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# The Effect of Cytochalasin B on Effector–Target Cell Interaction

## QUANTITATIVE AND ULTRASTRUCTURAL STUDY

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**Summary.** Interaction between pokeweed mitogen-stimulated secondary lymphocytes (PWM-lymphocytes) and target fibroblasts resulted in over 80 per cent adherence of the sensitized lymphocytes to the target cells. Adherence is by pseudopod penetration into target fibroblasts. The only lymphocyte cellular components found in the contact region were microfilaments. Cytochalasin B completely inhibited the specific adsorption of the PWM-secondary lymphocytes to the target cells. What adhesion did take place in the presence of cytochalasin B was found to be nonspecific. Ultrastructurally, the contact between lymphocyte and target cells was altered by the drug, when pseudopods were not observed.

Possible effects of cytochalasin B on lymphocyte-mediated cytolysis, mainly by its effect on microfilament function, is discussed.

## INTRODUCTION

Interaction between sensitized lymphocytes and antigen-bearing target cells, as manifest by the adherence of the lymphocytes to the target cells, is known to be an early stage in the lymphocyte-mediated cytolytic process (Brondz and Snegiröva, 1971; Golstein, Svedmyr and Wigzell, 1971). An *in vitro* system, demonstrating a high degree of specific adherence, was previously described (Hollander and Ginsburg, 1972) in which blast cells, obtained by stimulation of rat lymphocytes with pokeweed mitogen (PWM), redifferentiated after removal of the mitogen to small lymphocytes, termed 'secondary lymphocytes' (Ginsburg, Hollander and Feldman, 1971). These small lymphocytes represented a highly enriched population of cells with specific receptors for PWM; over 80 per cent of them adhered to target cells in the presence of PWM, as compared to 10–20 per cent adherence of normal lymphocytes (Hollander and Ginsburg, 1972).

Electron microscopical observations showed the lymphocytes to be attached to the target cells by pseudopods, which often penetrated into the target cells (to be published). The only cellular organelle in the vicinity of the contact region was found to be microfilaments and, in rare instances, microtubules. Other organelles such as the Golgi complex,

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lysosomes and vesicles were localized at a distance from the contact region. Several roles have been attributed to microfilaments, including cell movement (Wessels, Spooner, Ash, Bradley, Ludoena, Taylor, Wrenn and Yamada, 1971), capping of surface immunoglobulins (Taylor, Duffus, Raff and de Petris, 1971; Loor, Forni and Pernis, 1972; de Petris, 1974) and concavalin A (con A) receptors (de Petris, 1974). In view of these roles as well as the localization of the microfilaments in the vicinity of the contact region, it was of interest to study their involvement in adhesion and target cell lysis mediated by PWM-secondary lymphocytes.

The present communication reports on the effect of cytochalasin B, which is known to impair microfilament function (Wessels *et al.*, 1971), on the attachment of PWM-secondary lymphocytes to target fibroblasts at the ultrastructural level as well as quantitative study of the adherence and consequent lysis.

## MATERIALS AND METHODS

*Animals*

Lymphocytes and embryos for preparation of fibroblast monolayers were obtained from the Lewis strain rats.

*Materials*

A stock solution of PWM (Grand Island Biological Company) containing 1 mg dry weight per millilitre was kept frozen at −20°.

Cytochalasin B (ICI Research Laboratories) was dissolved in dimethyl sulphoxide (DMSO), and was maintained as a stock solution containing 1 mg/ml. Concentration of DMSO in the medium never exceeded 0·1 per cent.

*Fibroblast monolayers*

Monolayers were prepared and maintained as previously described (Berke, Ax, Ginsburg and Feldman, 1969).

*Stimulation of lymphocytes with PWM*

Lymphocyte suspensions were prepared from Lewis rat lymph nodes as previously described (Ginsburg and Sachs, 1965). The cells were centrifuged and resuspended in Dulbecco's modified Eagle's medium containing 15 per cent horse serum and 5 $\mu$g of PWM per millilitre medium. $30 \times 10^3$ lymphocytes in 4 ml of medium were cultured in 60 mm Falcon plastic Petri dishes and incubated for 4 days at 37° in a humidified incubator with a flow of 7 per cent $CO_2$ in air.

