CO-OPERATIVITY OF LECTIN BINDING TO FIBROBLASTS AND ITS RELATION TO CELLULAR ACTOMYOSIN

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SUMMARY

We have investigated the binding of $^{125}$I-concanavalin A ($^{125}$I-Con A) and $^{131}$I-succinyl concanavalin A ($^{131}$-s-Con A) to rat fibroblasts (16C line) as a function of the concentration of added lectin, and the alterations to this binding behaviour caused by drugs which modify the cytoskeleton. The changes in cell behaviour which occur at different levels of binding have also been studied. As shown previously for some other systems, the binding of Con A is complex and partly co-operative. Three phases can be distinguished in our system: (i) pre-nucleation binding, (ii) binding which shows a small positive slope in a Scatchard plot and a Hill coefficient greater than unity, and which therefore is incipiently co-operative, and (iii) post-co-operative binding.

The co-operative phase of binding is paralleled by progressive inhibition of EGTA-mediated cell detachment from substrata, with inhibition being complete when this phase of binding is complete. Likewise, the phagocytosis of latex spheres is progressively inhibited up to a threshold which coincides with the completion of co-operative binding. Thirdly, cells pretreated with Con A round up with colchicine ($10^{-6}$ M) if co-operative binding is complete, but adopt broad epithelial shapes if it is not. s-Con A does not show co-operative binding, and correspondingly does not inhibit EGTA-mediated cell detachment, or show a distinct threshold in the inhibition of phagocytosis, or promote the 2 types of shape change with colchicine.

The pattern of Con A binding is drastically altered by pretreatment of cells with cytochalasin B or azide. The Scatchard and Hill plots show that the co-operative phase remains and is complete at about the same level of binding, but that it is more readily nucleated and takes place against a changed number and/or distribution of receptors. Pretreatment of cells with colchicine causes changes in the pattern of binding which are different from those observed with cytochalasin B or azide and are more difficult to interpret.

We conclude that a reciprocal relationship exists between the cellular actomyosin and the state of cell surface receptors. Perturbation of actomyosin by cytochalasin B or azide can enhance the freedom of some receptors to participate in a co-operative rearrangement which facilitates the binding of further molecules of lectin. Vice versa, the co-operative event has a feedback influence on the cellular actomyosin to cause alterations of cellular response.

INTRODUCTION

For many animal cells it seems that the binding of multivalent ligands to the outer surface, can induce the formation of physical linkages to the internal actomyosin system (Koch & Smith, 1978; Flanagan & Koch, 1978; Ash, Louvard & Singer, 1977; Bourgignon & Singer, 1977). One particular form of surface cross-linking of fibroblasts is in the attachment to inert substrates which occurs through cell adhesion zones (Abercrombie, Heaysmann & Pegrum, 1971; Heath & Dunn, 1978). These
are specialized structures in which surface macromolecules are arranged with distinct 'side-to-side' periodicity and interact with actin microfilament bundles through a similarly ordered cytoplasmic protein plaque (Rees, Lloyd & Thom, 1977; Rees et al. 1978). Remote from the adhesion zones, a class of receptor is grouped over at least some areas of the actomyosin fibres and disperses when these fibres disperse, suggesting that it is also linked to the microfilament bundles (Badley, Woods, Carruthers, Smith & Rees, unpublished). Subsequent binding of multivalent ligands such as lectins or antibodies can cause other receptors to move towards the already organized actomyosin, apparently to form physical linkages (Ash & Singer, 1976). For cells in suspension it seems that similar interactions can occur between each of a series of subpopulations of membrane proteins and less visibly organized actomyosin structures (Bourgignon & Singer, 1977; Flanagan & Koch, 1978).

The detailed nature of the changes that follow receptor clustering by lectins depends to some extent on the origin and history of the cells, but general similarities do exist. Receptors form patches which then move together on the cell surface (ultimately to cap in some cells) in a process that is believed, because it is inhibited by azide, 2-deoxyglucose and cytochalasin B, to result from an active contraction (Taylor, Duffus, Raff & de Petris, 1971; Loor, Forni & Pernis, 1972; Ukena, Borysenko, Karnovsky & Berlin, 1974). For fibroblasts, this effect is greater for tumour cells in suspension (Nicolson, 1973) but it remains marked for substrate-attached cells after protease treatment or transformation (Rosenblith et al. 1973; Ukena et al. 1974; reviewed by Nicolson, 1974) and limited receptor redistribution is observed in at least some normal cells (Fernandez & Berlin, 1976; Ash & Singer, 1976). Shape changes occur which confirm the impression that the lectin has stimulated an internal contraction. These include the withdrawal of the cell periphery and distortions which are sensitive to metabolic inhibitors (Brown & Revel, 1976; Abertini & Anderson, 1977; de Petris, 1975; Storrie, 1974). The arrays of cross-linked receptors are considered to constrain further lateral movement and, as shown by electron microscopy and immunofluorescence (Rees et al. 1977), limit the dispersal of actomyosin bundles by trypsin.

