The Effects of Cytochalasins on Lymphocytes

IDENTIFICATION OF DISTINCT CYTOCHALASIN-BINDING SITES IN RELATION TO MITOGENIC RESPONSE AND HEXOSE TRANSPORT*

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Cytochalasin B inhibits phytomitogen-induced human lymphocyte proliferation with a $K_a$ of approximately $6 \times 10^{-8}$ M. Cytochalasins A, C, D, E, and H are also inhibitory with varying degrees of potency, whereas cytochalasins G, Chaetoglobosins A, B, C, E, F, and J are not at concentrations as high as 15 μM. Cytochalasin B also competitively inhibits carrier-mediated equilibrium exchange of hexose ($K_a$ of approximately $7 \times 10^{-7}$ M), but cytochalasin E is ineffective. Cytochalasin B binds reversibly to the lymphocyte at three distinct sites: L, M, and H. The ligand binding at L site shows the apparent dissociation constant ($K_a$) of 1 to $3 \times 10^{-7}$ M and total binding sites ($B_t$) of 6 to $8 \times 10^9$/cell, represents approximately 88% of the total saturable binding, shows a broad specificity interacting with cytochalasins C, D, and E, is not displaceable by D-glucose, is located mostly in a cytosol fraction, and exists in intimate relation to cytoskeleton actin. M site shows a $K_a$ of 2 to $4 \times 10^{-7}$ M and $B_t$ of 5 to $8 \times 10^9$/cell, represents about 8% of the total saturable binding, shows stringent specificity not being displaced by cytochalasins C, D, and E, is competitively displaced by α-glucose and phloretin, and is quantitatively recoverable in the plasma membrane fraction. The binding to H site shows a $K_a$ of 0.5 to $1.0 \times 10^{-7}$ M and $B_t$ of 4 to $5 \times 10^9$/cell, representing approximately 7% of the total saturable binding, shows a broad specificity, is insensitive to α-glucose, and is membrane bound. It is proposed that L site is actin and is involved in the inhibition of lymphocyte mitogenesis, whereas M site is associated with the hexose transport carrier. Structure-activity relationships of cytochalasin effects are also discussed.

Cytochalasin B, a fungal metabolite, affects many cell motility-related processes such as locomotion, cytokinesis, cytoplasmic streaming, axonal growth, clot retraction (1), cell morphology (2, 3), and microfilament assembly (4). Cytochalasin B is also known for its potent and specific inhibition of sugar transport in mammalian and avian cells (5–8). With lymphocytes, cytochalasin B affects mitogen-induced proliferation (9–11). At relatively low concentrations ($10^{-7}$ to $10^{-6}$ M), cytochalasin B enhances the early events of proliferation, such as lectin-induced stimulation of amino acid uptake, phospholipid turnover, and elevation of cellular cyclic AMP (9–11). At higher concentrations (above $10^{-5}$ M), on the other hand, cytochalasin B inhibits all these early events of lymphocyte proliferation as well as the late event of the DNA synthesis (9–11). The morphologic effects of cytochalasin B include the inhibition of the induction of capped of cell membrane receptors by ligands (12).

The molecular events underlying these cytochalasin B effects on lymphocyte proliferation are yet to be elucidated. As the first step toward this goal, we present this study to characterize different types of cytochalasin B-binding sites in human peripheral blood lymphocytes which may mediate distinct functional effects. Using a number of cytochalasins, two functional effects, the inhibition of mitogen-induced proliferation and glucose transport inhibition, have been studied and characterized in this paper. At least three distinct and independent types of cytochalasin B-binding sites are distinguished in terms of affinity, capacity, ligand specificity, sensitivity to glucose, solubility characteristics, and cellular localization. Based on these results, the present study tentatively assigns one of the binding sites to the inhibitory effect on lymphocyte proliferation. Furthermore, contrary to previous reports, the data demonstrate for the first time a distinct glucose transport-related cytochalasin B-binding site in the lymphocyte (13). These facts and other lines of evidence presented in this paper suggest that the inhibition of hexose permeation is not the mechanism by which cytochalasins inhibit proliferation, as previously proposed (14). Some aspects of structure activity relationship of the cytochalasin-induced inhibitory effect are also discussed. The possibility that the site-mediating inhibition of mitogenesis is in cytoskeletal actin is indicated.

EXPERIMENTAL PROCEDURES

Materials—Cytochalasins A, B, C, D, and E were obtained from Aldrich Chemical Co., Milwaukee, WI. Cytochalasin G was a gift from Professor Fortes Cameron, University of Glasgow, U. K. Cytochalasin H was a gift from Dr. G. C. Christoph, The Ohio State University, Columbus, OH. Chaetoglobosins A, B, C, E, F, and J were generously contributed by Professor S. Natori, National Institute of Hygienic Sciences, Tokyo, Japan. Concanavalin A was purchased from Calbiochem, San Diego, CA. Phytosemglaglucitin and pokweed mitogen were obtained from Burroughs Wellcome, Research Triangle Park, NC. 3-O-[14C]Methyl glucose, [3H]thymidine, and [3H]cytochalasin B were obtained from New England Nuclear.

Preparation of Peripheral Blood Mononuclear Cells—Venous blood was obtained from healthy hospital personnel. Mononuclear cell suspensions from blood specimens were prepared by centrifugation in Ficoll-Hypaque density gradients as detailed elsewhere (15). Briefly, 100 to 200 ml of peripheral venous blood was drawn either into ACD blood collection bags or EDTA-K3, “Vacutainer” tubes.
Cytochalasin Effects on Lymphocytes

Cyto-depleted Lymphocyte Preparations—Purified lymphocyte preparations were rapidly frozen in Medium 199 and freeze-dried in vials, and were stored at -70°C and the original cell suspension. The pellets were recovered by centrifugation at 160,000 x g for 20 min at 4°C (Beckman L-50 ultracentrifuge, Ti-50 rotor). This procedure consistently removed about 85% of the total cellular protein.

