Regulation of Lymphocyte Responses In Vitro: Potentiation and Inhibition of Rat Lymphocyte Responses to Antigen and Mitogens by Cytochalasin B*

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ABSTRACT Cytochalasin B, at concentrations between 0.02 and 0.2 μg/ml, was slightly stimulatory to lymph-node cells from normal rats and greatly potentiated their response to phytohemagglutinin and low concentrations of concanavalin A (mitogens for thymus-derived lymphocytes); it also potentiated the response of thymocytes to phytohemagglutinin. The response of lymph-node cells to lipopolysaccharide endotoxin (a mitogen for thymus-independent lymphocytes) was also enhanced, but only at concentrations in the usual inhibitory range, possibly by inhibition of a "suppressor T-cell" response. Sensitized lymphocytes responding to antigen were not stimulated at all, except at a very high cell density, where inhibition of a "suppressor cell" response was also considered likely. At concentrations of 5-10 μg/ml or higher, cytochalasin B inhibited all responses tested.

Cytochalasin B is a fungal product (1) widely used as an inhibitor of cell functions that involve contraction of microfilaments (2). Its target within the cell was undetermined (2) until recently; new evidence establishes firmly its ability to interact directly with the actin moiety of actomyosin, producing a loss of viscosity and of ATPase activity (4). Cytochalasin B, in a restricted low-concentration range (<1 μg/ml), stimulates chemotaxis (5) and phagocytosis (6), but inhibits these functions at higher concentrations (5 μg/ml). We report here its ability, in a concentration range of 0.02-0.5 μg/ml, to stimulate lymphocyte DNA synthesis and to potentiate the responses of lymphocytes to mitogens.

MATERIALS AND METHODS

Animals and Sensitization. Inbred DA rats of both sexes, 3-5 months of age, were sensitized by injection of ovalbumin (Nutritional Biochemicals Corp., Cleveland, Ohio), 100 μg in Freund's complete adjuvant in both hind foot pads (6). Their inguinal and iliac nodes, 9 days after sensitization, were used as sources of sensitized lymph-node cells (LNC). As sources of normal LNC, cervical, axillary, inguinal, and popliteal lymph nodes were harvested from untreated rats.

Cell Suspensions and Culture Media. LNC were gently squeezed between two sterile slides in ice-cold medium (0°C), passed through nylon mesh, washed twice by centrifugation, and diluted to a density of 2 × 10⁶ viable cells per ml (7). In some experiments thymocytes were used instead of LNC. Ham's F10 medium (Microbiological Associates Inc., Bethesda, Md.), containing 10% heat-inactivated (56°C, 30 min) fetal-calf serum (Grand Island Biological Co., Grand Island, N.Y.), 100 units of penicillin per ml, and 100 μg of streptomycin per ml, was used throughout.

Reagents. Ovalbumin, PHA (phytohemagglutinin P; Difco Laboratories, Detroit, Mich.), Con A (concanavalin A, twice crystallized, Nutritional Biochemicals Corp., Cleveland, Ohio), and lipopolysaccharide B, Escherichia coli 0111:B4 (Difco Laboratories, Detroit, Mich.) were dissolved in phosphate-buffered saline (pH 7.4) at various concentrations, and 0.1 ml of the solutions were added to appropriate cultures. Cytochalasin B (Imperial Chemical Industries Ltd., Macclesfield, Cheshire, England) was dissolved at 3 mg/ml in (CH₃)₂SO. Just before use, the stock solution was diluted with phosphate-buffered saline to the desired concentrations, and corresponding dilutions of (CH₃)₂SO were prepared as controls.

Estimation of DNA Synthesis. Uptake of [³H]thymidine (1.9 Ci/mmol. New England Nuclear Corp., Boston, Mass.) by LNC was determined as in ref. 8. Cultures, usually containing 2 × 10⁶ cells per ml, were incubated 72 hr at 37°C in a 5% CO₂ atmosphere and pulsed with [³H]thymidine during the last 24 hr. The counts in duplicate cultures regularly differed from the mean values by less than 15%.