To investigate further the mechanisms of these changes, we have examined the quantitative binding of concanavalin A (Con A), to correlate this with cellular behaviour and membrane properties. We chose to work mostly with a cell line that synthesizes little extracellular fibronectin, since the presence of large and perhaps variable amounts of this Con A receptor would obscure the behaviour of membrane components. We found, as others have in different systems, that binding is a complex process which in one phase shows some elements of co-operative character in Scatchard and Hill plots. Drugs which interfere with actomyosin function are found to facilitate nucleation of the co-operative event. Several cellular responses to surface binding are associated with this co-operative event, namely the inhibition of phagocytosis, the blockage of EGTA-mediated detachment from inert substrata and the effect on the alterations in cell shape induced by colchicine.
MATERIALS AND METHODS

Materials

Concanavalin A was obtained from Pharmacia (Uppsala, Sweden), succinyl concanavalin A (s-Con A) was obtained from L’Industria Biologique Francais. It had been prepared by the 2-stage derivatization procedure of Gunther et al. (1973) using succinic anhydride, contained an average of 10 succinyl groups per subunit of molecular weight 26000 and had a molecular weight of 56000 as determined in phosphate-buffered saline by ultracentrifugation. Colchicine, cytochalasin B and methyl α-D-mannopyranoside were from Sigma Chemical Co. (London) and polystyrene latex particles (0.794 μm diameter) from Serva Feinbiochemica (Heidelberg, West Germany). Sodium 125I-iodide (199 mCi/ml; code IMS 30) was obtained from the Radiopharmaceutical Centre, Amersham. All other reagents were of Analytical grade.

Cell culture

An established line of rat dermal fibroblasts (16C, Colworth strain) and a commercial preparation of rat embryo fibroblasts (Gibco-Biocult, Paisley, Scotland) were maintained and grown in 230-ml glass medical flat bottles in Dulbecco’s modification of Eagle’s medium (Gibco-Biocult, special formulation) containing 10% foetal calf serum and kanamycin (Flow Laboratories, Irvine, Scotland) 500 μg/ml, in a gas phase of 10% CO2 in air.

The rat embryo fibroblasts were frozen on arrival and fresh cultures were started every month from the frozen stock.

Preparation of 125I-lectins

125I-labelled Con A or s-Con A was prepared by lactoperoxidase-catalysed iodination by a method based on those of Arndt-Jovin & Berg (1971) and Weeks (1975). Typically the lectin (100 mg) was dissolved in phosphate-buffered saline (5 ml: PBS; containing in g per l., 8.0 NaCl, 0.2 KCl, 0.2 KH2PO4, 0.3 Na2HPO4·12H2O) pH 7.2, containing 0.1 M methyl-α-D-mannopyranoside and 100 μl of a solution (5 mg/ml) of lactoperoxidase (lyophilized powder from Sigma Chemical Co.) in PBS were added, followed by Na125I-iodide (1 mCi). A solution (25 μl) of 10 mM hydrogen peroxide in PBS was used to initiate the reaction. After 20 min at 20 °C, a further aliquot (25 μl) of the peroxide solution was added and the reaction was left for another 20 min. The reaction was stopped by addition of an ice-cold solution (50 ml) of 5 mM 2-mercaptoethanol in PBS and the solution was dialysed overnight against PBS.

The iodinated proteins were purified by affinity chromatography on a column, 2.5 x 90 cm, of Sephadex G50 (Agrawal & Goldstein, 1967). The specifically bound lectins were eluted with methyl α-D-mannoside (0.1 M in PBS), the sugar was removed by dialysis against PBS and the product was stored frozen in small aliquots at a concentration of 1 mg/ml.

We routinely confirmed that new preparations of iodinated Con A or s-Con A retained the native binding affinity by using a varying concentration of the 125I-lectin (20-100 μg/ml) made up to a constant total concentration with native lectin (100 μg/ml). In each case graphs of specific binding to 16C fibroblasts or trypsinized human red blood cells against the concentration of 125I-lectin gave a straight line through the origin.

Binding of iodinated lectins to fibroblasts

Rat 16C fibroblasts were seeded (6 x 10^6/bottle) in medical flat bottles (160 ml) and allowed to grow for 40 h to establish a dense, but not confluent, monolayer. Each bottle contained 10 ± 2.5 x 10^4 cells. The cells were rinsed (3 times) with Hanks’ buffered saline solution (HBSS; containing in g per l., 8.0 NaCl, 1.0 glucose, 0.4 KCl, 0.185 CaCl2·2H2O, 0.2 MgSO4·7H2O, 0.35 NaHCO3 and 0.0475 Na2HPO4), then 125I-lectin solutions (5 ml) at appropriate concentrations (in HBSS) were added. Non-specific binding of iodinated lectins to cells was determined by measurements in the presence of methyl α-D-mannoside (0.95 M).

After incubation at 37 °C for 30 min the solutions were decanted and each monolayer was washed for 3 10-min periods with HBSS. PBS (5 ml) was added to each bottle and the cells...
were scraped off using a rubber policeman, then shaken vigorously to achieve a single-cell suspension.