Equilibrium Binding of Lymphocyte Membranes—A method similar to that described previously was used (18). Briefly, washed, purified lymphocytes were suspended in ice-cold 1 mM NaHCO₃ solution at a concentration of 5 x 10⁸ cells/ml and stirred for 1 h. This led to the formation of relatively large membrane fragments, while microscopic examination failed to detect any intact cells at the end of this step. The suspension was centrifuged at 2,000 x g for 20 min. The supernatant fluid was discarded, and the pellet was resuspended in 10 ml of 55% (w/v) sucrose solution containing 5 mM MgCl₂. This was placed at the bottom of a 40-ml ultracentrifuge tube and overlaid successively with 10 ml of 45% sucrose, 10 ml of 40% sucrose, and 8 ml of 30% sucrose, all containing 5 mM MgCl₂. This gradient was centrifuged at 90,000 x g for 120 min. The material at the 40 to 45% sucrose interface was carefully removed, and diluted in chilled deionized water to a final concentration of 10% sucrose as determined by refractometry. The plasma membrane fragments found at this interface were briefly unencumbered by organelles or other particulate matter as revealed by electron microscopy (not shown). This was further centrifuged at 6,000 x g for 20 min and resuspended in 1 mM NaHCO₃. An aliquot was saved for protein measurement.

Cytochalasin Binding Assay—Equilibrium binding of cytochalasin B was measured using tritiated cytochalasin B as a tracer by a centrifugation method as described (7). One milliliter of purified peripheral blood lymphocyte suspension (4 x 10⁸ cells/ml in Medium 199, unless otherwise stated) or the membrane suspension, containing 0.1 nCi of [³H]-cytochalasin B and varying concentrations (10⁻¹⁰ M to 10⁻⁴ M) of unlabeled cytochalasin B, were incubated at 37°C for 30 min. Stock solutions of both cytochalasin B and tritiated cytochalasin B were prepared in ethanol so as to keep the final ethanol concentration less than 0.1%. At the end of this incubation, the cells were carefully separated by centrifugation (50,000 rpm) in a model L5-50 Beckman ultracentrifuge, Ti-50.1 rotor, for 20 min at 10°C, and the supernatants were carefully removed as described previously (7). The radioactivities were assayed by counting 0.3 ml of the supernatant and 0.3 ml of the pellet resuspended in 1% sodium dodecyl sulfate in a total volume of 1 ml. The radioactivities associated with the pellet and the supernatant were expressed as the percentage of total radioactivities of the supernatant plus pellet. The bound and free cytochalasin B concentrations were calculated from the percentage distribution. Binding experiments using glucose and phloretin were performed in glucose-free media.

Relative affinities of cytochalasins E, D, and B for binding to lymphocytes were determined by an equilibrium dialysis method as a function of the concentration of the displacing ligand. Cytochalasin B binding to the cytosol fraction of purified lymphocytes was studied in the presence and absence of cytochalasins E and D. Cytochalasin B concentration was kept fixed at 10⁻⁹ M. The presence of increasing concentrations of cytochalasin E and cytochalasin D displaced cytochalasin B binding with distinct dose dependency.

Purification of Lymphocyte Actin—A method similar to that previously described by Hartwig and Stossel was followed (19). Briefly, intact purified lymphocytes were homogenized in a buffer containing 0.34 M sucrose, 1 mM EDTA, 10 mM dithiothreitol, 0.5 mM ATP, and 10 mM Hepes at pH 7.5. This was then subjected to ultracentrifugation at 100,000 x g (Beckman SW 50.1 rotor) for 30 min. The supernatant (Supernatant I) was separated from the pellet (Pellet I). To Supernatant I was added MgCl₂ (to a concentration of 20 mM) and KCl (100 mM) and pH adjusted to 7.5, as described (19). This was subjected to a second ultracentrifugation at 200,000 x g for 10 min. This yielded Pellet II, as described in Table IV.

RESULTS

Inhibition of Lymphocyte Mitogenesis by Cytochalasins

Cytochalasin B and its analogs inhibit mitogen-induced lymphocyte proliferation with distinct dose dependencies (Figs. 1-3). The dose of each cytochalasin which inhibits 50% of the response (K₁) is given in Table I. The Hill plot of the curve for each cytochalasin indicated stochiometry of close to two, indicating some cooperative interaction (Table I). For
the mitogen concanavalin A and pokeweeds mitogen, cytochalasin E was found to be the most potent inhibitor, being about 30 times as potent as cytochalasin B. Cytochalasins A, C, D, and H were all at least approximately 5 times as potent as cytochalasin B. Furthermore, the dose-response curves for each mitogen were very similar, whether maximally stimulating or minimally stimulating doses of all three mitogen were used (data not presented). Cytochalasins G and chaetoglobosins A, B, C, D, E, and F were either ineffective or minimally effective in inhibiting proliferation in doses up to $1.5 \times 10^{-5}$ M.

**Inhibition of Glucose Transport by Cytochalasin B**

Fresh intact purified lymphocytes rapidly equilibrate 3-O-methyl-D-glucose across their surface membrane with a half-equilibration time ($t_{1/2}$) of 4.6 s at the sugar concentration of 2 mM at 25°C. This rapid equilibration was found to be specific for the sugar, since the isotopic equilibration of D-mannitol under identical conditions was negligible with observed $t_{1/2}$ of greater than 60 min (data not presented). The flux of the sugar was completely arrested in the presence of 5 mM HgCl$_2$ or of $10^{-4}$ M phloretin. The flux also showed typical saturation kinetics as a function of the sugar concentration (Fig. 4). The $K_m$ and $V_{max}$ (maximum flux rate) values of approximately 1.7 mM and 0.55 μmol/s/ml of cells, respectively, were found for the equilibrium exchange of 3-O-methyl glucose at 25°C.

**Cytochalasin B inhibits this apparently carrier-mediated sugar transport quite effectively (Fig. 5).** This inhibition was found to be competitive with respect to the sugar. The $K_i$ (concentration of the inhibitor which effects the half-maximum inhibition) was estimated to be $5.8 \times 10^{-5}$ M. Cytochalasin E did not inhibit this transport function (data not presented).