*Growth of blast cells on syngeneic fibroblast monolayers*

By the 4th day when cultures were rich in blast cells, the lymphoid cells were harvested, washed free of PWM and plated onto a Lewis fibroblast monolayer in 100 mm Petri dishes; $3 \times 10^6$ cells in 10 ml of medium per dish, containing 15 per cent horse serum. To obtain suspensions of secondary lymphocytes, these cultures were incubated for another 3–4 days.

*Adherence of lymphocytes to target monolayers*

$5 \times 10^6$ secondary lymphocytes in 3 ml of medium were plated onto Lewis fibroblast

monolayers. The non-adhering lymphocytes were collected intermittently by pipetting after which the monolayer was washed once with an additional 3 ml of medium which had been combined with the first harvest. This represented the non-adhering fraction. The remaining monolayer and adhering lymphocytes were overlaid with a trypsin solution and incubated for 30 minutes, after which the cell suspension representing the adherent fraction was collected. Lymphoid cells in both fractions were counted in a haemocytometer and mean values were obtained from eight counts of each sample. Adherence is expressed as the percentage of the total number of lymphoid cells obtained in the adherent fraction.

*Target cell lysis*

$3 \times 10^6$ lymphocytes in 1·5 ml of medium were plated on syngeneic $^{51}$Cr-labelled target fibroblasts. The cultures were incubated at 37° for 20 hours and the amount of radioactivity released into the medium was measured as previously described (Berke *et al.*, 1969). Lysis is expressed as the percentage of total radioactivity released into the medium after correcting for the spontaneous $^{51}$Cr release in control plates. The standard deviation of triplicate plates did not exceed 2 per cent.

*Electron microscopy*

Monolayers with adherent lymphocytes were washed vigorously with the culture medium and fixed for 2 hours in 2·5 per cent glutaraldehyde in 0·1 M cacodylate buffer, pH 7·2, post-fixed for 1 hour in 1 per cent osmium tetroxide, stained in block with 0·5 per cent uranyl acetate, dehydrated and embedded in Epon 812. Ultrathin sections were cut on a Reichert ultramicrotome, stained with uranyl acetate and lead citrate and examined with a JEM 100 B electron microscope.

## RESULTS

### EFFECT OF CYTOCHALASIN B ON ADHERENCE OF PWM-SECONDARY LYMPHOCYTES TO FIBROBLAST MONOLAYERS

To study the effect of cytochalasin B on the adsorption of PWM lymphocytes to target cells, $5 \times 10^6$ secondary lymphocytes were placed on syngeneic fibroblast monolayers in medium containing 10 $\mu$g of PWM per millilitre. The cells were incubated either with or without 7 $\mu$g/ml of cytochalasin B (concentration at which maximum inhibition was obtained with minimal damage to the fibroblasts) and adherence was determined at various intervals. The results (Table 1) indicate that cytochalasin B inhibited PWM-induced adherence of PWM-secondary lymphocytes. Adherence was seen as early as 30 minutes after plating on fibroblast monolayers and increased until a plateau was reached at about 4 hours. In addition to its inhibitory effect, cytochalasin B also altered the kinetics of adherence; a sharp increase in adherence was observed at 4 hours with cytochalasin B, when a plateau was reached without the drugs. Two possible explanations could account for this altered kinetics of adsorption. The inhibitory effect of cytochalsin B may be transient and lymphocytes recover their adhering capacity after being blocked for a certain period; or this late adsorption may be a manifestation of a non-specific process unrelated to the specific PWM-induced adsorption. When the two possibilities were tested the second was found to be the case (Table 2): 20–35 per cent of the lymphocytes were adsorbed to the monolayer after 6 hours of incubation in presence of cytochalasin B. However, this was a

TABLE 1
EFFECT OF CYTOCHALASIN B ON PWM-INDUCED ADHERENCE OF PWM SECONDARY LYMPHOCYTES

| Time (hour) | Percentage adherence* | |
|---|---|---|
| | In presence of cytochalasin B† | In absence of cytochalasin B† |
| ½ | 8·9 ± 1·2 | 19·2 ± 1·5 |
| 1 | 18·8 ± 3·9 | 31·9 ± 2·7 |
| 2 | 17·0 ± 1·8 | 46·9 ± 3·9 |
| 4 | 17·2 ± 2·1 | 60·7 ± 4·8 |
| 6 | 29·7 ± 3·7 | 64·4 ± 5·0 |
| 8 | 33·0 ± 4·3 | 60·9 ± 5·1 |

* In medium containing 10 μg of PWM per millilitre.
† Seven micrograms per millilitre of medium.