Bound radioactivity was measured in aliquots (1 μl) of the cell suspension (series 5000 auto-α-spectrometer; Packard Instrument Co., Illinois). Cell number was determined by analysis of the protein precipitated by ice-cold 5% trichloroacetic acid from duplicate aliquots (1 μl) of the same suspension, using the method of Lowry, Rosebrough, Farr & Randall (1951). The relationship between protein concentration and cell number, as determined by a Coulter Electronic Particle Counter (model ZB, Coulter Electronics Limited, Harpenden), had been determined previously using similar cell cultures. The average number of 1125-I-lectin molecules specifically bound per cell was calculated and plotted as shown in Fig. 2, p. 123. Points were read off from the best curve and replotted using both Scatchard (1949) and Hill (1913) treatments. The concentration of free lectin at each of these points was calculated from the difference between the total number of molecules added to the bottle and the number bound by the 10^9 cells. For the Hill treatment it is necessary to know the total number of lectins bound/cell at saturation; this was obtained by extrapolation from the Scatchard plot.

To measure the binding at 0 °C, cells were first cooled rapidly by the addition of a large volume (25 ml) of ice-cold HBSS and then incubated on ice for 10 min. The buffer was decanted and prechilled, ^1125^-Con A in HBSS was added. The bottles were incubated on ice for 30 min and cells were washed with ice-cold HBSS.

The effect of colchicine on ^1125^-Con A binding was determined by preincubating the cells with colchicine in HBSS (10^-6 M; 5 μl/ml) for 1 h at 37 °C. The ^1125^-Con A solutions also contained 10^-6 M colchicine (in HBSS). Similar procedures were used for cytochalasin B and sodium azide treatment at concentrations of 10 μg/ml and 10^-4 M, respectively, with the only modification being that PBS was used as buffer for all solutions containing azide.

The effect of Con A and s-Con A on cell behaviour

Phagocytosis. Phagocytosis and its inhibition by Con A and s-Con A were measured using polystyrene latex particles (0.794 μm diameter). Cells were harvested with trypsin and seeded into 230-ml medicine flat bottles (1.5 × 10^8 cells/bottle) and allowed to settle and spread in complete medium for 16 h. Monolayers were washed (3 times) with HBSS and incubated with appropriate concentrations of Con A or s-Con A in HBSS for 20 min at 37 °C. Latex particles (2 × 10^10/bottle) were added and incubated with the cells for 30 min at 37 °C, which were then thoroughly washed. Excess beads were removed by washing the monolayer with HBSS (3 times) and cells were harvested with tetracaine (4 mM in Ca^2+-and Mg^2+-free HBSS) and collected and washed on the centrifuge (5–6 times) until there was no visible evidence of beads in the supernatant. The final pellet was extracted overnight with 1 ml dioxan and after centrifugation the level of phagocytosis was determined from the extinction coefficient of the supernatant solution at 259 nm, taking into account the results of control experiments in which cells were extracted with dioxan without prior incubation with latex particles.

In preliminary experiments the final pellet of washed, sedimented cells which had been incubated with latex particles according to our standard procedures was examined in the electron microscope to determine the distribution of the beads. We found no evidence of beads outside the cells; all the beads were observed to be in endocytosed vesicles within the cytoplasm of the cells (Fig. 1). We concluded that our washing regime was sufficient to remove all particles non-specifically associated with the cell surface and that the latex measured in our experiments represents endocytosed material.

Inhibition of cell detachment. The rate of detachment of cells by ethylene glycol bis-(β-aminoethyl ether), N,N’-tetraacetic acid (EGTA) and the influence of lectin binding on this process was measured by a method similar to that previously described (Rees et al. 1977). Cells were harvested with trypsin and seeded in 230-ml medicine flat bottles (1.5 × 10^6 cells/bottle) and allowed to settle and spread in complete medium for 16 h. Each monolayer was washed (3 times) with HBSS and incubated with the appropriate lectin solution in HBSS for 20 min at 37 °C, before decanting off the solution and rinsing with Ca^2+- and Mg^2+-free HBSS. EGTA (0.05% in Ca^2+- and Mg^2+-free HBSS) was added and incubated with the cells at 37 °C for periods up to 60 min. From time to time, a bottle was removed and the easily detachable cells were collected by gently rolling the medium several times over
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Fig. 1. Typical thin section of 16C cells containing phagocytosed latex particles. All the particles were observed to be within endocytosed vesicles (inset) in the cytoplasm of the cells and there was no evidence of particles outside the cells. The 16C cells were incubated with latex particles, washed, harvested and thoroughly rewashed by centrifugation as described in the Materials and methods section. The final cell pellet was fixed for 30 min at 37 °C in glutaraldehyde (3 %, v/v) in 0.01 M cacodylate buffer, pH 7.4, washed in 0.1 M cacodylate buffer and postfixed for 1 h at room temperature with osmium tetroxide (1 %, w/v) in 0.1 M cacodylate buffer, pH 7.4, followed by aqueous (1 %, w/v) uranyl acetate for 1 h. Dehydration was through a graded series of ethanol concentrations. The sample was embedded in Spurr's resin, sectioned, stained with uranyl acetate (3 %, w/v) in aqueous ethanol (50 %, v/v), followed by lead citrate (Reynold's); cell, ×8000; inset showing membrane enclosed particles, ×33000.
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the monolayer, then counted using a Coulter counter. The remainder of the cells were harvested with tetracaine (4 mM in Ca²⁺- and Mg²⁺-free HBSS) and also counted, thus enabling the percentage of cells detached by EGTA to be calculated.