**Cytochalasin B Binding to Lymphocytes**

**Cytochalasin B Binding to Intact Cells**—Fresh intact lymphocytes bind cytochalasin B in a saturable manner at cytochalasin B concentrations of $10^{-5}$ M or lower (Figs. 6 and 7). The saturable portion of this binding revealed nonlinearity on Scatchard analyses (Fig. 7a), indicating the presence of at least two types of cytochalasin B binding sites.

**Fig. 2. The effects of cytochalasins (Cyt) E and D on lymphocyte transformation.** Details of culture and analysis are as in the legend to Fig. 1. ▲—▲, cultures done with cytochalasin E using concanavalin A (1 μg/well) as the mitogen. △—△, cultures done with cytochalasin D using concanavalin A (1 μg/well) as the mitogen. ○—○, the same, for cytochalasin D using phytohaemagglutinin (1.0 μg/well) as the mitogen.

**Fig. 3. The effects of cytochalasin A (CA).** Details of culture and analysis are as in the legend to Fig. 1. △—△, dose response of inhibition using concanavalin A (1 μg/well) as mitogen. ○—○, the same, using minimally stimulating doses of phytohaemagglutinin (0.1 μg/well). ○—●, the same, using maximally stimulating doses of phytohaemagglutinin (1 μg/well). These dosages have been determined in our laboratory (15, 16).

**TABLE I**

**Relative potency of different cytochalasins in inhibiting lymphocyte transformation and the Hill coefficients of the dose-response curves**

<table>
<thead>
<tr>
<th>Concentration giving 50% inhibition ± S.E.</th>
<th>Hill coefficients</th>
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<tr>
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<tr>
<td>Con A*</td>
<td>PWM*</td>
</tr>
<tr>
<td>Cytochalasin A</td>
<td>1.26 ± 0.06 (3)</td>
</tr>
<tr>
<td>Cytochalasin B</td>
<td>5.06 ± 0.17 (3)</td>
</tr>
<tr>
<td>Cytochalasin C</td>
<td>0.4 (1)</td>
</tr>
<tr>
<td>Cytochalasin D</td>
<td>0.53 ± 0.13 (3)</td>
</tr>
<tr>
<td>Cytochalasin E</td>
<td>0.18 ± 0.02 (2)</td>
</tr>
<tr>
<td>Cytochalasin G</td>
<td>&gt;15</td>
</tr>
<tr>
<td>Cytochalasin H</td>
<td>0.40 ± 0.20 (2)</td>
</tr>
</tbody>
</table>

*Con A, concanavalin A. PWM, pokeweeds mitogen. PHA, phytohaemagglutinin.*
least two distinct classes of binding sites. The high affinity site (H sites) showed a $K_d$ of less than $10^{-6}$ M and $B_s$ of not more than 8 pmol/10^6 cells, whereas the low affinity site (L sites) showed a $K_d$ of greater than $1.5 \times 10^{-6}$ M and $B_s$ of 90 to 110 pmol/10^6 cells (Figs. 6 and 7a).

A major part of the saturable cytochalasin B binding was displaced by the presence of $10^{-5}$ M cytochalasin E (Fig. 6). This cytochalasin E-sensitive portion was also found to be heterogeneous on the Scatchard plot (Fig. 7b). Thus, at least two distinct classes of cytochalasin E-sensitive sites were identified, one with a $K_d$ of less than $10^{-7}$ M and $B_s$ of 7 pmol/10^6 cells, and the other with a $K_d$ of greater than $2 \times 10^{-6}$ M and $B_s$ of approximately 70 pmol/10^6 cells (Fig. 7b). It should be noted that these binding parameters are essentially the same as those of the H and L sites, respectively, observed in the absence of cytochalasin E as described above.

A small but significant portion of the saturable cytochalasin B binding remained unaffected in the presence of an excess ($10^{-3}$ M) of cytochalasin E (Fig. 6). This cytochalasin E-insensitive cytochalasin B binding revealed an apparent linearity on the Scatchard plot (Table II), and displayed an affinity which is significantly lower than that of H sites but signifi-

![Fig. 4](image)

**Fig. 4.** Determination of the kinetic parameters for equilibrium exchange of 3-O-methyl glucose (3OMG). Fresh lymphocytes were incubated with 2, 5, 10, or 20 mM 3-O-methyl glucose. They were equilibrated for 45 min at 37°C with constant shaking prior to the flux measurement. The time course for exchange was followed at 21°C, from which $t_{1/2}$ was calculated as described under "Experimental Procedures." Flux rate ($\nu$) was calculated using the relationship $\nu = 0.693(S) V/t_{1/2}$, where (S) and V denote the sugar concentration and total cellular water space, respectively. Data were presented as a double reciprocal plot of $1/\nu$ versus $1/(S)$. ---, $Y = 3.0 + 1.8$, which is drawn to best fit to data.

![Fig. 5](image)

**Fig. 5.** Competitive inhibition of 3-O-methyl glucose equilibrium exchange exerted by cytochalasin B (CB). Fresh lymphocytes were preincubated with 2 and 20 mM 3-O-methyl glucose for 2 h at 37°C with constant shaking. At the end of this preincubation, cytochalasin B was added to the final concentration of 0, 10^{-4}, 10^{-5}, 3 \times 10^{-6}, and 10^{-6} M and incubated further for 15 min. A time course of isotopic equilibration of 3-O-methyl glucose was then followed by adding isotopic tracer. The data were plotted according to the equation $(1 - v_0/t_{1/2})^{-1} = 1 + K_c(1 + (S)/K_c)(D)^{-1}$, where $v_0$ and $t_{1/2}$ denote flux rate with and without cytochalasin B, respectively, $K_c$ is the inhibition constant; and (D) is the cytochalasin B concentration. With 2 mM (○—○) and 20 mM (■—■) 3-O-methyl glucose.

