube No. 15 to 23. QAE II fraction: Tube No. 36 to 46. QAE III fraction: Tube No. 49 to 57. ▲: Absorbance at 225 nm. ●: Ionic strength (mMHO). □: Activity of cell aggregation.

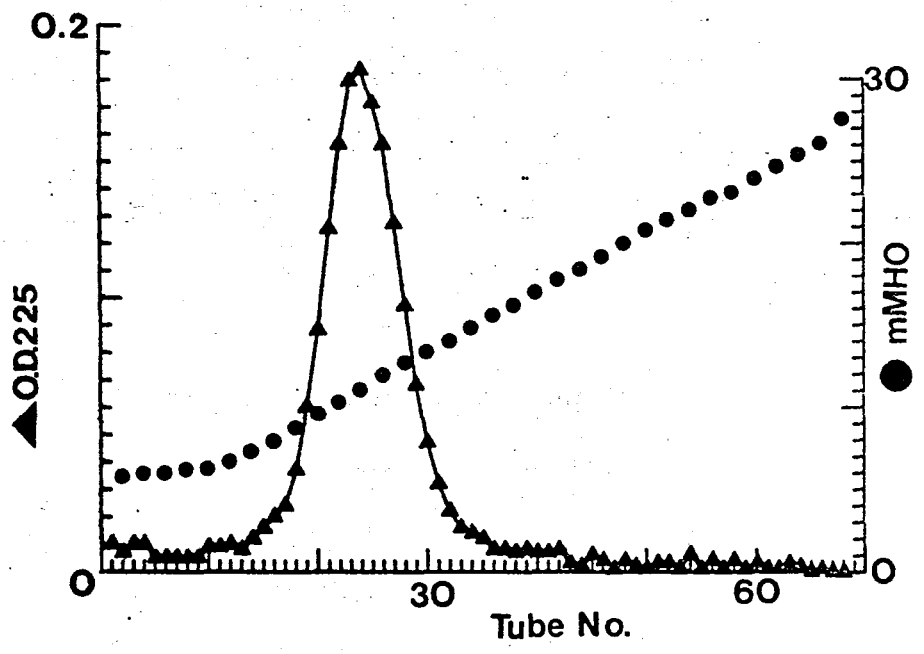
0.2
0
30
60
Tube No.
▲O.D.225
● mMHO
30
0

Fig. 5 a

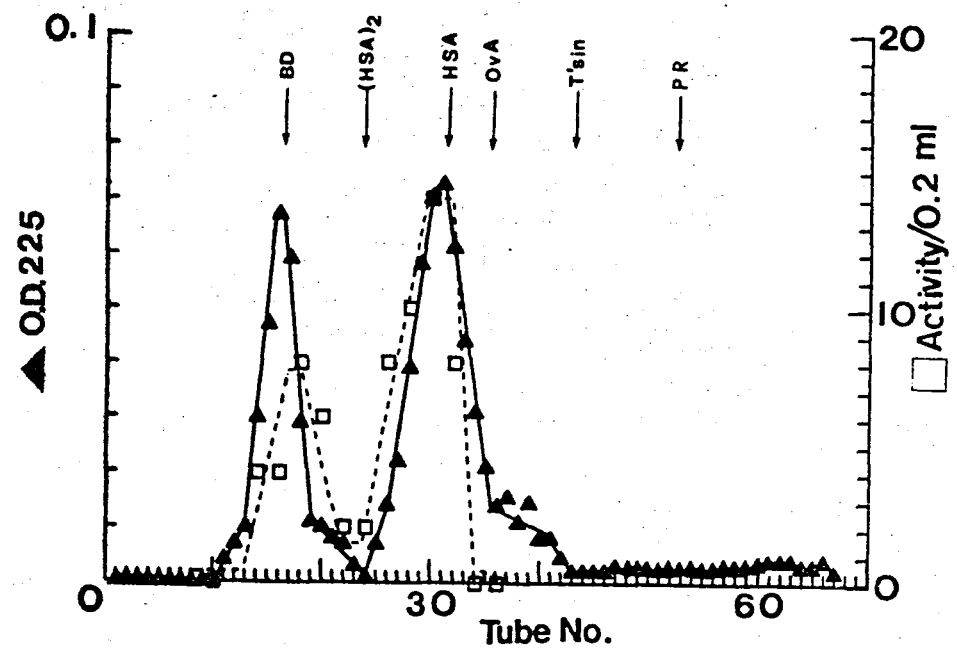
0.1
0
30
60
Tube No.
▲ O.D.225
□ Activity/0.2 ml
20
10
0
BD
(HSA)2
HSA
OvA
T'sin
P R

Fig. 5 b

Figure 5. Elution profiles of FQ I.

a) Elution profile of QAE I fraction by QAE Sephadex (A-50) column chromatography. QAE I fraction (Fig. 4) derived from G II fraction was re-chromatographed on QAE Sephadex (A-50) column (2 x 12 cm) with a linear gradient from 100 ml of 0.06 M to 100 ml of 0.5 M NaCl in 10 mM phosphate buffer (pH 7.5). Fraction size was 3 ml per tube. The fraction containing active macromolecule was pooled from tube No. 19 to No. 26, condensed by negative pressure dialysis and followed by Sephadex G-200 column chromatography.

b) Elution profile of FQ I on Sephadex G-200 column. 2.5 ml of purified macromolecule from QAE I fraction (Fig. 5a) was applied to Sephadex G-200 column (2 x 65 cm) and eluted with PBS(-). Fraction size was 2.5 ml per tube. Cell-aggregation activity was tested with 0.2 ml of each fraction as described in Materials and Methods.

▲: Absorbance at 225 nm. ●: Ionic strength (mMHO). □: Activity of cell aggregation. BD:Blue Dextran. $(HSA)_2$:dimer of human serum albumin. HSA:human serum albumine. OvA:ovalbumin. T'sin:trypsin. PR:phenol red.