TABLE 2
EFFECT OF CYTOCHALASIN B ON NON-SPECIFIC ADHERENCE OF PWM-SECONDARY LYMPHOCYTES

| Expt no. | Percentage adherence* | | | |
|---|---|---|---|---|
| | + PWM† − cyt.B† | + PWM† + cyt.B‡ | − PWM + cyt.B‡ | − PWM − cyt.B |
| 1 | 65·0 ± 4·9 | 29·7 ± 1·5 | 22·9 ± 3·2 | 9·3 ± 1·2 |
| 2 | 67·2 ± 7·0 | 40·7 ± 3·3 | 34·4 ± 4·0 | 16·8 ± 1·7 |
| 3 | 69·1 ± 5·1 | 24·9 ± 1·8 | 22·7 ± 1·9 | 12·7 ± 0·9 |

* Determined after 6 hours of incubation.
† Ten micrograms per millilitre.
‡ Cyt.B = cytochalasin B (7 μg/ml).

non-specific effect of cytochalasin B, the specific PWM-induced adherence being completely inhibited.

### EFFECT OF CYTOCHALASIN B ON CYTOTOXIC ACTIVITY OF PWM-SECONDARY LYMPHOCYTES

It has been shown that the first step in target cell destruction involves contact between the lymphocytes and the target cells (Brondz and Snegiröva, 1971; Golstein *et al.*, 1971). Since cytochalasin B is known to inhibit lymphocyte-mediated cytolysis, and has been shown to prevent the early stage of interaction between effector and target cells, it was reasoned that cytochalasin B would have no or very little effect on cytotoxic activity, if lymphocytes were allowed first to adhere and cytochalasin B added only after optimal adherence had been reached. If, on the other hand, cytochalasin B also affected later events, it would inhibit cytolysis even when added after contact between lymphocytes and target cells had been established.

$3 \times 10^6$ PWM-lymphocytes were plated in medium containing 10 μg of PWM per millilitre onto $^{51}$Cr-labelled syngeneic target fibroblasts. Cytochalasin B (7 μg/ml) was added at various times and $^{51}$Cr release measured after 20 hours' incubation. As can be seen in Table 3, cytochalasin B present from time zero caused 76 per cent inhibition of lysis. $^{51}$Cr release was not more pronounced when the drug was added later; even if added at 6 hours, when plateau of adherence had been established, cytochalasin B caused 60 per cent inhibi-

TABLE 3
EFFECT OF CYTOCHALASIN B ON CYTOTOXIC ACTIVITY OF PWM-SECONDARY LYMPHOCYTES

| Cytochalasin B* added at (hours) | Percentage $^{51}Cr$ release† | Percentage inhibition of lysis |
|---|---|---|
| 0 | 11·6 | 76·0 |
| 1 | 11·9 | 75·4 |
| 2 | 14·7 | 69·3 |
| 4 | 19·2 | 60·3 |
| 6 | 20·3 | 58·0 |
| Not added | 48·3 | |

* Cytochalasin B (7 μg/ml) was added at different times after the beginning of the incubation.
† Determined after 20 hours of incubation.

tion of lysis. It therefore appears that the lymphocyte–target cell interaction occurring within the first 6 hours is not sufficient for subsequent $^{51}Cr$ release, and more cycles of interaction or other events which are inhibited by cytochalasin B are required.