![Fig. 6](image)

**Fig. 6.** Binding of cytochalasin B (CB) at equilibrium to human lymphocytes as a function of cytochalasin B concentration in the absence (○) and in the presence of $10^{-6}$ M cytochalasin E (■), 500 mM D-glucose (□), or $10^{-5}$ M cytochalasin E plus 500 mM D-glucose (△). Each point represents a single binding assay. Reaction mixture for each binding assay contained 2 × 10^6 cells (98% pure), [H]cytochalasin B (0.02 μCi), cytochalasin B at a varying concentration, and a given additive or additives where specified, in Medium 199, pH 7.4, in a total volume of 1.0 ml. The bound [H]-cytochalasin B is plotted in the vertical axis, while the concentration of unlabeled cytochalasin B is on the horizontal axis.

![Fig. 7](image)

**Fig. 7.** Scatchard analysis of cytochalasin B (CB) binding to lymphocytes. The data of Fig. 1 are used. a, overall cytochalasin B binding after correction for nonsaturable binding. ---, $y = 0.165 - 0.0083x$ and $y = 0.71 - 0.00034x$. b, cytochalasin E-sensitive, cytochalasin B binding as calculated from the difference in the cytochalasin B binding in the absence and in the presence of $10^{-6}$ M cytochalasin E for each cytochalasin B concentration in assay mixture. The value of unbound cytochalasin B used in this calculation was an average of those in the presence and absence of cytochalasin E. ---, $y = 0.142 - 0.0095x$ and $y = 0.081 - 0.00042x$. The bound/free ratio is expressed in milliliters per mg units.
cytochalasins E and D as well, while the M sites show a much more stringent specificity in distinguishing the subtle difference in the structures of different cytochalasins, thus not accepting both cytochalasins E and D.

A small yet significant portion of the saturable cytochalasin B binding was displaced in the presence of 0.5 M D-glucose at cytochalasin B concentrations of $3 \times 10^{-7}$ M or higher (Fig. 6). L-Glucose was not effective in this regard, the displacement of D-glucose was not effective in this regard, the displacement thus being specific for D-glucose. It is noted that there was a 100% displacement of the cytochalasin B binding which is not displaceable by an excess of D-glucose, e.g. the D-glucose-sensitive cytochalasin E-insensitive cytochalasin B-binding components, e.g. one with a $K_d$ of less than $10^{-7}$ M and a $B_t$ of 6 to 8 pmol/10$^6$ cell equivalents, and another having a $K_d$ of higher than $2 \times 10^{-6}$ M and a $B_t$ of 40 to 60 pmol/10$^6$ cell equivalents (Fig. 8). Comparison of these binding parameters with those of intact cells indicates that the H sites of intact cells are quantitatively retained in the cytosol-depleted preparation, while more than 50% of the L sites are not recovered in this preparation. A significant portion of the cytochalasin B binding to the cytochalasin E-insensitive cytochalasin B-binding sites or the M sites. The $B_t$ of this glucose-sensitive site appears somewhat less than that of the cytochalasin E-insensitive component. A significant portion of the cytochalasin B binding to cytosol-depleted lymphocytes was not displaced by an excess (0.5 M) of D-glucose. This glucose-insensitive portion was found to be nonlinear on the Scatchard analysis.

**Table II**

<table>
<thead>
<tr>
<th>Component</th>
<th>$K_d$ (M)</th>
<th>$B_t$ (pmol/10$^6$ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>0.7 x 10$^{-7}$</td>
<td>7</td>
</tr>
<tr>
<td>Overall</td>
<td>2.6 x 10$^{-7}$</td>
<td>90</td>
</tr>
<tr>
<td>Overall</td>
<td>3.8 x 10$^{-7}$</td>
<td>8</td>
</tr>
<tr>
<td>Overall</td>
<td>1.3 x 10$^{-6}$</td>
<td>110</td>
</tr>
<tr>
<td>Overall</td>
<td>0.6 x 10$^{-7}$</td>
<td>6</td>
</tr>
<tr>
<td>CE insensitive</td>
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<td>108</td>
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<tr>
<td>CE insensitive</td>
<td>2.8 x 10$^{-7}$</td>
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</tr>
<tr>
<td>CE insensitive</td>
<td>4.3 x 10$^{-6}$</td>
<td>8</td>
</tr>
<tr>
<td>Cytochalasin D</td>
<td>4.4 x 10$^{-7}$</td>
<td>8</td>
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<tr>
<td>insensitive</td>
<td>1.0 x 10$^{-7}$</td>
<td>7</td>
</tr>
<tr>
<td>sensitive</td>
<td>2.3 x 10$^{-6}$</td>
<td>86</td>
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<tr>
<td>Cytochalasin D</td>
<td>9.1 x 10$^{-7}$</td>
<td>10</td>
</tr>
<tr>
<td>sensitive</td>
<td>3.2 x 10$^{-6}$</td>
<td>110</td>
</tr>
<tr>
<td>*$K_d$ values were obtained from the Scatchard plot. Where the plot showed nonlinearity, it was further analyzed as a sum of two linear processes with two distinct $K_d$ values.</td>
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</tr>
<tr>
<td>*One picomole per 10$^6$ cells corresponds to 6.2 x 10$^5$ molecules per cell, and 10$^6$ cells represent approximately 0.25 mg of protein.</td>
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**Table III**

<table>
<thead>
<tr>
<th>Component</th>
<th>$K_d$ (M)</th>
<th>$B_t$ (pmol/10$^6$ cells)</th>
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<tbody>
<tr>
<td>Overall</td>
<td>1.1 x 10$^{-7}$</td>
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</tr>
<tr>
<td>Overall</td>
<td>2.3 x 10$^{-6}$</td>
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</tr>
<tr>
<td>Overall</td>
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<td>6</td>
</tr>
<tr>
<td>CE insensitive</td>
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<td>58</td>
</tr>
<tr>
<td>CE insensitive</td>
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<td>CE insensitive</td>
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<td>4</td>
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<tr>
<td>CE insensitive</td>
<td>2.5 x 10$^{-7}$</td>
<td>9</td>
</tr>
<tr>
<td>CE sensitive</td>
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</tr>
<tr>
<td>sensitive</td>
<td>1.6 x 10$^{-6}$</td>
<td>42</td>
</tr>
<tr>
<td>Glucose insensitive</td>
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<tr>
<td>Glucose sensitive</td>
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<tr>
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<tr>
<td>Phloretin sensitive</td>
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<td>5</td>
</tr>
<tr>
<td>sensitive</td>
<td>3.5 x 10$^{-7}$</td>
<td>2</td>
</tr>
</tbody>
</table>