Fig. 6 a

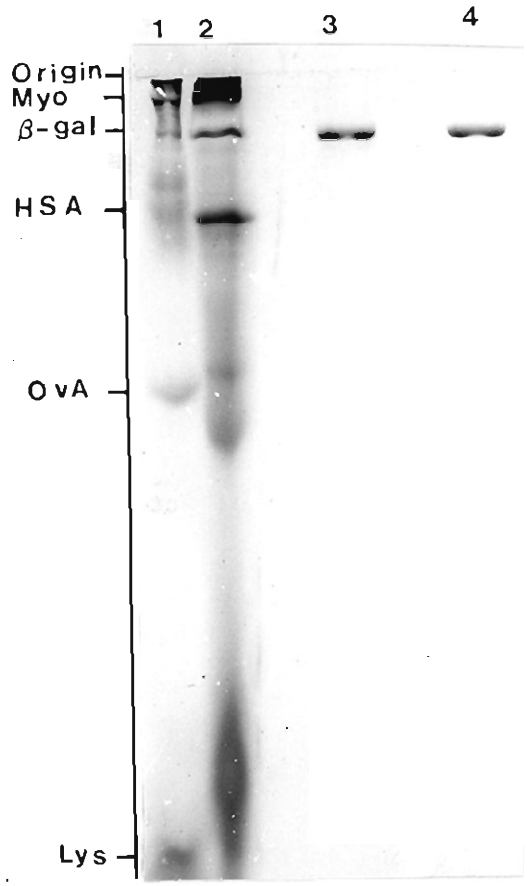
1
2
3
4
Origin
Myo
β-gal
HSA
OvA
Lys

Fig. 6 b

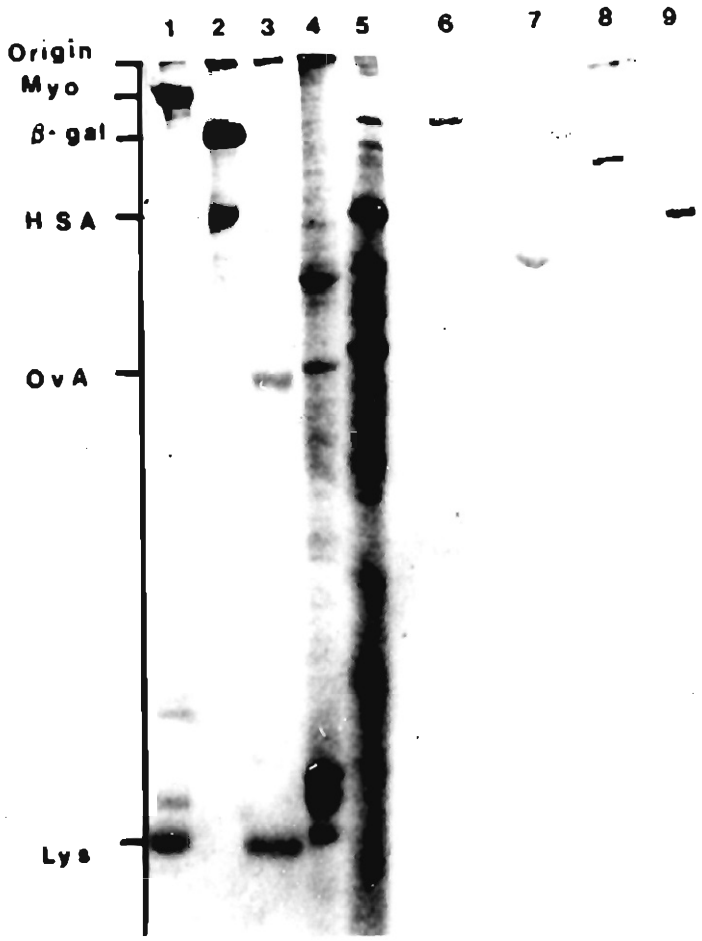
1
2
3
4
5
6
7
8
9
Origin
Myo
β-gal
HSA
OvA
Lys

Figure 6. SDS-polyacrylamide gel electrophoresis(SDS-PAGE)

The slab gel was performed and stained with Coomasie Brilliant Blue for protein as described in Materials and Methods. Following standard markers were used; myosin(Myo), β-galactosidase(β-gal), human serum albumin (HSA), ovalbumin(OvA) and lysozyme(Lys).

a) SDS-PAGE of FG I sample on Sephadex G-200 column.

1: Myosin, β-galactosidase, human serum albumin, ovalbumin and lysozyme.

2: Conditioned medium from CHL 36 cells designated as crude factor(30μg).

3: FG I sample(5μg) in included fraction on Sephadex G-200 column(Fig. 3b).

4: FG I sample(5μg) in excluded fraction on Sephadex G-200 column.

b) SDS-PAGE of purified factors.

1: Myosin and lysozyme. 2: β-galactosidase. 3: Human serum albumin and ovalbumin. 4: Whole cell of CHL 36(30μg of protein). 5: Conditioned medium , i.e., crude factor from CHL 36 cells(40μg of protein). 6: FG I(5μg of protein) purified from G I fraction on Sephadex G-200 column(Fig. 2). 7: FQ I(5μg of protein) purified from QAE I fraction on QAE Sephadex(A-50) column(Fig. 4). 8. FQ II(5μg of protein) purified from QAE II fraction on QAE Sephadex(A-50) column (Fig. 4). 9: FQ III(5μg of protein) purified from QAE III fraction on QAE Sephadex(A-50) column(Fig. 4).

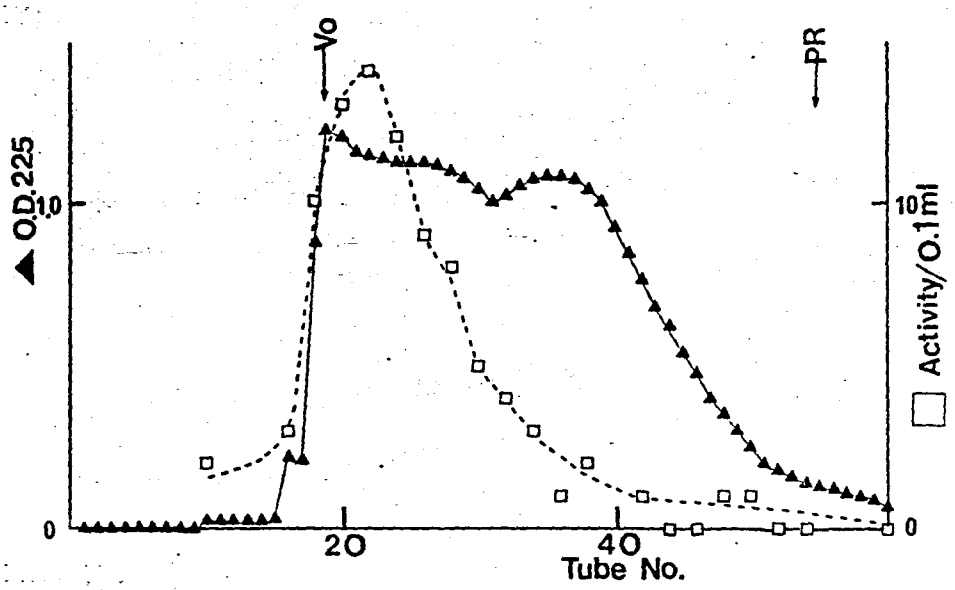
Vo
PR
OD 225
1.0
0
20
40
Tube No.
10
0
Activity/0.1ml

Fig. 7a

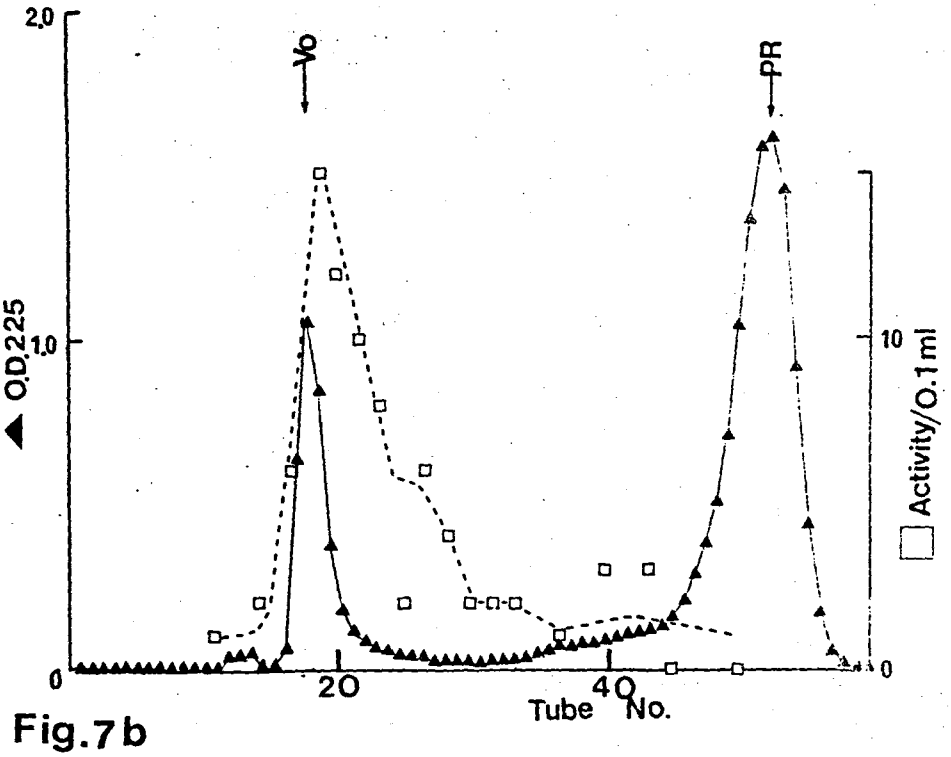
2.0
Vo
PR
OD 225
1.0
0
20
40
Tube No.
10
0
Activity/0.1ml