### EFFECT OF CYTOCHALASIN B ON THE ULTRASTRUCTURE OF THE INTERACTING CELLS

A typical attachment of PWM-secondary lymphocyte to the monolayer is presented in Figs 1 and 2. Lymphocyte pseudopods are seen mainly on the monolayer side, often penetrating the fibroblast target cell. In most cultures the most intimate contact between lymphocyte and target cells appeared to be in the region of the penetrating pseudopods. The major cellular element observed in the pseudopods or its vicinity is microfilament. Most lymphocyte organelles such as Golgi elements, endoplasmic reticulum, lysosomes and various vacuoles and vesicles were found adjacent to the nucleus or in the uropod, but never close to the contact region. In cultures with cytochalasin B added there was a marked morphological change in lymphocyte target cell attachment (Figs 3 and 4). There is almost

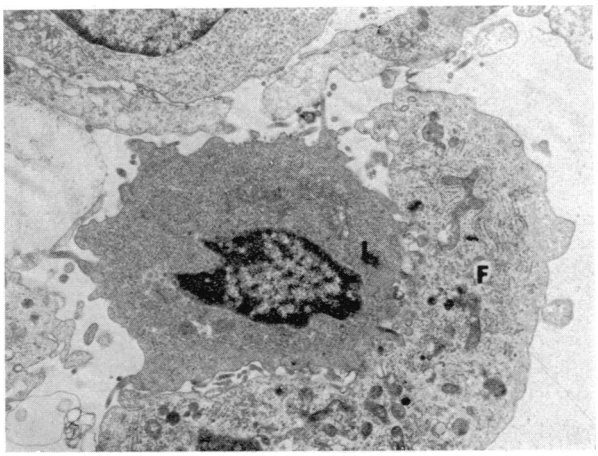


FIG. 1. A low power electromicrograph of a PWM-secondary lymphocyte (L) adhered to a target fibroblast (F). Note the many lymphocyte pseudopods, some of them appear to penetrate the target fibroblast. Preparation was made after 4 hours' adhesion in culture. (Magnification ×4000.)

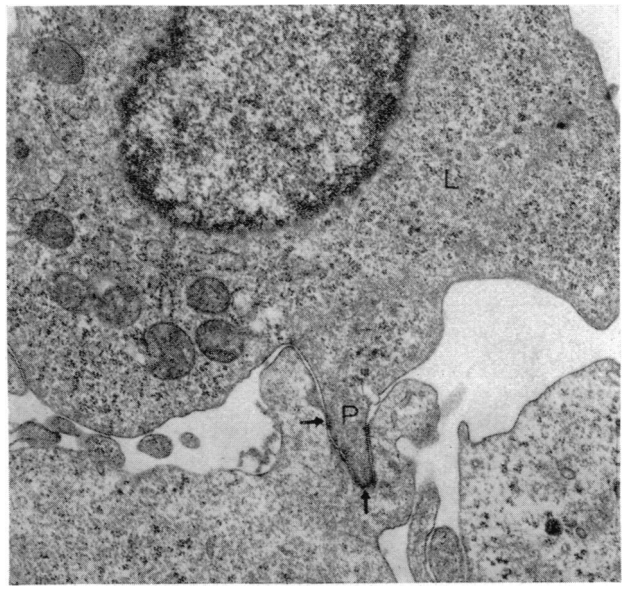


FIG. 2. A higher magnification of a PWM-secondary lymphocyte (L) attached to a target fibroblast by a penetrating pseudopod (P); contact region can be observed (arrows). Note that the pseudopod is devoid of any cellular organelles except microfilaments. Preparation was made after 4 hours' adhesion in culture. (Magnification × 12,500.)

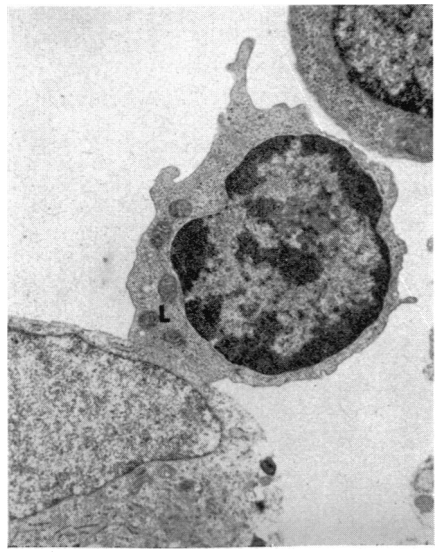


FIG. 3. A low power electronmicrograph of a PWM-secondary lymphocyte (L) attached to a target fibroblast after treatment with cytochalasin B. Note that the contact region is devoid of any pseudopods. Preparation was made after 4 hours' adhesion in culture; cytochalasin B was added at zero time. (Magnification × 4000.)