*10$^6$ cells after cytosol depletion represent approximately 0.087 mg of total protein. Others as in Table II. |

*CE, cytochalasin E. |
Cytochalasin Effects on Lymphocytes

FIG. 9. Effects of sugars on equilibrium binding of cytochalasin B (CB) to cytosol-depleted lymphocytes as a function of the sugar concentration. The cytochalasin B concentration was fixed at 10^{-7} M. For each binding assay, 7 x 10^6 cells, first freeze-thawed, then washed, were used in the final volumes of 1.0 ml of isotonic saline, pH 7.4, adjusted with 10 mM Tris-HCl, containing varying concentrations of D-glucose (●) or L-glucose (○).

FIG. 10. Effect of phloretin on equilibrium binding of cytochalasin B to cytosol-depleted lymphocytes as a function of concentrations of cytochalasin B. Cells, first freeze-thawed, then washed, were suspended (6.5 x 10^6 cells/ml) in 1/10 balanced salt solution, pH 7.4, containing 0.02 μCi of tritiated cytochalasin B and varying concentrations of cytochalasin B, without (●) or with (△) 10^{-5} M phloretin. The cytochalasin B binding without phloretin at pH 3.2 (□) is also shown.

Phloretin was found to competitively displace cytochalasin B binding to cytosol-depleted lymphocytes. This effect showed a distinct dose dependence (Figs. 10 and 11) with a K_d of approximately 10^{-5} M. Unlike the D-glucose effect, this phloretin effect was evident even at a cytochalasin B concentration as low as 1.6 x 10^{-5} M (Fig. 10).

FIG. 11. Effects of phloretin on equilibrium binding of cytochalasin B to cytosol-depleted lymphocytes as a function of phloretin concentrations. Experimental conditions are identical with those of Fig. 10 except that a fixed (10^{-7} M) concentration of cytochalasin B was used with varying concentrations of phloretin.

Cytochalasin B Binding to Lymphocyte Plasma Membrane Preparations—Semipurified lymphocyte membrane preparations retained a significant amount of saturable cytochalasin B-binding activity (Fig. 12). This was unaffected in the presence of an excess (10^{-2} M) of cytochalasin E except where very low concentrations of cytochalasin B were used (10^{-9} M). The Scatchard plot of this cytochalasin E-insensitive cytochalasin B binding revealed apparent linearity with a K_d of approximately 2 x 10^{-5} M (Fig. 12, inset). The B_c was approximately 53 pmol/mg of protein. A major fraction (75 to 90%) of this cytochalasin B binding was displaceable by an excess of D-glucose (Fig. 13), while L-glucose had no effect. This glucose-displaceable cytochalasin B binding revealed a single binding component on the double reciprocal plot (Fig. 13, inset) with...
Fig. 13. Equilibrium binding of cytochalasin B (CB) to partially purified surface membranes as a function of cytochalasin B concentration in the absence (○, □) and in the presence of 500 mM D-glucose (●) or 500 mM L-glucose (■). For each binding assay, surface membranes of 3.0 mg of membrane protein equivalent were used. Inset, a double reciprocal plot of D-glucose-sensitive cytochalasin B binding as estimated by the difference in binding in the absence and in the presence of 500 mM D-glucose at each cytochalasin B concentration in the binding assay. The average value of the free cytochalasin B concentrations of the two was used. The solid line is drawn best fit to data and gives $K_d = 4.3 \times 10^{-7}$ M and $B_t = 43$ pmol/mg of protein.

$K_d$ and $B_t$ values of approximately $4.3 \times 10^{-7}$ M and 43 pmol/mg of protein, respectively. The sensitivity to glucose and the value for the $K_d$ are characteristics of the M sites.

A small portion (less than 5%) of the cytochalasin B binding was not displaceable by the presence of an excess of D-glucose (Fig. 13). This glucose-insensitive portion had a $K_d$ of approximately $5.1 \times 10^{-8}$ M, a value typical for the H sites. These $K_d$ and $B_t$ values of approximately $4.3 \times 10^{-7}$ M and 43 pmol/mg of protein, respectively. The sensitivity to glucose and the value for the $K_d$ are characteristics of the M sites.

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Fig. 14. Effect of Triton X-100 treatment on equilibrium binding of cytochalasin B (CB) to cytosol-depleted lymphocytes. Freeze-thawed and washed lymphocytes were suspended (3.8 $\times 10^8$ cell equivalents/ml) in 1/10 balanced salt solution containing 0.5% Triton X-100 and incubated for 20 min at 21°C. The residue of the Triton extraction was recovered by a centrifugation (Sorvall RC-2B, in an SS 34 rotor) for 20 min at 4°C, without (○) and with (□) the Triton treatment. Inset, a double reciprocal plot of Triton X-100-sensitive portion of the cytochalasin B binding. The straight line represents best fit to data and gives $K_d = 4.1 \times 10^{-7}$ M and $B_t = 33$ pmol/3.8 $\times 10^8$ cell equivalents.

$K_d$ and $B_t$ values of approximately $4.3 \times 10^{-7}$ M and 43 pmol/mg of protein, respectively. The sensitivity to glucose and the value for the $K_d$ are characteristics of the M sites.

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Fig. 15. Structural features of cytochalasins A, B, D, and E, and basic skeleton of chaetoglobosins. The central core perhydrosiindol ring is heavily lined. To the left of this is the C-10 substituent, while to the right is the macrocyclic ring. R = O for cytochalasin A, and R = OH for cytochalasin B. The structure of chaetoglobosin A is depicted in order to illustrate the basic skeleton of chaetoglobosins.