Fig. 7b

Figure 7. Elution profiles of G 0 fraction by Sepharose 4B column chromatography.

a) G 0 fraction excluded by Sephadex G-200 column chromatography (Fig. 2) was concentrated by negative pressure dialysis and centrifuged at 3,000 rpm for 30 min. The supernatant (2.5 ml) was applied to Sepharose 4B column (2 x 65 cm) and eluted with PBS(-). Cell-aggregation activity was tested with 0.1 ml of each fraction.

b) G 0 fraction treated with pronase (200 μg/ml) at 37°C for 24 hr, heated at 100°C for 30 min and centrifuged at 3,000 rpm for 30 min. The supernatant was applied to Sepharose 4B column (2 x 65 cm) and eluted with PBS(-). Cell-aggregation activity was tested with 0.1 ml of each fraction. The fraction size was 3 ml per tube. Vo:Void fraction. It was determined by using Blue Dextran. PR: Phenol red. ▲:Absorbance at 225 nm. □: Activity of cell aggregation.

Title: Studies on Cell Aggregating Factors by Using Fixed Cells.

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SUMMARY

Cell-aggregation promoting factors derived from the conditioned medium of CHL 36 cells were studied by using cells fixed with glutaraldehyde. The level of cell aggregation was determined by measuring the disappearace of single cells in suspension. By using this methode, the assay of the cell aggregation was performed quantitatively, and same aggregation factors could be detected as in the case of using live cells.

Characterizing these factors, we found the existence of some co-operative effects between them on the fixed cell aggregation. From the experiment using the fixed cells treated previously with the GO or GI factor, we showed the binding of GI factor to the fixed cells is indispensable for the synergistic effect before GO factor binds to GI factor or the fixed cells.

INTRODUCTION

Adhessiveness of higher animal cells is generally believed to play a key role in cell-to-cell interaction, such as metastasis (Nicolson, G.L. and Winkelhake, J.L., 1975), contact inhibition of cell movement (Yamada, K.M., et al., 1976), cell growth (Lloyd, A.C. and Josefina, F. B., 1976), cell differentiation (Martin, G. R.,1975). and the pattern formation of tissue construction (Ade, D. A.,1975). Little information is, however, available at present on the molecular basis of cell-to-cell adhesion underlying these phenomena.

We have previously reported the presence of five factors which promote cell aggregation in the conditioned medium derived from CHL 36 cells and succeeded in purifying four of them (Tanaka, A., 1976). However, by using live cells, it is difficult to study the cell aggregation promoted by the factors quantitatively and to clarify the precise mechanism of cell aggregation promoted by each factors since the live cells produce substance(s) effective to cell aggregation by themselves during the assay procedure and having an ability to aggregate spontaneously as well. Thus, the data obtained by using live cells become some complicated ones and some novel method was needed to be developed for the quantitative and more detailed study of cell aggregation.

In the present study, we show a method for measuring the level of cell aggregation quantitatively by using the cells fixed with glutaraldehyde instead of live ones. The interaction between the factors and the cells fixed with glutaraldehyde was also investigated.

MATERIALS and METHODS

Cell and Cell Preparation

Established cell line, CHL36 derived from Chinese hamster lung cells, was used. CHL36 cells were passaged with 0.25%(w/v) trypsin and cultured in Eagle's minimal essential medium (MEM) containing 10%(v/v) calf serum at 37°C for 40 hr. Cells at growing stage were treated with 0.12%(w/v) trypsin at 37°C for 30min and washed four times with Dulbecco's phosphate buffer (Ca^{++},Mg^{++}free) [PBS(-)] containing 1%(v/v) Ca^{++},Mg^{++}-free calf serum, which was dialyzed against PBS(-). 2.5ml of 4% glutaraldehyde (Ladd Res. Ind. Inc.) in 10mM phosphate buffer (PH 5.0) was added to the cells suspended in 2.5ml of PBS(-) containing 1%(v/v) Ca^{++},Mg^{++}-free calf serum and the cells were fixed in ice for 30min. After this treatment, the cells were washed four times with PBS(-) containing 1%(v/v) calf serum, suspended in PBS(-) containing 1%(v/v) calf serum and 500μg of kanamycine per ml and stocked at 4°C. These fixed cells showed 80 to 90% of single cell rate and were used for the cell-to-cell binding assay.

Cell-to-cell Binding Assay

The cells fixed with 2% glutaraldehyde designated as GA-cells were washed with PBS(-) and suspended in Ca^{++}-free MEM bufferized with 10mM hydroxyethyl piperazine ethan sulfonic acid (HEPES) [pH7.5] and the medium finally contained 1mM $CaCl_2 \cdot 2H_2O$, 5%(v/v) dialyzed calf serum and appropriate volume of tested sample solution. Each tested sample was performed in duplicate and the cell concentration was adjusted to 30×10^4 cells per 1.5ml of the medium in each 10-ml Erlenmeyer flask. All samples were shaken on a gyratory shaker (70rpm) at 37°C and removed at the indicated times (generally 24hr).

The level of cell aggregation was measured with haemocytometer and expressed by the function of single cell number per unit volume of suspension medium (Curtis, A. S. G. and Greaves, B, M., 1965. Orr, C. W. and Roseman, S., 1969). This method measures the number of residual single cells in suspension, from which the number of disappeared single cells can be calculated. The latter number directly reflects the level of cell aggregation. In this paper, the level of cell aggregation was shown as follows.

$$\% \text{ of residual single cells} = \frac{Ns}{No} \times 100$$

$$\% \text{ of disappeared single cells} = 100 - \frac{Ns}{No} \times 100$$

(No: Single cell number per unit volume in control solution at zero time. Ns: Single cell number in tested solution at an appropriate time.)

In the case of using live cells, cells were prepared as described in "Cell and Cell Preparation", suspended in HEPES-buffered MEM containing 1mM $CaCl_2.2H_2O$, 5%(v/v) calf serum and 1%(w/v) glutamine, and tested. The level of cell aggregation was determined in order of the increasing size of cell aggregate by microscopical and macroscopical observations as mentioned in the previous paper (Tanaka, A., 1976).

Preparation of Conditioned Medium

The serum-free conditioned medium from CHL 36 cells was prepared as follows. Cells at confluent stage were incubated for 24 hr in fresh medium, washed four times with PBS(-) and cultured in serum-free MEM at 37°C for 3days. The conditioned media were collected, filtrated through glass fiber filter (Whatman GF/F), concentrated in cellulose tubings by negative pressure dialysis and centrifuged at 3,000rpm for 30min. The supernatant designated as crude factor was used for column chromatography or dialyzed against PBS(-) for 3days and used for experiments.

Measurements of Protein and Carbohydrate

Protein concentration was measured by Lowry's method (Lowry, O. H., et al., 1951). The amount of carbohydrate was estimated by measuring uronic acid (Matsumura, T., 1969) to determine GO concentration, by employing glucuronic acid as a standard sample.