The effect of colchicine and cytochalasin B on the inhibition of EGTA-induced detachment by Con A and s-Con A, was analysed by pre-treating the cells for 60 min at 37 °C with colchicine (10⁻⁶M) and/or cytochalasin B (10 μg/ml) in Dulbecco’s modified Eagle’s medium (DMEM; Gibco-Biocult) containing 10% (v/v) foetal calf serum. Cytochalasin B (10 μg/ml) was dissolved in ethanol and diluted to 10 μg/ml with DMEM for these experiments. Control experiments showed that the low concentration (0.1%) of ethanol in the final medium had no visible effect on the cells. In the experiments involving Con A the cells were pretreated with the drug, followed by Con A solution in which the drug was present at the same level.

Colchicine-induced changes in morphology. Cells were harvested with trypsin, seeded onto glass coverslips (22 mm diameter; 2 × 10⁴ cells per coverslip) and incubated in complete medium for 40 h at 37 °C. They were rinsed (3 times) with HBSS, pH 7.4, and then incubated in lectin solution (1-100 μg/ml in HBSS) for 20 min at 37 °C, again rinsed (3 times), and incubated in colchicine (10⁻⁶ M) in HBSS for 30 min at 37 °C. After further washing, the cells were fixed with 1% glutaraldehyde in PBS and mounted for examination under a Leitz Ortholux II microscope and photographed on Ilford FP-4 film.

RESULTS

The binding of ¹²⁵I-Con A and s-Con A

Lactoperoxidase-catalysed iodination gave labelled products having typical activities of 1 × 10⁴ cpm/μg and 7 × 10³ cpm/μg for Con A and its divalent derivative, s-Con A, respectively. Self-iodinated lactoperoxidase was removed by the affinity chromatography step. For quantitative measurements of lectin binding the incubation conditions chosen were 30 min at 37 °C because: (i) previous kinetic studies (Schmidt-Ullrich, Wallach & Hendricks, 1976; Gachelin, Buc-Caron, Lis & Sharon, 1976; Weeks, 1975) have shown that, over the concentration range used here, binding of lectins to cells is virtually complete within 10 min and is certainly complete in 30 min; and (ii) this incubation period is close to that used in our earlier studies of lectin inhibition of cell detachment by proteases and chelating agents (Rees et al. 1977, 1978). The variation of lectin binding to monolayers of rat (16C) fibroblasts with the concentration of Con A and s-Con A is shown in Fig. 2. At 0 °C, where the evidence is that Con A exists as a dimer (Huet, Lonchamp, Huet & Bernadac, 1974; Huet, 1975), rather than the usual tetramer, the binding gave a hyperbolic curve which plateaued at ~ 55% of the amount of Con A bound at 37 °C. The apparent similarity of the binding curves for Con A at 0 °C and s-Con A is superficial, since molecular weights of 110000 and 55000 were used for Con A and s-Con A respectively, so that the binding of dimeric iso-Con A at 37 °C and of the proposed dimer at 0 °C actually differ by a factor of two. Perhaps more interestingly, the binding curve for Con A at 37 °C showed a sigmoidal form (Fig. 2) at low concentrations (< 10 μg/ml) which was not observed at 0 °C or for s-Con A.

Plotting the results according to Scatchard (1949) emphasizes the differences in the binding processes. At 37 °C the binding of ¹²⁵I-Con A shows 3 separate phases (Fig. 3), namely (i) binding to sites of highest affinity which for reasons to be explained we call 'pre-nucleation binding', (ii) binding which shows a small positive slope in the Scatchard plot and a coefficient of 1.29 in a Hill plot (not shown) and which is
Cellular response to lectin binding

Fig. 2. Concentration-dependent binding of $^{125}$I-Con A and $^{125}$I-succinyl Con A to rat (16C) fibroblasts; A, 5-100 μg/ml lectin; B, more detailed investigation at low (< 10 μg/ml) concentrations of lectin. Binding experiments were carried out with Con A at 37 °C (○—○) and 0 °C (■—■) and s-Con A at 37 °C (□—□) on cells grown for 40 h in flat (160-ml) medicine bottles. On average the bottles contained $10^6$ cells. Non-specific binding was determined in the presence of methyl α-D-mannoside (0-05 M) and deducted from the total binding. Each point is the average of 4 independent binding assays.

Fig. 3. Correlation of the binding of $^{125}$I-Con A at 37 °C to rat (16C) fibroblasts with its effect on cell behaviour. A, binding; B, protection; C, phagocytosis. The binding data have been plotted according to Scatchard (1949) and to facilitate the comparison the concentration of lectin used in the analysis of binding is also given on the abscissa. The rate of detachment of the cells by EGTA (0-05 % in Ca$^{++}$- and Mg$^{++}$-free HBSS at 37 °C) and the inhibition of this by Con A was measured by a method closely analogous to that previously described (Rees et al. 1977). In B, curves A, B, C, D show control and media containing 2-5, 5-0 and 10-0 μg/ml Con A, respectively. Phagocytosis and its inhibition by Con A were measured using latex spheres (0-8 μm; 2 x 10$^{10}$/bottle). Cell monolayers were washed with HBSS (3 times) and incubated with the appropriate concentrations of Con A solutions in HBSS for 20 min at 37 °C. After rewashing with HBSS (3 times), the monolayers were incubated with the spheres for 30 min (37 °C), thoroughly washed again with HBSS and, following detachment with tetracaine (4 mM in Ca$^{++}$- and Mg$^{++}$-free HBSS), they were collected and rewashed on the centrifuge. The cell pellet was extracted with dioxan (1 ml) overnight and, after removal of the cells by centrifugation, phagocytosis of latex particles was estimated from the extinction coefficient at 259 nm.
therefore incipiently co-operative, and (iii) a post-co-operative phase in which binding apparently becomes progressively more difficult – as we would expect if fewer sites remain, possibly of lower affinity. In contrast the binding of s-Con A (Fig. 4) or of Con A at 0 °C shows no evidence of a co-operative phase but only binding to at least 2 classes of sites with different affinities.