RESULTS

Effect of Cytochalasin B on Nonstimulated Cells. Normal LNC (2 × 10⁶/ml) were incubated with cytochalasin B at concentrations between 0.01 and 2.0 μg/ml or with corresponding amounts of (CH₃)₂SO (0.67-3.4%). The latter did not affect the baseline level of thymidine incorporation. However, 0.02-0.5 μg of cytochalasin B per ml stimulated DNA synthesis slightly (maximal incorporation above baseline, at 0.1 μg/ml, was only 562 cpm), while 1.0 and 2.0 μg of cytochalasin B per ml inhibited DNA synthesis, the decrease being directly proportional to the amount of cytochalasin B on a logarithmic scale.

Effect of Cytochalasin B on Normal Lymphocyte Responses

Abbreviations: Con A, concanavalin A; LNC, lymph node cells; PHA, phytohemagglutinin; B-lymphocytes, thymus-independent or "bone marrow-derived" lymphocytes; T-lymphocytes, thymus-derived lymphocytes.

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Used as a 5-ml solution in distilled water, according to Difco's description sheet. Amounts used are thus expressed in microliters.
to Mitogens. The dose–response curve of normal LNC (2 × 10^6/ml) exposed to various concentrations of PHA showed increasing thymidine incorporation between 0.1 and 10 μl/ml, a peak or a plateau at 10–30 μl/ml, and a decreasing response at higher concentrations (Fig. 2). In the presence of 0.1–3 μg of cytochalasin B per ml, the response to PHA was enhanced, the highest cpm being obtained with 10–30 μl of PHA and 0.1–0.3 μg of cytochalasin B. At 10 μg of cytochalasin B per ml, the response was inhibited throughout the PHA concentration range. The peak with a smaller amount of cytochalasin B (0.1 μg/ml) required more PHA; conversely with a higher amount of cytochalasin B (1 μg/ml), less PHA was required. Detailed dose–response curves at fixed high or low concentrations of PHA, 25 and 1 μl, respectively (Fig. 3), illustrate this shift of the peak with PHA concentration and show further that inhibition at the higher concentration was achieved with less cytochalasin B (5 μg/ml). The maximum thymidine incorporation with both concentrations of PHA was that normally attained with optimal Con A stimulation (see below).

The normal thymocyte response to a low concentration of

**Fig. 1.** Effect of cytochalasin B on incorporation of [H]-thymidine by normal LNC. Background values observed in the absence of cytochalasin B with or without addition of (CH2)2SO are shown in the shaded zone.

**Fig. 2.** Effect of various concentrations of cytochalasin B on the dose–response curve obtained with PHA and normal LNC. Each point is the average of duplicate values: ——— without cytochalasin B; ———, ———, ———, ——— rep- resent 0.1, 0.3, 1, 3, and 10 μg of cytochalasin B, respectively.

PHA (1 μl) was more than doubled in the presence of cytochalasin B; the peak occurred at a concentration of 0.2 μg of cytochalasin B per ml and almost complete inhibition at a concentration of 5 μg/ml (Fig. 4).

0.3–3 μg of Con A per ml stimulated increasing levels of DNA synthesis in normal LNC, a maximal response was obtained at concentrations between 3 and 30 μg of Con A per ml, and the response was inhibited at still higher concentrations. A titration of the effect of cytochalasin B at maximal and at low concentrations of Con A, 10 and 0.5 μg, respectively, is shown in Fig. 5. In the presence of maximal stimulation, it produced slight inhibition throughout the range of concentrations tested, but marked inhibition at a concentration of 2 μg/ml and total inhibition at 10 μg/ml. With 0.5 μg of Con A, which stimulated DNA synthesis to the same degree as moderate concentrations (3–10 μl) of PHA, there was a substantial potentiation of the response by cytochalasin B, reaching a peak at a concentration of 0.2 μg to cytochalasin B per ml, and inhibition at 2–10 μg/ml.

Lipopolysaccharide endotoxin is a pure B-lymphocyte mitogen (9, 10). We found that it stimulated LNC maximally at concentrations between 3 and 30 μg/ml, but the amount of DNA synthesis was extremely low in comparison with responses to PHA or Con A. With 10 μg of lipopolysaccharide, thymidine incorporation was clearly enhanced by 1–2 μg of cytochalasin B per ml and inhibited by 10 μg/ml (Fig. 6). The enhancing range of cytochalasin B concentrations was obviously higher and narrower than with either PHA or Con A.