Fig. 16. Relative affinity of cytochalasin E, D, and B (CB) for the L site as determined by equilibrium dialysis. Lymphocyte cytosol supernatant after 100,000 $\times g$ centrifugation (Supernatant I, as in Table IV) containing 2 mg/ml of total protein was incubated in the presence of 0.2 $\mu$Ci/ml of $[^3H]$cytochalasin B at a concentration of $5 \times 10^{-8}$ M. Cytochalasins B (●), D (■), or E (▲) in concentrations varying between $5 \times 10^{-8}$ M to $1.5 \times 10^{-7}$ M were added to the protein side. The buffer side contained buffer alone. Equilibrium dialysis was carried out at room temperature, and samples taken at 12 and 18 h exhibited similar binding. Nonspecific binding of 15%- was subtracted from all curves of the 12th-h data presented here. Scatchard plot of data yielded a $K_d$ of $5 \times 10^{-7}$ M and $B_t$ of approximately 3000 pmol/mg.
results indicate that the cytochalasin B binding to the lymphocyte plasma membrane is mainly due to the presence of M sites and only to a very small extent due to H sites. L sites are totally lacking in these lymphocyte membrane preparations.

More than 90% of the cytochalasin B binding to purified lymphocyte membrane was lost when the membrane preparations were treated with 0.67% Triton X-100 (Fig. 14). The double reciprocal plot of the detergent-sensitive portion of cytochalasin B binding indicate that the detergent treatment selectively abolished the M sites. The small portion (5 to 10%) of the cytochalasin B binding which was retained in the membrane preparation after detergent treatment revealed a high binding affinity and probably represents contaminating H sites. An EDTA extraction, which is known to extract extrinsic membrane proteins, on the other hand, did not reduce cytochalasin B binding to the purified lymphocyte membranes. These results suggest that both M and H sites are intrinsic membrane proteins and, that while M sites are extractable with detergent, H sites are resistant to both detergent and EDTA extractions (Fig. 14, inset).

Cytochalasin B Binding to Lymphocyte Cytosol Preparations—As shown in Fig. 16, a saturable, high affinity cytochalasin B-binding site with the binding properties (K, = 5 × 10⁻⁹ M) similar to the L site was identified in lymphocyte cytosol by equilibrium dialysis technique. Furthermore, the hierarchy of binding affinity was found to be cytochalasin E > cytochalasin D > cytochalasin B, which is identical with the hierarchy of activity of these agents in inhibition of lymphocyte mitogenesis.

**TABLE IV**

<table>
<thead>
<tr>
<th>Fractions*</th>
<th>Volume</th>
<th>Protein</th>
<th>B,</th>
<th>K,</th>
<th>Specific Activity</th>
<th>Fold pure*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact cells</td>
<td>10</td>
<td>2.0</td>
<td>2,200</td>
<td>2.0</td>
<td>1,100</td>
<td>1.0</td>
</tr>
<tr>
<td>Supernatant I</td>
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<td>2,000</td>
<td>2.2</td>
<td>3,500</td>
<td>3.2</td>
</tr>
<tr>
<td>Pellet I</td>
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<td>20</td>
<td>1.0</td>
<td>476</td>
<td>0.4</td>
</tr>
<tr>
<td>Pellet II</td>
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<td>0.02</td>
<td>450</td>
<td>3.6</td>
<td>21,428</td>
<td>19.5</td>
</tr>
</tbody>
</table>

*Fractions are defined under “Experimental Procedures.”

**Fig. 17.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis of lymphocyte actin preparations. Details are described under “Experimental Procedures.” The purification of cytochalasin B-binding activity is summarized in Table IV. Gel a represents that from Supernatant I, and Gel b that from Pellet II. The major protein peak of Pellet II is shown to co-migrate with purified rabbit muscle actin (Gel c). The method of Fairbanks et al. (28) was followed for sodium dodecyl sulfate polyacrylamide gel electrophoresis.

**Fig. 18.** DNase I affinity chromatograph of lymphocyte cytosol fraction. a, protein profile of fractionation. A 5-ml DNase column was prepared by attachment of 50 mg of DNase I (Sigma) to N-hydroxysuccinimide-linked, controlled pore glass beads (Corning) with mean diameter of 550 Å. A 100,000 × g supernatant of lymphocyte cytosol (Supernatant I, as in Table IV) was brought to pH 5.1 with a buffer containing 100 mM KCl and 20 mM MgCl₂. The column was equilibrated with the same buffer. After application of the cytosol preparation and washing (I) of the column with this buffer, it was washed twice (II and III) with pH 7.0 buffer containing 100 mM KCl and 20 mM MgCl₂. The material remaining attached to the column was eluted with 2% sodium dodecyl sulfate in pH 7.0 buffer. b, polypeptide profile of subfractions. Samples of the eluates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Gel 1 is the applied material. Gels 2 to 4 represent Wash I. Gels 5 to 9 represent Washes II and III. Gels 10 to 13 are from the 2% sodium dodecyl sulfate elution of the column, showing an enrichment in a protein that co-migrates (upper arrow) with purified rabbit skeletal muscle actin. The lower arrow represents the dye front.
Cytochalasin Effects on Lymphocytes

Partial Purification of the L Site—The L site is practically quantitatively recoverable in the cytosol fraction and seems to co-purify with a protein which migrates with actin in sodium dodecyl sulfate polyacrylamide gel electrophoresis (Table IV, Figs. 17 and 18). When the lymphocyte cytosol was subjected to precipitation with 100 mM KC1 and 20 mM MgCl2, to which were attached DNase I by covalent linkage, sodium dodecyl sulfate polyacrylamide gel electrophoresis of the precipitate showed that the protein peak of Pellet I was co-migrating with a purified cytoskeletal protein (21). As shown in Fig. 17, the major protein peak of Pellet II was found to co-migrate with purified rabbit muscle actin. To provide further evidence of the relationship of L sites to actin, lymphocyte cytosol preparations, following treatment with buffer containing 100 mM KC1 and 20 mM MgCl2, pH 5.1, were passed through glass bead columns, to which were attached DNAse I by covalent linkage (Fig. 18). The material that passed through contained virtually no saturable cytochalasin B-binding activity. When the column was then eluted with 2% sodium dodecyl sulfate in pH 7.0 buffer, the eluate was found to have a protein profile on sodium dodecyl sulfate polyacrylamide gel electrophoresis remarkably similar to that of Pellet II material (Fig. 18b).