RESULTS

Effects of Factors on GA-cell Aggregation

Crude factor derived from CHL36 cells was shown to have the activity to promote cell aggregation (Tanaka, A., 1976). However, it was rather difficult to measure the level of cell aggregation quantitatively by using live cells since the cells aggregate spontaneously. Therefore, we tried to use the cells fixed with glutaraldehyde (Capaldi, R. A., 1973) for the more quantitative measurement of cell aggregation promoted by the factor. It was observed by macroscopical and microscopical observations that the number of larger particles increased as the number of single cells in suspension decreased with the time in the presence of the factor, and the assay involved measuring the decrease in single cells as a function of time or of dose of crude factor as described in MATERIALS and METHODS. Fig. 1a shows that the relationship between the logarithm of residual single cell number in suspension and time is linear from 8 to 24-hr shaking. The slope of the lines related to the concentration of added crude factor: i.e., the rate of GA-cell aggregation was a function of factor concentration. The effect of increasing amounts of crude factor on the rate of aggregation is shown in Fig. 1b. The relationship between the logarithm of the factor concentration and disappeared single cell number in suspension was linear over the range from 1 to 120 μg of protein per ml in 16 to 24-hr shaking. But, in 8-hr shaking, that was linear from 10 to 120 μg protein of the factor per ml. On the basis of these findings, this method is indicated to be very useful for quantitative studies of cell aggregation promoted by the factor.

In order to examine what kind of substances promote the GA-cell

aggregation and whether these substances correspond to the factors to promote the level of live-cell aggregation (Tanaka, A., 1976), separation of activity substances was carried out as described below. Crude factor was applied to Sephadex G-200 column (3 x 100 cm) and eluted with PBS(-) (Fig. 2a). And cell-aggregation promoting activity in each fraction was determined by using live cells or GA cells (Fig. 2b). In the case of live cells, the activity was determined by ordering the aggregate size (Tanaka, A., 1976), while the level of GA-cell aggregation was determined as described above. Three regions (designated as GO, GI and GII fractions) having activity of cell aggregation which were determined by using live cells corresponded to those by using GA cells. This may indicate that both live and GA-cell aggregation are promoted by the same factors. Each fraction on QAE Sephadex (A-50) column of GII fraction and the factors (designated as FGI,FQI,FQII and FQIII) purified by using live cells were also examined about the activity by using GA-cells. Fig.3 shows the activity profile of GII fraction on QAE Sephadex (A-50) column. The profile was very similar to that determined by using live cells (Tanaka, A., 1976), and aggregation of GA-cells was also promoted by the purified factors (Table 1). GO fraction excluded by Sephadex G-200 column chromatography contains acid mucopolysaccharide substances, whose activity is resistant to pronase treatment and sensitive to hyaluronidase (Tanaka, A., 1976). The GO fraction treated with pronase E (designated as GO sample prepared as described in legend to Fig.4) also had the activity to promote the level of GA-cell aggregation (Fig.4) same as live cells. These data suggest that the aggregation of GA-cells can be promoted by the same factors detected by using live cells. These findings obtained by using GA cells suggest that the above-mentioned factors play a role in cell-to-cell adhesion on the cell surfaces.

Effects of the Mixtures of the Factors on GA-cell Aggregation.

We recently demonstrated the existence of five factors which promoted the aggregation of live cells (Tanaka, A., 1976). However, the mechanism how these factors contribute to the cell aggregation has not been elucidated in detail. In the previous sections, we described that the factors could be analyzed quantitatively. In the present study, we further investigated the effect of the mixtures on GA-cell aggregation. The effects of GO, GI, GII sample and their mixtures on GA-cell aggregation are shown in Fig. 4. It was evident that GO sample equivalent to 0.9 μg/ml of glucuronic acid was less effective to GA-cell aggregation whereas GO sample equivalent to 3.6 μg/ml of glucuronic acid was more effective than control sample. Thus, it is likely that GO sample could promote the level of GA-cell aggregation depending on its concentration as well as GI and GII samples.

The aggregation level in the case of the mixture of (GO + GI) or (GO + GII) was observed to be higher than that in the case of GO, GI or GII sample, but not in the case of the mixture of (GI + GII). Next, we precisely examined the effects of the mixtures of GO, QAEI, II and III sample separated by QAE Sephadex(A-50) column chromatography(Fig. 3). In the case of the mixture of GO and QAEII or III sample, the aggregation level of GA-cells at the rather low concentration was higher than that in the case of GO, QAE II or III sample. While that at rather high concentration of the mixture was observed to be lower(Fig. 5-II and III). In the case of the mixture of GO and QAE I sample, similar effect on GA-cell aggregation were observed at rather low concentration(Fig. 5-I). The inhibitory effect on GA-cell aggregation in the case of the rather

high concentration of the mixture was similar to that in the case of (QAE I +QAEII), (QAEI + QAEIII) or (QAEII + QAEIII). Such effect of the mixture may lead to the suggestion that there exists some co-operative effect between factors on GA-cell aggregation.

Effects of the Previous Treatment of the Factors on GA-cell Aggregation

The mixture of GO and some factors suggested the existence of co-operative effects on the aggregation of GA-cells as described in the previous section. To investigate the co-operative effects further, we examined the effect of the factors on aggregation of GA-cells treated previously with another factor. GA-cells, thus treated previously with GO, GI sample or control solution, were washed with PBS(-) and subsequently incubated in media containing another factors. GA-cells treated previously with GO and GI samples were incubated with GI and GO samples, respectively, while GA-cells treated previously with control solution were incubated with GO, GI sample or their mixture. These experimental conditions were designated as [GO→GI], [GI→GO], [O→GO], [O→GI] and [O→GI + GO], respectively. Fig. 6 shows diagramatically the responses of thus treated GA-cells. The promotion effect of [GO→GI] is considered depending solely on GI activity since the aggregation promoting level of [O→GI] was similar to that of [GO→GI] at rather high concentration while some difference exists at lower concentration of GI sample. This effect is supposingly due to GO bound to GA-cells during the pre-incubation, according to the fact that the effect is similar to the inhibitory effect of GO sample at the lower concentration (Fig. 4). The promotion of cell aggregation observed in the case of [GI→GO] apparently shows the synergistic effect by GI and GO samples. The level of cell aggregation promoted by the treatment by [GI→GO] was similar to that by

[0→GI + GO]. These data suggest that the previous binding of GI factor to GA cells is indispensable for the synergistic effect before GO factor binds either to GI or GA-cells.

DISCUSSION

Adhesive properties of cells play a essential role in biological activity, and thus had been intensively investigated in many laboratories (Curtis, A. S. G., 1967). Although the entire mechanism of this process remains unsolved at present, one of the major interests for the investigators engaging in the study have been unravelling the complex tapestry involving many steps of the process. These steps may even be sequential, although not necessarily synchronous among all the cells in an aggregate, or among different aggregates in the common suspension. Thus, in the studies of using live cells, many complicated phenomenon are involved altogether to form stable cell aggregate, such as macromolecular synthesis (Moscana, M. H. and Moscona, A. A., 1966), microtubules (Goldman, R. D., 1971), microfilaments (Appleton, J. C. and Kemp, R. B., 1974), calcium and magnesium ions (Curtis, A. S. G., 1967) and cyclic AMP (Johnson, G. S. and Pastan, I., 1972).

Fixed cells could be reasonably employed for such studies eliminating the effects mentioned above since it is well known that fixed cells aggregate in the presence of factors (Balsamo, J. and Lilien, J., 1974). The difficulties were, however, in preparing fixed cells in a fully dissociated condition. It was also hard to prepare the fixed cells conserving an aggregation level which could be estimated by the single cell disappearance method, which was one of the best methods for quantitative studies of the mechanism of cell-to-cell binding. These difficulties seemed to be due to the pH and/or the presence of calcium and magnesium ions in the preparation of cells fixed with glutaraldehyde. We were

successfuly able to measure the GA-cell aggregation level promoted by the factors according to the method described in MATERIALS and METHODS. By this method, the standard deviation of the proportion of single cells fell into the similar range to that for live cells(Fig. 1).