**Fig. 4.** Correlation of the binding of 125I-succinyl Con A to rat (16C) fibroblasts at 37 °C with its effect on cell behaviour. The binding data (A), inhibition of EGTA-mediated cell detachment (B) and inhibition of phagocytosis (C) were measured as described under Fig. 3. In B, O, △, and ● show control and medium with 10 and 100 μg/ml of s-Con A, respectively.

**Correlation of lectin binding with cellular response**

We have shown elsewhere (Rees et al. 1977, 1978) that EGTA causes fibroblast detachment primarily through its action on the cytoskeletal components responsible for cell flattening and that rounding and detachment is blocked by prior binding of Con A (Rees et al. 1977). We now show that this inhibition is much diminished at low levels of bound Con A and that complete inhibition coincides with completion of the co-operative phase of binding (Fig. 3). Similar effects are not seen with s-Con A (Fig. 4). Secondly, the phagocytosis of latex spheres is progressively inhibited as the concentration of Con A increases until a threshold is reached which again correlates with completion of the co-operative phase of binding (Fig. 3). Above

**Fig. 5.** The influence of Con A and s-Con A on colchicine-induced changes in the morphology of rat (16C) fibroblasts. The cells were grown on glass coverslips for 40 h at 37 °C, washed and incubated with the lectin solutions for 20 min at 37 °C. After further washing they were incubated in the colchicine solution (10^-6 M in HBSS) for a further 30 min at 37 °C before being fixed, mounted and examined under phase optics using a Leitz Ortholux II microscope. Details are in the Materials and methods Section; A, control, untreated 16C fibroblasts; B, 16C fibroblasts treated with colchicine (10^-6 M); C, treated with 2 μg/ml Con A followed by the colchicine treatment; D, as C but using 5 μg/ml Con A; E, as C but using 100 μg/ml Con A; F, treated with 1 μg/ml s-Con A followed by the colchicine treatment; G, as F but using 20 μg/ml s-Con A; H, as F but using 100 μg/ml s-Con A. Bars equal 10 μm.
Cellular response to lectin binding
this concentration little further inhibition of phagocytosis is observed. Thirdly, when 
pretreated at levels of Con A insufficient to complete the co-operative phase of 
binding, 16C fibroblasts do not show the usual rounding when treated with colchicine 
($10^{-6}$ M). Instead (Fig. 5) they spread and flatten, adopting broad epithelial shapes 
somewhat reminiscent of these cells treated with a mixture of colchicine, dibutyryl-
c-AMP and theophylline (Lloyd, Smith, Woods & Rees, 1977). When co-operative 
binding is complete, however, pronounced rounding is again observed (Fig. 5) and 
indeed may even be enhanced relative to control cells.

Such correlations are not limited to 16C fibroblasts. As shown in Fig. 6, rat 
embryo fibroblasts also have a distinct endpoint for the inhibition of phagocytosis 
by Con A which can be correlated with the concentration dependence of ‘protection’ 
against detachment by EGTA. A third cell type, BHK cells, also showed distinct 
evidence of such a correlation, although the initial level of phagocytosis was con-
siderably lower and the cells showed day-to-day variations which made exact quanti-
fication difficult.

Further evidence that co-operativity in binding is somehow linked to the modi-
fications of cellular response is that s-Con A for which co-operative binding is not 
detectable (Fig. 4) correspondingly does not cause a distinct threshold in the inhibition 
of phagocytosis (Fig. 4), or the 2 types of shape change with colchicine (Fig. 5), or 
the inhibition of detachment of cells from their substrate by EGTA (Fig. 4). The 
binding of s-Con A causes only a very gradual and relatively inefficient inhibition 
of phagocytosis, increasing with concentration of lectin. With colchicine, s-Con A-
treated cells change to flattened, epithelial shapes analogous to those observed for 
concentrations of Con A which are insufficient to complete the co-operative phase 
of binding (Fig. 5).

Con A is also known to inhibit trypsin-mediated cell detachment and we found 
that this inhibition is also dependent upon the solution concentration of the lectin.
Cellular response to lectin binding

However, the threshold occurs at a lower concentration than is necessary to complete the co-operative binding to the unperturbed cell – actually at 0.1–0.5 of this concentration.