DISCUSSION

Inhibition of Lymphocyte Mitogenesis by Cytochalasins

Cytochalasin B inhibits mitogen-induced lymphocyte mitogenesis (Fig. 1). The inhibition shows a distinct dose dependency with a stoichiometry of approximately two. This would indicate some cooperativity in this inhibitory interaction. All cytochalasins tested inhibited the mitogenesis, with the exception of cytochalasin G, with varying potencies and the stoichiometry significantly greater than one (Table I).

In the light of the potential anti-tumor activities of this class of compounds, information on structure-function relationships are of interest (Table I and Fig. 15).

a: The Nature of the Aromatic Substituent at C-10—This is very important in determining efficacy since all potent compounds have a phenyl substituent. Chaetoglobosins and cytochalasin G, having an indol group at this position, are relatively ineffective.

b: The Macrocyclic Ring—Firstly, this has a strong influence since cytochalasin A is more potent than cytochalasin B. These two compounds differ only in the substituent at C-20. Secondly, the substituents at C-17 and C-18 may not be important, since cytochalasins D and H are approximately equipotent. The former differs from the latter in having a keto oxygen at C-17 and in having opposite stereochemistry at C-18. Thirdly, the size of this ring may not be important by itself in determining potency. Thus, among the agents with 11-member macrocyclic rings, cytochalasins C, D, and H are not markedly different from one another in potency, but cytochalasin G is practically ineffective. Among those with 13-member macrocyclic rings, cytochalasin E is one of the most potent, while chaetoglobosins are extremely weak. Cytochalasins A and B both have 14-member macrocyclic rings but are markedly different in potency.

c: Core Cyclohexane Ring—Substitution pattern in this ring may not be critically influential. Cytochalasins C and D are approximately equipotent, although they have markedly different structures, the former having a double bond between positions 5 and 6 while the latter has, instead, a CH3 substituent at 6.

These structure-activity relationships on lymphocyte responses are in marked contrast with those reported on the cytotoxic effects of cytochalasins on nucleated cell lines as determined by growth inhibition (20, 21). Thus, in HeLa cells, Minato and co-workers observed that the phenyl group at C-10 and the C-7 hydroxyl groups are essential for cytotoxicity. They also conclude that, while the macrocyclic ring is required to be intact, modifications of the ring such as acylation of hydroxyl groups and reduction of double bonds, do not substantially alter cytotoxic potency (20). Beno and co-workers have rationalized that the biologic effects may not vary much with changes in macrocyclic ring, which may serve only to provide bulk and some sort of shielding function after binding (21). Further, the core peryhydrosindol ring may be critically important in binding, with the rigidly positioned 1-C═O, 2—NH, and the C-7 hydroxyl groups determining binding. Our observations are in sharp contrast to those of Natori, who concluded that replacement of the phenyl group by an indolyl group does not significantly influence cytotoxicity in cultured mammalian cells (HeLa and cultured kidney cells) (22).

Inhibition of Glucose Carrier by Cytochalasins

The uptake of hexose by human peripheral blood lymphocytes is a carrier-mediated process (Fig. 4), and cytochalasin B inhibits this carrier function with a one-to-one stoichiometry (Fig. 5). The inhibition is competitive with respect to the permeating sugar. Cytochalasin E, a close structural analog of cytochalasin B, is ineffective. This indicates that the inhibition of glucose carrier results from interaction of cytochalasin B to specific sites with a stringent stereospecificity. All of these findings are very similar to the inhibition by cytochalasin B of glucose carrier in human erythrocytes (7, 23) and nucleated cells such as Ehrlich ascites tumor cells (24).

Heterogeneity of Cytochalasin-binding Sites and Their Functional Relevances

In the present study, we have identified three distinct types of cytochalasin B-binding sites, L, M, and H, in human peripheral blood lymphocytes. The cytochalasin B binding to L site (Kd of 1 to 3 x 10^-9 M and B, of 6 to 8 x 10^7/cell) represents the lowest affinity binding, yet is the most abundant, accounting for about 85% of the total saturable binding of intact cells. It displays broad specificity, also accepting cytochalasins C, D, and E. Cytochalasin B bound to this class of sites is not displaced by an excess of D-glucose. Most of the binding appears to be in the cytosol compartment. The broad specificity and insensitivity to D-glucose of this site is similar to Site II of human erythrocytes (7, 23) and to presumably motility-related, high affinity cytochalasin B-binding sites observed in animal cells (25). However, the affinity of L site to cytochalasin B is much (10- to 100-fold) lower than these sites.

We propose that the binding of cytochalasins to L sites leads to inhibition of lymphocyte mitogenesis. The following observations made in the present study support this contention. 1) The dissociation constant for cytochalasin B to L sites (Kd = 1 to 3 x 10^-9 M) and the concentration of cytochalasin B that effects 50% inhibition on lymphocyte mitogenesis (Kd = 5 x 10^-9 M) are remarkably similar. The Kd for the M and H sites are at least 1 order of magnitude lower, and at these low concentrations, cytochalasin B does not significantly inhibit lymphocyte mitogenesis. 2) The relative potency of different cytochalasins as mitogenesis inhibitors (cytochalasin B > cytochalasin D > cytochalasin B) is identical with the relative affinity for binding to L site. 3) The M site cannot be involved since cytochalasins C and D, as well as E, do not bind to this site, although they are potent inhibitors of lymphocyte mitogenesis.