By using GA-cells, we tried to investigate in details of the mechanism of cell aggregation promoted by the factors derived from conditioned medium of CHL36 cells. The activity profiles of the factors on live cells corresponded to those on GA-cells (Fig. 3), though an exceptional activity peak existed for GA-cells. This exceptional peak may be due to the difference in the assay sensitivity. Accordingly, in the present study, we investigated the activity of the peaks which manifested either for live cells or the fixed cells.

To study the effects of mixtures of factors suggested to be the presence of co-operative effects(Fig.4 and 5). And the effect of the previous treatment of GI, GO, on GA-cell aggregation was further studied(Fig. 6), suggesting that there exists synergistic effect of the mixture on GA-cell aggregation. Such synergistic effect seems to be due to the previous binding of GI to GA-cells followed by the binding of GO factor. Considering the fact the main component of GO factor is hyaluronic acid (Tanaka, A., 1976), which is a well-known substance to bind protein (Matsumura, T., 1970), we speculate the mechanism of such synergistic effect as follows:

(1) In case a GO factor has multiple binding sites to such protein, the mixture of GO and GI sample results in more stable binding by the direct interaction between these factors,

(2) after the completion of binding of GI factor to GA-cells, GO factor binds to such site resulting in the stabilization of the cell-to-cell adehesion,

(3) the rearrangement of membrane macromolecules directly relating to cell-to-cell binding may result from the adsorption of GI factor to GA-cells followed by the binding of GO factor, just as an well-known patching phenomenon in higher animal cells(Edelman, G. M., et al., 1973).

This problem remains unsolved and further study is in progress.

Acknowledgment

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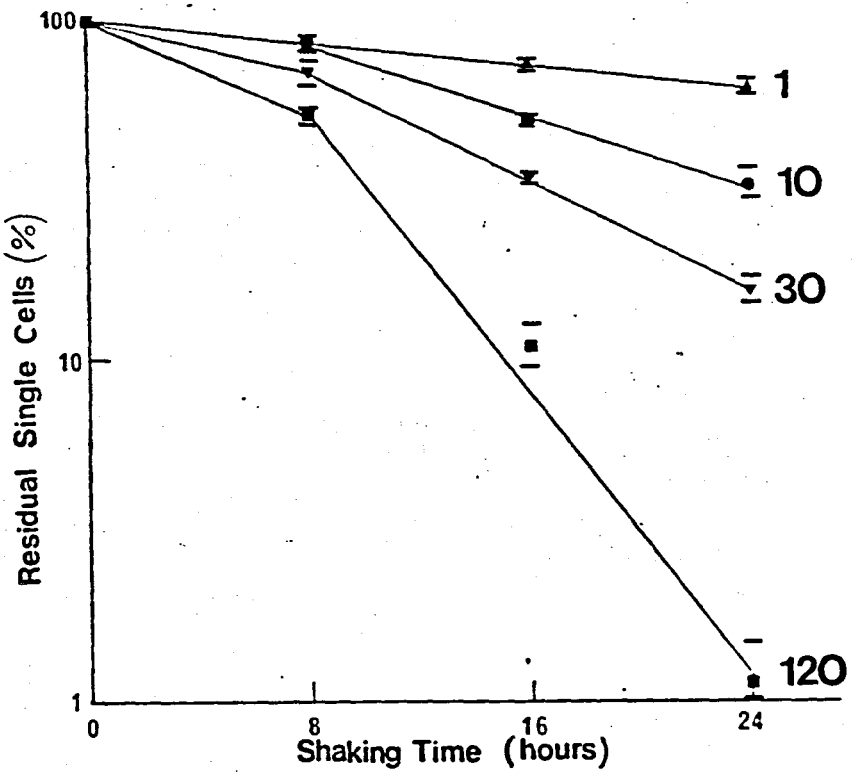
100
10
1
Residual Single Cells (%)
0
8
16
24
Shaking Time (hours)
1
10
30
120

Fig. 1a

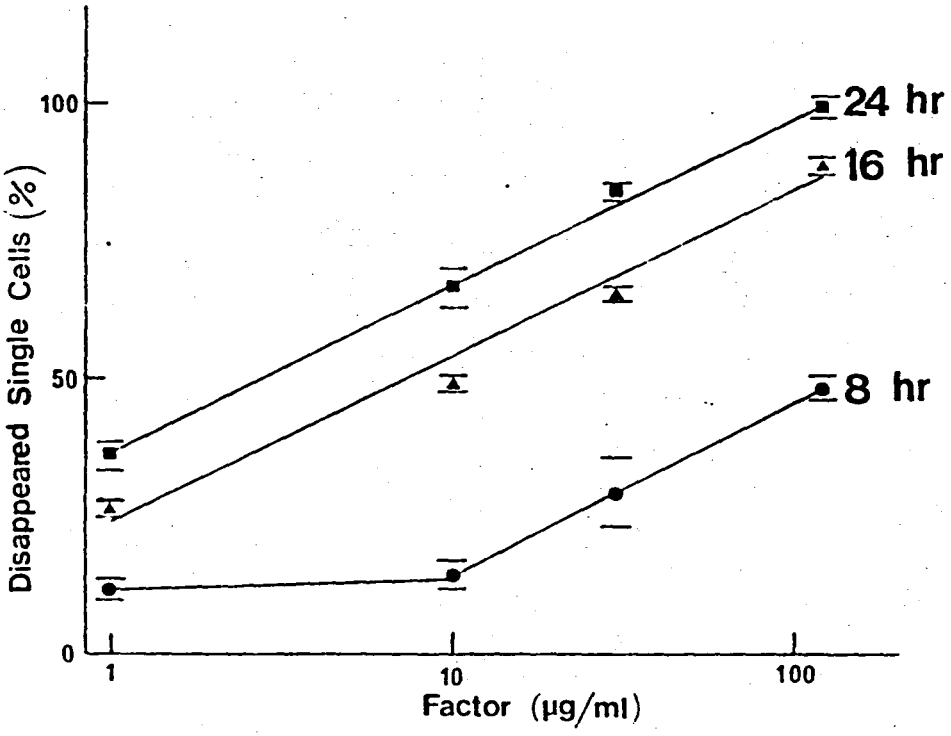
100
50
0
Disappeared Single Cells (%)
1
10
100
Factor (µg/ml)
24 hr
16 hr
8 hr

Fig. 1b

Figure 1. Effect of crude factor on GA-cell aggregation.

Flasks containing following concentration of crude factor per ml of HEPES-buffered MEM, i.e., 1(▲), 10(●), 30(▼) or 120(■) μg of protein were shaked for 8(●), 16(▲) and 24hr(■). The level of cell aggregation in each flask was measured as described in MATERIALS and MRTHODS. The net difference between the control and the test system was calculated and plotted as described below. Each value was the mean in duplicate and the deviation of the value were expressed by bars.

a) Aggregation kinetics of GA cells. Ordinate: The logarithm of percentage of residual single cell number in each flask. Abscissa: Shaking time(hr).

b) Dose effect of crude factor on GA cell aggregation. Ordinate: The level of cell aggregation expressed by percentage of disappeared single cells. Abscissa: The logarithm of concentration of crude factor (μg of protein per ml).