The influence of cytochalasin B and sodium azide

Insight into the relation between the molecular events occurring in the pre-nucleation and co-operative phases of lectin binding can be derived from the binding behaviour of cells treated with cytochalasin B or azide which interfere with actomyosin function (Fig. 7). The total number of lectin molecules bound per cell (estimated by extrapolation from the Scatchard plot) increased from 9.5 x 10⁷ for untreated cells to 28 and 20 x 10⁷ after cytochalasin B and azide respectively, and the shapes of the Scatchard plots (Figs. 8, 9) suggest that the increase is due to the exposure of a new class of receptor with low affinity for Con A. The Scatchard plots and the Hill plots (not shown) provide clear evidence of a co-operative phase in the binding, and the Hill coefficients which characterize this (1.29, 1.35 and 1.21 respectively for control, cytochalasin-B-treated and azide-treated cells) were very similar for the cells in all 3 states. We deduce that the molecular events underlying co-operative behaviour in the drug-treated cells are probably analogous to those in untreated cells but that they take place against a changed number and/or distribution of other membrane receptors. A dramatic change (Fig. 8) for cytochalasin-B-treated cells was that the pre-nucleation phase was not observed.

Comparison of the solution concentrations of Con A at which the maximum occurs in the Scatchard plot (6 and 1.5 μg/ml, respectively, for untreated and
Fig. 8. Correlation of the binding of $^{128}$I-Con A (A) to rat (16C) fibroblasts at 37 °C in the presence of cytochalasin B (10 μg/ml) with the inhibition of EGTA-mediated cell detachment (B) under the same conditions. The cell monolayers were preincubated with cytochalasin B in HBSS at 37 °C for 1 h and appropriate $^{128}$I-Con A solutions (in HBSS) also contained the drug at the same concentration. Binding was measured as described under Fig. 6 and has been replotted according to Scatchard. The rate of detachment of cytochalasin B-treated cells by EGTA and its inhibition by Con A were measured as described under Fig. 2: ○—○, untreated control cells; •—•, cells pretreated with cytochalasin B; △—△, cells pretreated with a Con A 0.75 μg/ml solution; ▲—▲, cells pretreated with cytochalasin B followed by a 0.75 μg/ml Con A solution which also contained the drug; □—□, cells pretreated with a 20 μg/ml Con A solution; ■—■, cells pretreated with cytochalasin B followed by a 20 μg/ml Con A solution which also contained the drug.

Fig. 9. Scatchard plot of the binding of $^{128}$I-Con A to rat (16C) fibroblasts at 37 °C in the presence of sodium azide (10$^{-3}$ M). Binding was measured as described under Fig. 6 and points were read off from the best curve and replotted according to Scatchard.
Cellular response to lectin binding

Fig. 10. Correlation of the binding of 125I-Con A(A) to rat (16C) fibroblasts at 37 °C in the presence of colchicine (10⁻⁶ M) with the inhibition of EGTA-mediated cell detachment (B) under the same conditions. The cell monolayers were preincubated with colchicine in HBSS at 37 °C for 1 h and appropriate 125I-Con A solutions (in HBSS) also contained the drug at the same concentration. Binding was measured as described under Fig. 6 and plotted according to Scatchard. The rate of detachment of colchicine-treated cells by EGTA and its inhibition by Con A were measured as described under Fig. 2: ○—○, control untreated cells; •—•, cells pretreated with colchicine; △—△, cells pretreated with a 4 μg/ml Con A solution; ▲—▲, cells pretreated with colchicine followed by a 4 μg/ml Con A solution which also contained the drug; □—□, cells pretreated with a 20 μg/ml Con A solution; ■—■, cells pretreated with colchicine followed by a 20 μg/ml Con A solution which also contained the drug.

Fig. 11. The rate of EGTA-mediated detachment of 16C fibroblasts which have been pretreated with colchicine (10⁻⁶ M) in conjunction with cytochalasin B (10 μg/ml), and its inhibition by Con A. Cell monolayers were preincubated with the drugs in HBSS at 37 °C for 1 h and the Con A solutions (in HBSS) also contained the drugs at the same concentration. Detachment by EGTA was measured as described under Fig. 2: ○—○, control untreated cells; •—•, cells pretreated with cytochalasin B and colchicine; △—△, cells pretreated with a 20 μg/ml Con A solution; ▲—▲, cells pretreated with cytochalasin B and colchicine followed by a 20 μg/ml Con A solution which also contained the drugs.
cytochalasin B-treated cells) or the free Con A concentrations at the mid-points of the co-operative phases in Hill plots (7.5 and 0.45 µg/ml, respectively) also show that co-operative binding is more easily induced after cytochalasin B treatment. However, the extent of binding at the maximum in the Scatchard plot was very similar to that in untreated cells (2.2.5 x 10^7 molecules of Con A per cell).

As shown in Fig. 8, EGTA remains effective in detaching cells treated with cytochalasin B, and Con A inhibits this completely but only if the co-operative phase of binding is complete.

For azide-treated cells the results were more complicated by the changed number of binding sites but it was still very clear (Fig. 9) that the co-operative character was present. The mid-point of the co-operative transition as measured from the Hill plot was around 2.9 µg/ml free Con A, showing that the co-operative event was again more easily nucleated in the treated relative to control cells.