How the cytochalasin binding to L sites would lead to...
inhibition of lymphocyte mitogenesis is yet to be explained. Cytochalasin B affects cell motility-related membrane function and morphology in animal cells only at concentrations similar to the \( K_d \) of L site (greater than 2 to 5 \( \times 10^{-7} \) M). Results of the present study also indicate that the L site may be the actin component of the cytoskeleton (site infra). The cytochalasin B binding to this structure may lead to cessation of motility of interacting cells, and this, in turn, restricts the cell-to-cell collaboration necessary for the mitogenic response (16, 26).

The H site \( (K_d = 0.5 \) to 1 \( \times 10^{-7} \) M and \( B = 6 \) to 8 \( \times 10^{-9} \) cell), the highest affinity site to cytochalasin B, represents around 7% of total saturable cytochalasin B binding to intact lymphocytes. The site appears to be exclusively membrane bound, is insensitive to an excess of D-glucose, and displays broad specificity, accepting other cytochalasins. The functional significance of this particular class of sites remains uncertain at this time. Cytochalasin B at concentrations similar to the \( K_d \) of the H site stimulates, rather than inhibits, lymphocyte mitogenesis with concomitant stimulation in solute transport and DNA synthesis in early response to mitogenic stimuli (11, 13). This would indicate that H site binding may also play a role in lymphocyte mitogenesis.

The M site \( (K_d = 2 \) to 4 \( \times 10^{-7} \) M and \( B = 5 \) to 8 \( \times 10^{-6} \) cell) represents about 8% of the total saturable binding of intact lymphocytes. Cytochalasin B bound to these sites is competitively displaced by D-glucose and phloretin, typical glucose transport substrate and inhibitor, respectively, with similar affinities. It is not affected by L-glucose. The M site further shows a narrow specificity, not accepting cytochalasins C, D, and E that are without effect in inhibiting glucose transport. They accept cytochalasin B with a \( K_d \) which is very similar to the value of its \( K_d \) (4 to 6 \( \times 10^{-7} \) M) as a glucose transport inhibitor (Fig. 5). The M site is almost quantitatively recoverable in a plasma membrane fraction and is sensitive to Triton X-100. These findings strongly suggest that the M site is the glucose transport-related site.

Our detection of the glucose-displaceable cytochalasin B-binding site is at variance with data reported by Parker et al. (13), who were unable to detect this site. Differences in experimental protocol, e.g., the cold washing procedure that they used, may explain this. They also measured binding under nonequilibrium conditions. Alternatively, M sites, representing only 8% of total binding, may have been undetected in the face of an overwhelmingly large L site binding since intact lymphocytes only were checked. Their estimate of the total number of "high affinity" cytochalasin B-binding sites is also much lower compared to our estimate of the overall binding. It is possible that the "high affinity" cytochalasin B binding Parker et al. studied may be the L site binding of the present study. The relative order of binding affinity among different cytochalasins, cytochalasin E > cytochalasin D > cytochalasin B, reported by Parker et al. (13) is indeed identical with the relative order of the binding affinity to the L sites in the present study. Our identification of M sites may provide a means to isolate the carrier involved in facilitated glucose transport in lymphocytes and to detect any possible changes brought about on these following mitogenic stimulation.

**Biochemical Identity of the L Site**

The results presented in this paper strongly suggest that the L site is the cytoskeletal protein, actin. (i) The L site co-purifies with actin. (ii) When lymphocyte cytosol preparation is passed through a DNase I column, virtually all the L site binding activity is lost. It is well known that DNase binds actin. (iii) The observed relative affinity of binding to the L site, e.g., cytochalasin E > cytochalasin D > cytochalasin B (Fig. 16) is the same as their relative potencies of binding to an actin-spectrin-containing complex of erythrocytes (25) and in inhibiting polymerization of muscle actin induced by this complex (25).

A clear idea of the molecular events leading to the morphologic and motility effects on eukaryotic cells has not yet emerged. Ultrastructural studies show disruption and disappearance of actin filaments from intact cells exposed to micromolar concentrations of cytochalasin B (27). Recently, it has been shown that F actin, but not G actin, contains the high affinity cytochalasin B-binding site (29). Submicromolar concentrations of cytochalasins can inhibit nucleus-induced polymerization and elongation of muscle and slime mold actin by binding to sites located most likely at the elongating end of the filaments (30, 31). The structure of purified actin filaments in vitro is disrupted by cytochalasin B (32).

Assuming that the L site is indeed actin, the stoichiometry in purified actin fractions was approximately one site per actin monomer (ranging from 0.7 to 1.3:1 based on three independent measurements). The stoichiometry calculated on the basis of binding data in intact cells is not less than one binding site per 10 actin monomers (33) (Table III). In mammalian muscle and slime mold actin, the stoichiometry of cytochalasin B binding is less than one site per 50C actin monomers (29, 31). The relatively high \( K_d \) and the one-to-one stoichiometry of the cytochalasin B binding to L sites of the human lymphocyte observed here thus represents a novel mode of binding, perhaps related to differences in the actin species being examined. At least six different actin polypeptides are expressed in differentiated cells of the higher mammal (34). The skeletal muscle actin is a tissue-specific polypeptide of the \( \alpha \)-electrophoretic species, while non-muscle cells from higher mammals contain \( \beta \) and \( \gamma \) forms (33). Although we did not define the electrophoretic species of actin that we examined, replicating human T-lymphocytes have been reported to contain primarily \( \beta \) and smaller amounts of \( \gamma \) species (35). It should also be noted that low affinity cytochalasin B binding (>1 \( \mu \)M) has not been extensively studied. We cannot rule out the possibility that the L site exists as a contaminant co-purifying with actin.

**Acknowledgments**—We express our thanks to Drs. S. Natori, G. G. Christoph, and Fortes Cameron for their gifts of cytochalasins. The superb technical assistance of Mrs. Joanne Ballard, Camille Cataldo, and Charles Berenski, and the secretarial assistance of Ms. Helen K. Haddock are acknowledged. We thank Professor Carl Bentzel for performing electron microscopy.

**REFERENCES**