**Fig. 3.** Effect of cytochalasin B on normal LNC activated with 25 μl (——) and 1 μl (——) of PHA. Activation in the absence of cytochalasin is shown by shaded zones.

Effect of Cytochalasin B on Antigen-Stimulated Cells. In preliminary experiments, it was found that LNC, 9 days after sensitization, were activated by ovalbumin throughout the
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**DISCUSSION**

Cytochalasin B produced a striking enhancement of DNA synthesis by LNC of normal rats stimulated with PHA at both high and low concentrations and by Con A at suboptimal concentrations. Both are mitogens for T-lymphocytes (10–13). There may be a pool of T-cells that can be stimulated by Con A or by PHA plus cytochalasin B, some of which cannot be stimulated by PHA alone or by low concentrations of Con A. Such a heterogeneity is strongly suggested by the known variability of T-cells with respect to their responses to PHA and Con A (13).

Fig. 4. Effect of cytochalasin B on thymocytes (5 × 10⁶ cells per ml) activated with 1 μl of PHA. Activation in the absence of cytochalasin is shown by shaded zone.

The effect of cytochalasin B was titrated in a series of tubes containing ovalbumin at final concentrations of 2.5, 25, and 250 μg/ml. As shown in Fig. 7, DNA synthesis in antigen-stimulated LNC was not potentiated at any concentration of cytochalasin B, but was clearly inhibited at concentrations that potentiated responses to PHA or Con A. The inhibition was roughly proportional to the dose of cytochalasin B on a logarithmic scale, being seen with as little as 0.1 μg/ml.

In a narrow range of low cytochalasin B concentrations, the data suggested a slight enhancement of DNA synthesis, for instance, at concentrations between 0.05 and 0.1 μg/ml of cytochalasin B and 250 μg/ml of ovalbumin. That this effect was real could be brought out by culturing larger numbers of cells. Incubation of increasing numbers of sensitized LNC with a fixed amount (25 μg/ml) or a proportionally increasing amount (25 μg/ml/2 × 10⁶ cells) of antigen in the presence of cytochalasin B gave essentially similar patterns of response; only data obtained with a fixed amount of antigen are given here (Fig. 8). DNA synthesis increased with increasing numbers of cells up to 8 × 10⁶/ml, and cytochalasin B was inhibitory throughout this range. A sharp inhibition of DNA synthesis was observed with higher numbers of cells, and, in this range, cytochalasin B enhanced the response. Titration of the effect of cytochalasin B at a cell density of 16 × 10⁶/ml with 25 μg of ovalbumin per ml (Fig. 9) showed a maximal effect at concentrations between 0.1 and 0.2 μg/ml of cytochalasin B, with a less than 2-fold enhancement of DNA synthesis.

Incubation of sensitized LNC with 1 μl of PHA resulted in a somewhat lower thymidine incorporation than was usually observed with normal LNC (shaded zone in Fig. 10). Cytochalasin B enhanced DNA synthesis in the same concentration range (0.2–2 μg/ml) that was effective with normal LNC stimulated by PHA, but the enhancement was substantially less than that attained with normal LNC.

Fig. 5. Effect of cytochalasin B on LNC activation with 10 and 0.5 μg of Con A. The responses elicited in the absence of cytochalasin B are shown by the shaded zones. ▲——, LNC with 10 μg Con A and cytochalasin B; ▲——, LNC with 0.5 μg Con A and cytochalasin B.

Fig. 6. Effect of cytochalasin B on LNC activated with lipopolysaccharide. Activation with 10 μg of lipopolysaccharide alone is shown by shaded zone.
Fig. 7. Inhibition of DNA synthesis in antigen-stimulated LNC by cytochalasin B. Stimulation by various amounts of antigen without cytochalasin B is shown by shaded zones. The doses of antigen were 250 μg (—), 25 μg (●—●), and 2.5 μg (■—■).

It is also remarkably similar to their heterogeneity in response to PHA alone and to PHA plus the "lymphocyte-activating factor" released by macrophages (8, 14). It was suggested (8) that this factor might potentiate PHA responses by acting simply as an additional mitogen, or perhaps by changing the level of responsiveness of certain cells so that they could now be triggered by normally submitogenic stimuli. These possibilities apply to the case of cytochalasin B potentiation of the response to PHA. One must consider the alternative possibility that cytochalasin B potentiates a stimulation of B-lymphocytes by PHA or Con A. These mitogens can stimulate B-cells if bound to a rigid surface (15, 16) or by release of a humoral factor (15). This possibility clearly does not apply in the case of thymocytes, whose response to PHA was also potentiated by cytochalasin B. It remains open in the case of lymph-node cells.