The influence of colchicine

After treatment with colchicine, the binding behaviour of 16C fibroblasts (Figs. 7, 10) shows 3 distinct phases similar to those of control cells. The total number of lectin molecules bound per cell (12.5 x 10^7) was again increased and the Hill plot indicates that the co-operative phase may extend over a wider concentration range and that its mid-point is therefore shifted to higher free Con A concentrations (~ 10 µg/ml). Colchicine treatment does not alter the susceptibility of 16C cells to EGTA-mediated detachment (Fig. 10) or prevent its inhibition by sufficiently high concentration of Con A or the relationship of this inhibition to the co-operative phase of binding (Fig. 10). Treatment with mixtures of colchicine and cytochalasin B also failed (Fig. 11) to prevent this Con A-mediated protection against cell detachment by EGTA.

DISCUSSION

Earlier work with a number of cell types, namely T lymphocytes (Bornens, Karsenti & Avrameas, 1975, 1976; Pruiansky, Ravid & Sharon, 1978), fat cells (Cuatrecasas, 1973), erythrocytes (Reisner, Lis & Sharon, 1976), and teratocarcinoma cells (Gachelin et al. 1976), has given evidence for co-operativity in the early phase of binding of a number of different lectins to cell surfaces. The effect seems to be retained in the isolated membranes when cells are lysed and cytoplasmic components removed (Riordan & Slavik, 1974; Schmidt-Ullrich et al. 1976; Carraway, Jett & Carraway, 1975) but not to be observed for isolated receptors (Riordan & Slavik, 1974) or model saccharides. In this last respect this phenomenon therefore differs from the co-operative binding of the oligosaccharide moiety of the ganglioside receptor to cholera toxin (Sattler et al. 1978).

Because the co-operativity of lectin binding to cells and to membranes is abolished by prior fixation (Bornens et al. 1976; Schmidt-Ullrich et al. 1976), is promoted by increased valency of the lectin without known change in binding specificity (Bornens et al. 1976; Pruiansky et al. 1978), and because the patching and capping
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of bound lectin are so well documented, it has been proposed that the origin of co-operativity is in rearrangement and clustering of receptors in the membrane (Bornens et al. 1976; Schmidt-Ulrich et al. 1976; Prujansky et al. 1978). Binding to T lymphocytes is drastically modified by pretreatment with a mixture of colchicine and cytochalasin B (although the influence of either drug alone is much less marked); therefore it has been suggested that submembranous proteins linking receptors to the cytoskeleton can assist the co-operative process by their own tendency to aggregate (Karsenti, Bornens & Avrameas, 1977).

It has been pointed out that the cellular responses to lectin stimulation often occur at levels much below saturation — in the range where co-operativity is seen (Cuatrecasas, 1973; Riordan & Slavik, 1974; Bornens et al. 1976; Gachelin et al. 1976; Carraway et al. 1975). Therefore causal connexions could exist between the co-operative events and the biological stimuli. The consequences include insulin-like stimulation of fat cells, mitogenesis of lymphocytes, agglutination of erythrocytes, and a suppression or enhancement of enzyme activities in the membrane. Recent studies (Rees et al. 1978; Prujansky et al. 1978) have confirmed that cellular response can indeed be closely correlated with co-operative binding of lectins. Mitogenic stimulation of neuraminidase-treated mouse and rat lymphocytes was observed only when binding of a range of lectins (soy bean agglutinin, peanut agglutinin, each of these lectins in glutaraldehyde-polymerized form, and leucoagglutinin), to the cells was co-operative (Prujansky et al. 1978). In one case, the same lectin (peanut agglutinin) was mitogenic for rat cells to which it bound co-operatively but not for mouse cells to which it did not. These and other considerations led to the proposal that the co-operativity results from a change in the membrane structure which is an essential event in the stimulation process.

Our own initial studies (Rees et al. 1978) and the results which we now discuss are for a cell type for which such studies have not so far been reported, namely substrate-attached fibroblasts. We have been able to derive further insight into the nature of the co-operative event, its relation to cytoplasmic structures and its implications for altered cellular response. Our results and interpretations are entirely consistent with the observations for lymphocytes, although in addition we emphasize the involvement of the cellular actomyosin in the response to lectin stimuli. It seems likely that co-operative grouping of membrane receptors might represent a rather general phenomenon which triggers a variety of cellular responses and that many of the conclusions will be found to apply to other cell types as well.

The existence of co-operativity shows that the first molecules of Con A to bind to the fibroblast surface somehow facilitate the binding of further molecules — presumably at least in part by making it possible for the later molecules to satisfy more valencies as they attach. Theoretical analyses of model systems (Hill, 1967; Hill & White, 1967; Wagner, 1969), confirm that co-operativity could arise in this way and point to a possible mechanism. This is that initial binding induces a changeover from a side-to-side spacing of receptors which may be regular or irregular, to a new spacing which is more favourable for the multivalent attachment of the lectin molecules that are bound subsequently. The change would occur at a point where
the total binding energy just compensates for the energetic and/or entropic dis-
advantage that the new spacing would have in the absence of cross-linking. The
fact that co-operativity was not observed in the binding of s-Con A at 37 °C or
Con A at 0 °C is consistent both with the requirement for a rearrangement resulting
from efficient cross-linking and with the origin of the binding enhancement being
in the multivalent character of Con A. The initial phase of binding would then
include the formation of lectin-receptor associations before the critical stage at
which the switch occurs to the new spacing. These first associations serve as units
in the later rearrangement – and it is for this reason that we refer to their formation
as the ‘prenucleation phase’. In other work we have confirmed that the physical
basis of co-operativity could indeed be in receptor rearrangement because the well
known patching phenomenon parallels co-operative binding under a variety of
conditions (Badley, Woods & Rees, unpublished).