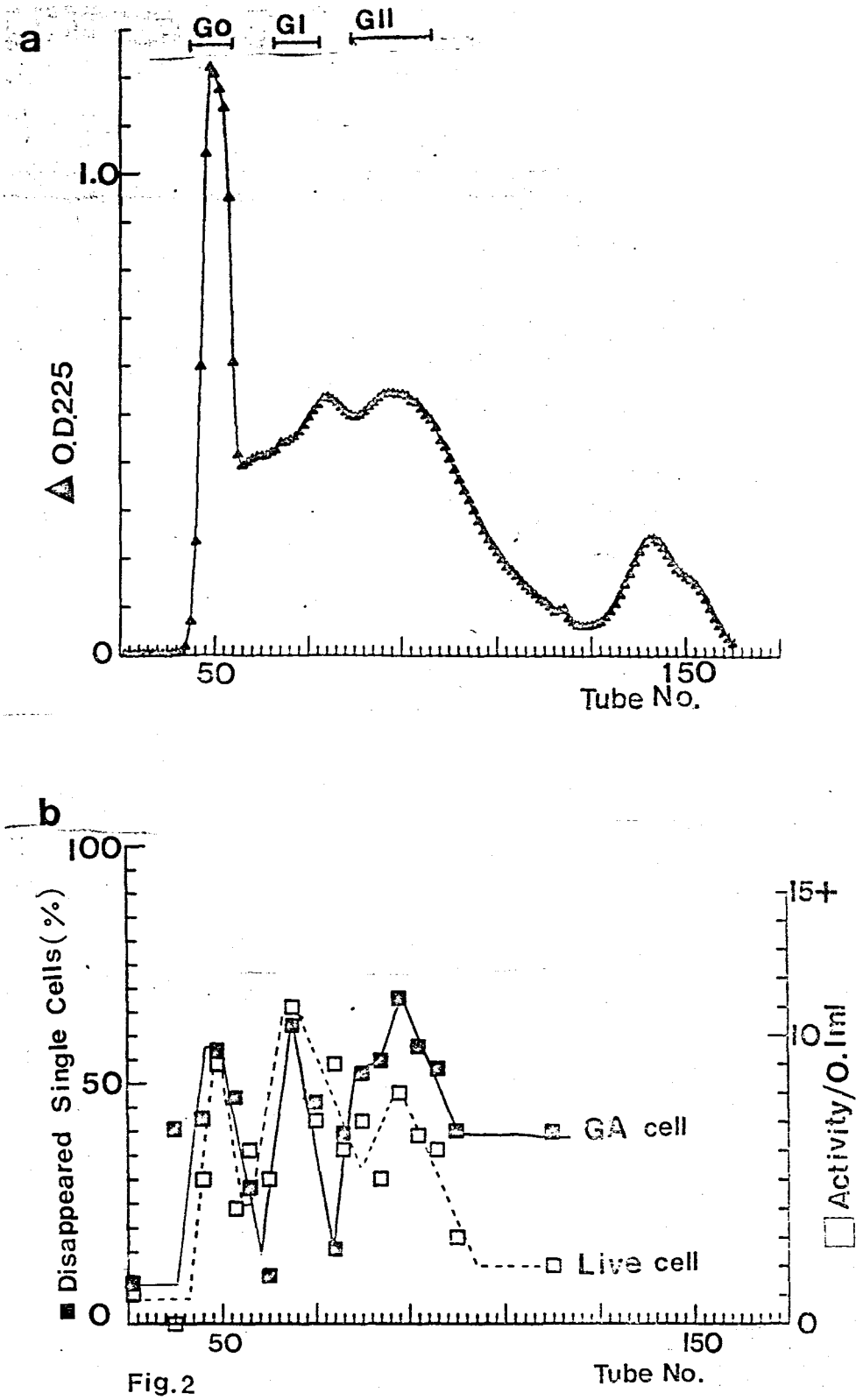
a
Go
GI
GII
1.0
0
Δ O.D.225
50
150
Tube No.
b
100
50
0
Disappeared Single Cells (%)
15+
10
0
Activity/0.1ml
GA cell
Live cell
50
150
Tube No.

Fig. 2

Figure 2. Activity profiles of conditioned medium from CHL 36 cells on Sephadex G-200 column.

Conditioned medium, i. e., crude factor prepared as described in MATERIALS and METHODS was applied to Sephadex G-200 column(3 x 100cm) and eluted with PBS(-). The fraction size was 6ml/tube. G0 fraction was collected from tube No. 45 to 54. GI fraction was from No. 61 to 72. GII was from No. 78 to 95.

a) Elution profile on Sephadex G-200 column. ▲: Absorbance at 225nm.

b) Activity profile of cell aggregation on the same column of Sephadex G-200. The cell aggregating activity was tested with 0.1ml of each fraction by using live cells(□) and GA cells(■), and measured as mentioned in MATERIALS and METHODS.

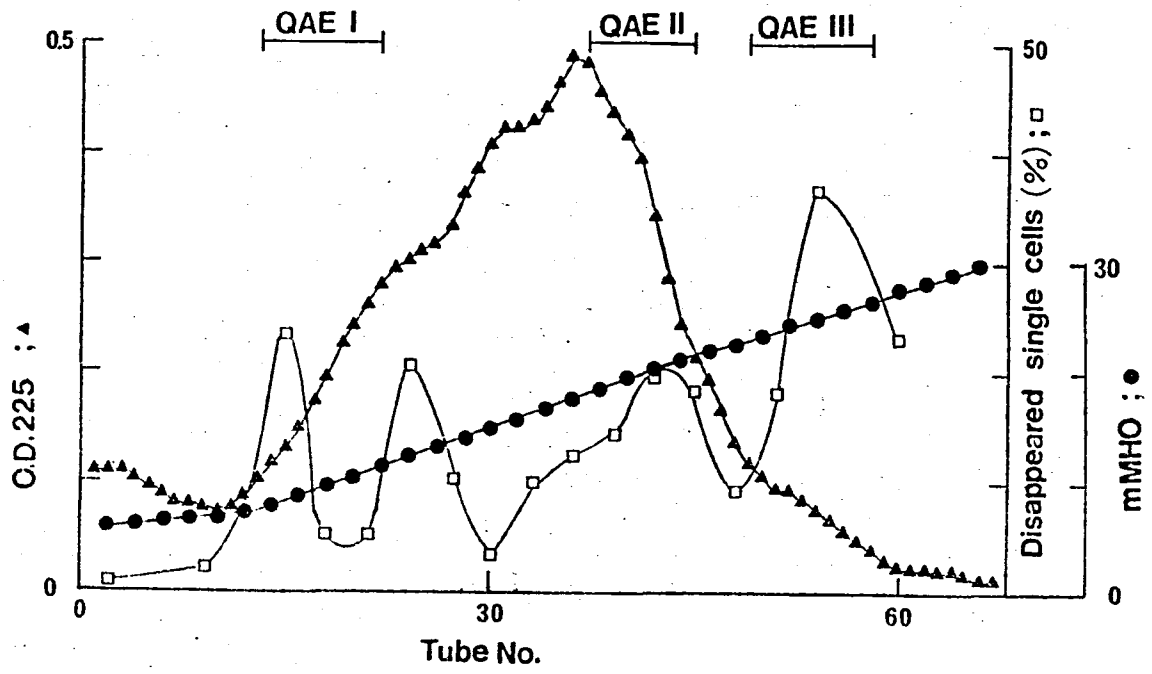

Fig.3

Figure 3. Activity profile of G II fraction on QAE Sephadex (A-50) column.