Fig. 9. Effect of cytochalasin B on crowded cultures (16 × 10⁶/ml) activated by antigen (25 μg/ml). Activation in the absence of cytochalasin B is shown by the shaded zone.

With lipopolysaccharide endotoxin, a mitogen that stimulates B-lymphocytes (9, 10, 13), cytochalasin B potentiated the mitotic response in a narrow concentration range. Here the level of DNA synthesis attained was higher than could be obtained with endotoxin alone. We have shown in a previous paper (7) that when adherent cells are removed from lymphoid-cell suspensions, the response to endotoxin increases to a level comparable to that attained here. This change appears to result from removal of macrophages and the consequent elimination of a macrophage-dependent "suppressor T-cell" effect, since it is not observed with cells of thymus-deprived animals. Possibly, the potentiation action of cytochalasin B is mediated by an effect on such "suppressor" cells. The higher dose of cytochalasin B required, which approaches the range in which there is inhibition of responses of T-lymphocytes, is consistent with this possibility.

With stimulation of sensitized lymph-node cells by antigen, cytochalasin B under most conditions failed to enhance the response, although a positive finding was obtained when the cell concentration in culture was greatly increased. When these lymph-node cells were exposed to PHA, they gave a substantially lower degree of DNA synthesis than normal LNC, and the potentiated response to PHA plus cytochalasin B was correspondingly lower than that of normal LNC. The simplest view of this observation is that only the unsensitized cells in the suspension respond, the sensitized cells being relatively insensitive to stimulation by either PHA or cytochalasin B. We harvested these cells 9 days after challenge, when they had just completed a cycle of blast transformation and several cell divisions in the draining lymph nodes (17-19); they might be expected to differ physiologically from their unstimulated precursors. Sensitized cells have been described as intensely pyronophilic (20-22), medium-sized lymphocytes (3, 5, 18, 23) of low to moderate density (24), possessing a uropod unlike their precursors (25, 26) and ultrastructural features resembling those of a cell activated by mitogen (26). Thus, they may be regarded as already partially activated, and this would account for their decreased sensitivity to PHA or cytochalasin B.
The apparent potentiation of the response to antigen at very high cell density was observed only in a range of diminished responses, which we believe to depend on "suppressor T-cell" effects (**). Cytochalasin B may be acting to eliminate activation of these cells. However, this effect was observed at a low cytochalasin concentration (0.05-0.5 \mu g/ml), and its interpretation must remain in doubt.

In sufficiently high concentration, cytochalasin B inhibits all triggering of DNA synthesis and, as we showed earlier (27, 28), lymphotoxin release. Inhibition is seen with 5 \mu g of cytochalasin B per ml or more with both thymocytes and LNC and with all mitogens tested as well as with antigen. This is the level found to be inhibitory in other systems such as chemotaxis (5), phagocytosis (1, 22, 29), and exocytosis (30). With low levels of stimulation, e.g., with 1 \mu l of PHA acting on LNC, inhibition required a higher concentration of cytochalasin B (10 \mu g/ml). The dose range for inhibition of phagocytosis varies similarly with the strength of the stimulus (§).

The present study establishes that cytochalasin B can stimulate both T-lymphocytes, e.g., thymocytes, activated by PHA, and B-lymphocytes, since lipopolysaccharide endotoxin acts only on the latter (9, 10). The mechanism of this stimulation and its relation to the inhibition observed at higher concentrations in this system are unknown. Since the inhibitory effect of cytochalasin B appears to result from its interaction with microfilament protein (2, 4), it is possible that at low concentration cytochalasin enhances the contractility of these cellular elements. However, recent work (31-33) shows that it also affects membrane transport of a number of biologically significant small molecules, such as glucose. Thus, while our observations suggest that some form of cell movement, possibly related to the movements responsible for chemotaxis and phagocytosis, may form an essential part of the lymphocyte triggering mechanism, such a possibility requires substantiation by other experimental means.

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