Support for this interpretation comes from several observations on cells treated
with cytochalasin B. Despite the dramatic disappearance of the initial phase with
negative slope on the Scatchard plot, the number of molecules of Con A bound at
completion of the co-operative binding is very similar to that in control cells. This
suggests that the same family of receptors can show either single-phase (co-operative)
or 2-phase (prenucleation then co-operative) behaviour, depending on whether the
cells have been pretreated. That co-operative binding was indeed facilitated by
cytochalasin B was also shown by the lower concentrations of Con A required in
solution to bring about this phase and its completion. Somewhat similar to this
effect is the reported influence of prior trypsinization of human erythrocytes on the
binding of soy bean agglutinin (Reisner et al. 1976). Co-operative behaviour moved
to lower lectin concentrations but the absolute number of sites occupied at completion
was about the same as in untrypsinized cells.

The disappearance of a detectable prenucleation phase after cytochalasin B treat-
ment must mean that this agent diminishes the resistance to the lateral rearrangement
of the surface receptors. We cannot be certain whether the relevant action is on
extracellular or cytoskeletal components because it is known to cause the release of
well as perturbing the actomyosin system. The similar influence of azide does, how-
ever, suggest that the action is at least in part on the actomyosin and therefore that
functioning actomyosin actually hinders nucleation of the co-operative event. This
is probably to be explained in terms of a decreased freedom to rearrange submem-
branous components associated with the receptors, caused either by an increase in
associated F-actin and/or by increased actomyosin-driven membrane activity. The
prenucleation phase of binding has never been reported in previous work (see above
for references), which has been entirely with cells in suspension or with isolated
membranes. Perhaps the actomyosin structures that are well known to develop with
fibroblast spreading on substrata (e.g. Rees et al. 1978), offer especially enhanced
resistance to the co-operative process and hence are responsible for the visible pre-
nucleation phase. The relevant action of cytochalasin B and azide would then be
to impair the function of these particular actomyosin structures, and to diminish
their resistance to receptor rearrangement.
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Impairment of the function of microtubules by treatment with colchicine had a different and less clear-cut effect on the binding behaviour. No change was observed in the pattern of prenucleation and co-operative binding, except that the co-operative phase extended over a wider range of Con A concentrations. We note that the action of drugs on our spread fibroblasts differ somewhat from those reported for lymphocytes in suspension (Karsenti et al. 1977).

The well-known shape changes (Storrie, 1974; Albertini & Anderson, 1977) and withdrawal of patches of bound lectin from the cell margins (Ukena et al. 1974; Koh, Toh, Gallichio & Elrich, 1978) provide evidence that the binding stimulates an actomyosin contraction. Since this contraction is inhibited by cytochalasin B (Ukena et al. 1974; Koh et al. 1978) and azide (Ash et al. 1977) and we found both these agents to facilitate rather than hinder nucleation of the co-operative event, it follows that the co-operative event is not driven by the contraction. On the contrary, several separate findings could be explained on the basis that an actomyosin contraction follows from the co-operative event. Thus, with α-Con A or with insufficient Con A to enter the co-operative phase, our evidence suggests that microtubule disruption with colchicine promotes uncontrolled cell spreading; once co-operative binding is initiated, however, the dominant influence becomes a strong contraction that rounds the cells. Similarly, the progressive inhibition of phagocytosis in parallel with co-operative binding could result from such a contraction which modifies the membrane activity necessary for adhesion to and intake of the latex beads.

At first sight it is puzzling that prevention of cell detachment by EGTA closely follows the co-operative binding, whereas the prevention of detachment by trypsin is fully effective long before the co-operative phase is complete. However, there is evidence that the mechanisms of action of these 2 agents are fundamentally different (Rees et al. 1977, 1978; Badley et al. 1978). EGTA leaves essentially intact the pattern of membrane contacts with the glass seen by interference-reflexion microscopy, while dispersing stress fibres in a manner that is not inhibited by Con A. Prevention of detachment requires inhibition of the blebbing and cell rounding. This could occur either by contraction of the actomyosin meshwork to keep the cell braced to the substratum and/or by direct physical cross-linkage between cell and substratum; whichever is the case, our evidence suggests that co-operative binding of lectin is a necessary prerequisite. In contrast, trypsin alters the pattern of membrane contacts with the glass as well as causing dispersal of stress fibres and Con A is able to limit both these changes. The relation to co-operative binding is less clear and is under investigation.

In summary, we conclude that interactions between the actomyosin system of fibroblasts and the Con A receptors can be seen in the co-operative phase of binding and at least some of its consequences. Actomyosin impedes the rearrangement of receptors into relationships to favour the multivalent attachment of lectin molecules. Once this resistance has been overcome and co-operative binding can proceed, an influence is seen in the opposite direction in that actomyosin contraction is induced to alter several aspects of cellular response.
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