GII fraction of Sephadex G-200 column was applied to QAE Sephadex (A-50) column (2x12cm) and eluted with a linear gradient from 0.06M to 0.5M Nacl in 10mM phosphate buffer (PH7.5). The fraction size was 3ml/tube. Each fraction was dialyzed against PBS(-) for 3 days for eliminating the effects of ionic strength on cell aggregation, and the cell aggregating activity was measured with o.1ml of each fraction. Tube No.15 to 23, No.36 to 46 and No.49 to 54 were collected as QAEI, II and III samples, respectively.

□ : Activity measured by using GA cells.

▲ : Absorbance at 225nm. ● : Ionic strength (mMHO).

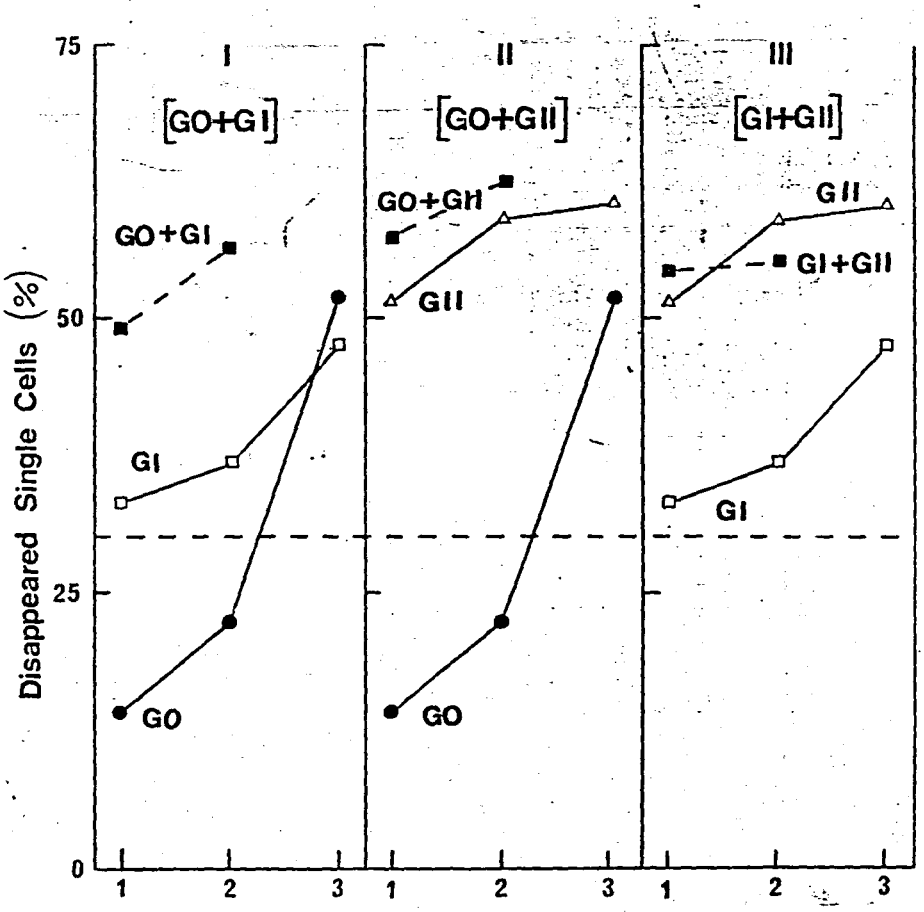
75
50
25
0
Disappeared Single Cells (%)
I
[GO+GI]
II
[GO+GII]
III
[GI+GII]
GO+GI
GI
GO
GO+GII
GII
GII
GI+GII
GI
GO
1
2
3
1
2
3
1
2
3

Fig. 4

Figure 4. Effects of G0, GI, GII sample or their mixtures on GA-cell aggregation.

Activity peaks, i.e., G0, GI and GII fractions on Sephadex G-200 column (Fig.2) were collected and the effects of each factor and mixtures of the factors on GA-cell aggregation were examined. ●: G0 sample prepared as follows. G0 fraction (Fig.2) was treated with pronase E (500 μg/ml) at 37°C for 24hr, heated at 100°C for 30 min and centrifuged at 3,000 rpm for 30 min. And the supernatant was dialyzed against PBS(-) for 3 days and used for experiments. The G0 concentration equivalent to the amount of glucuronic acid as described in MATERIALS and METHODS was as follows. 1: 810 μg/ml. 2: 2 μg/ml. 3: 4 μg/ml. □: GI sample on Sephadex G-200 column. Protein concentration was as follows. 1: 3.3 μg/ml. 2: 10 μg/ml. 3: 25 μg/ml. △: GII sample on Sephadex G-200 column. Protein concentration was as follows. 1: 4.1 μg/ml. 2: 8.3 μg/ml. 3: 25 μg/ml. ■: the incubation mixture prepared as follows. Each sample shown as 1 or 2 was mixed with another one shown as 1 or 2, respectively. I: the mixture of pronase-treated G0 sample with GI sample. II: the mixture of pronase-treated G0 sample with GII sample. III: the mixture of GI sample with GII sample.

-----: the level of cell aggregation in control solution.

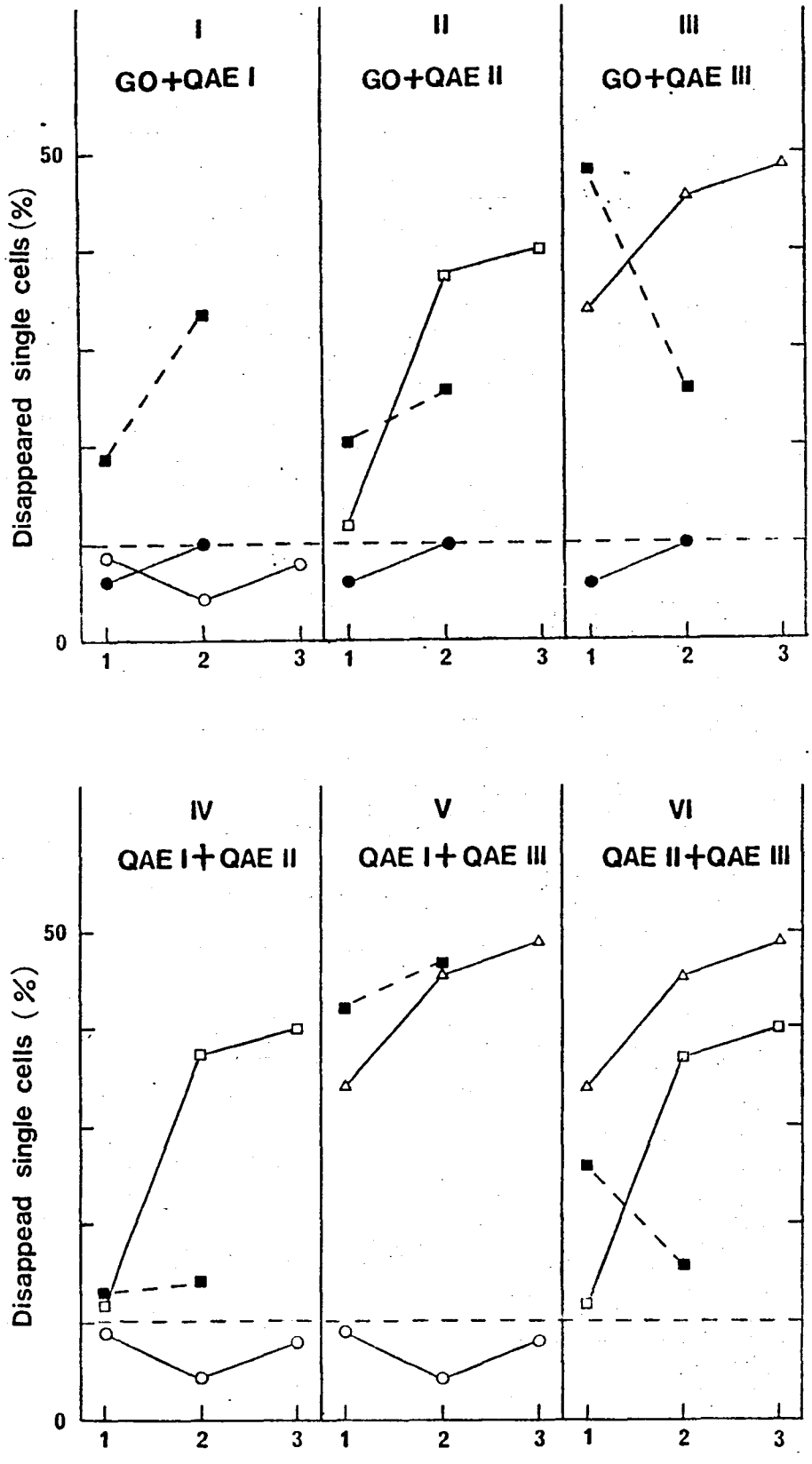
I
GO+QAE I
II
GO+QAE II
III
GO+QAE III
IV
QAE I+QAE II
V
QAE I+QAE III
VI
QAE II+QAE III
Disappeared single cells (%)
Disappead single cells (%)
50
0
1
2
3

Fig.5

Figure 5. The mixing effects of each factor on GA-cell aggregation.

Activity peacks, i.e., QAE I , II and III fractions on QAE Sephadex column (Fig. 3) were collected and dialyzed against PBS(-) for 3 days. These samples and G0 samples were used for experiments. ● : G0 sample prepared as described in the legend to Fig.4. The G0 concentration equivalent to the amount of glucuronic acid was as follows. 1: 2 μg/ml. 2: 4 μg/ml. Protein concentrations of 1, 2 and 3 on abscissa corresponded to 0.7, 2.1 and 7 μg/ml in the case of QAE I sample (○), 1 , 3 and 10 μg/ml in the case of QAE II sample (□) and 0.6, 1.8 and 6 μg/ml in the case of QAE III sample (△). The mixtures (■) of those factors were prepared as follows. One sample shown as 1 and 2 on abscissa was mixed with another one shown as 1 and 2, respectively. I, II and III were the mixtures of G0 sample with QAE I , QAE II and QAE III samples, respectively. IV and V were the mixtures of QAE I sample with QAE II and QAE III samples, respectively. VI : the mixture of QAE II sample with QAE III sample.

-----: the level of cell aggregation in control solution.

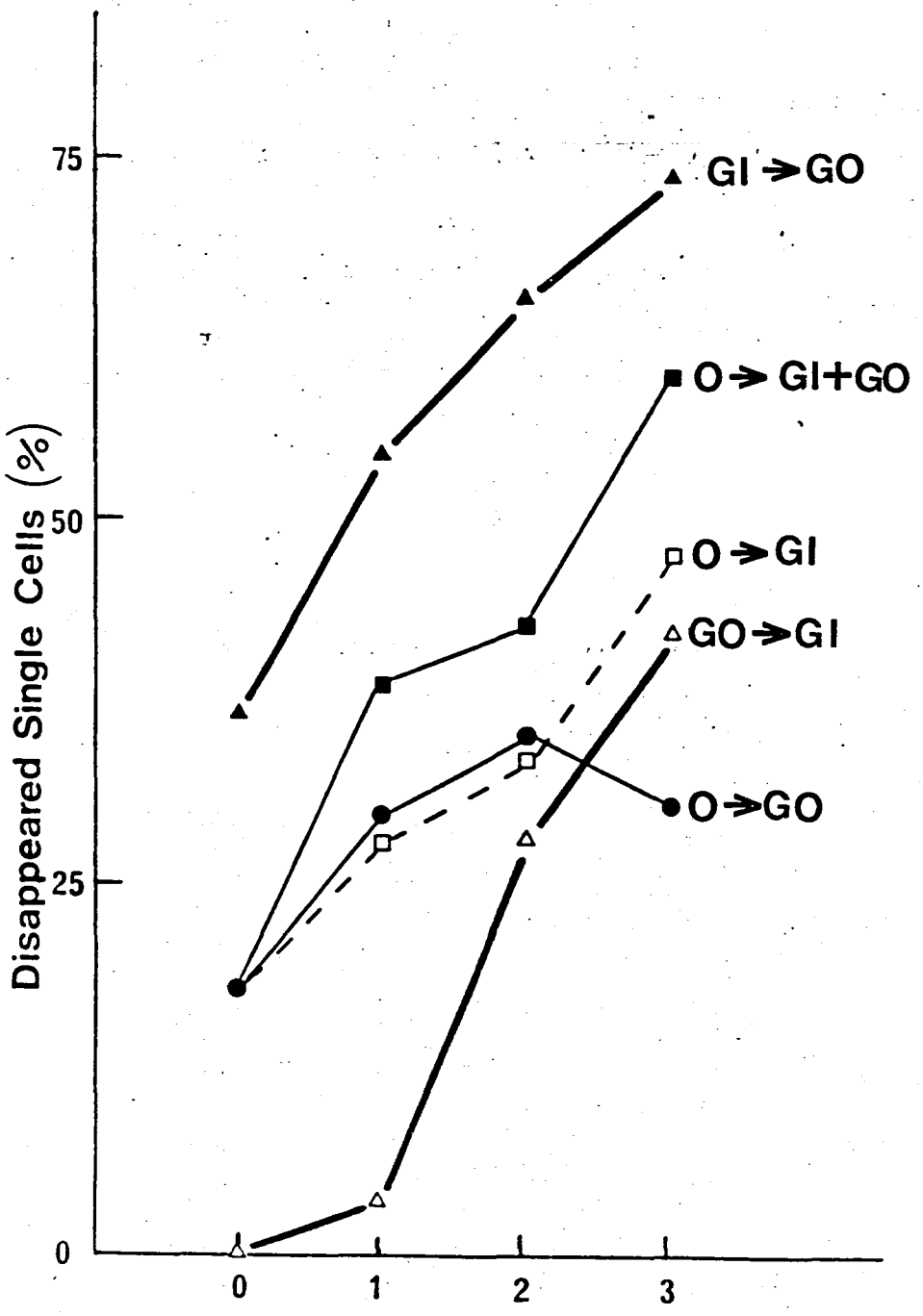
75
50
25
0
Disappeared Single Cells (%)
0
1
2
3
GI → GO
O → GI+GO
O → GI
GO → GI
O → GO

Fig. 6

Figure 6. Effects of factors on aggregation of GA cells which were treated previously with a factor.

GA cells were shaked on a gyratory shaker (90 rpm) at 37°C for 24hr in the first mediume without factors (□, ■ , ●) or in medium containing factors, i.e., G0 sample (△, 2 μg glucuronic acid per ml) and G I sample (▲, 10 μg protein per ml). Cells were collected and washed with PBS(-). Cells were then shaked (70 rpm) at 37°C for 24hr in medium containing factors, i.e., G I sample, G0 sample or their mixture. Abscissa in the figure represents arbitrary concentration of factors in the second medium; 0, 1, 2 and 3 correspond to 0, 3, 10 and 20 μg protein of G I sample per ml (□, △), respectively, and 0. 0.6, 2 and 4 μg glucuronic acid of G0 sample per ml (▲, ●), respectively. Each G I sample shown as 0, 1, 2 and 3 was mixed with each G0 sample shown as 0, 1, 2 and 3, respectively and the mixtures (■) were shown by 0, 1, 2 and 3, respectively.