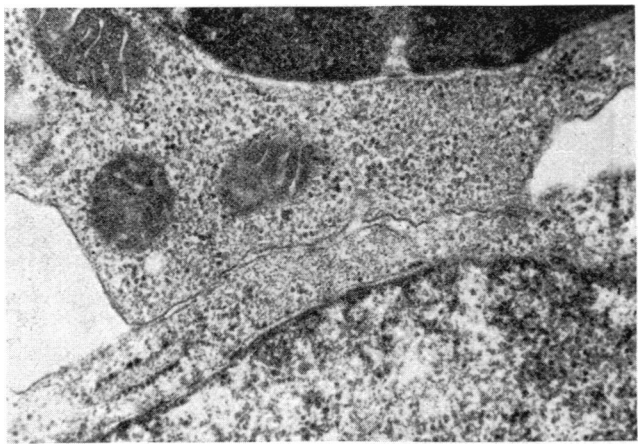

FIG. 4. A higher magnification of the contact region observed in Fig. 3. Note that the membranes of both cells are parallel to each other and no pseudopods are seen in this region. (Magnification × 25,000.)

a complete disappearance of the lymphocyte pseudopods. The membranes of both the lymphocytes and target cells are positioned parallel to each other, and in some instances electron-dense material appeared between the two membranes (Fig. 5). No cellular elements except microfilaments could be seen in this region, similar to the picture described above without cytochalasin B.

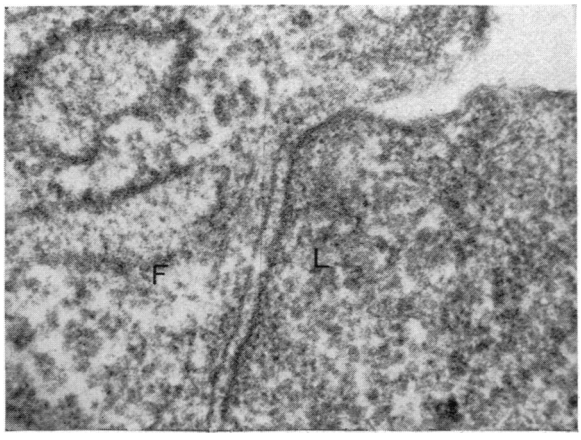


FIG. 5. Cytochalasin B treated PWM-lymphocyte (L) attached to fibroblast target cell (F). Note the electron dense intercellular material between the cells. Preparation as in Fig. 3. (Magnification × 47 500.)

## DISCUSSION

The initial step in the cytolytic process is the interaction between effector lymphocytes and antigen-bearing target cells. In the system employed, effector cells are secondary lymphocytes bearing receptors for PWM, and target cells are syngeneic fibroblasts to which PWM is spontaneously conjugated when added to the culture medium. The ad-

vantages of this system are the high proportion of cells (60–70 per cent) which undergo specific adhesion, and the whole process from adhesion and to $^{51}Cr$ release is carried out on the same target fibroblasts. This seems to be an advance over the system employed by Henney and Bubbers (1973), in which fibroblast monolayers were used as targets for adherence and mastocytoma as targets for $^{51}Cr$ release.

The results in the present study clearly indicate that cytochalasin B inhibits lysis by preventing lymphocyte–target cell interaction. This finding is consistent with previous studies on the effect of cytochalasin B in lymphocyte-mediated cytolysis (Henney and Bubbers, 1973; Plaut, Lichtenstein and Henney, 1973). In view of the known inhibitory effects of this drug upon cell motility (Wessels *et al.*, 1971), capping of surface immunoglobulin (Taylor *et al.*, 1971; Loor *et al.*, 1972; de Petris, 1974) or con A receptors (de Petris, 1974), cytoplasmic streaming (Bradley, 1973) and macromolecules secretion (Schofield, 1971; Thomas, Lutzae and Manavathu, 1974), several mechanisms can be suggested by which cytochalasin B affects lymphocyte-mediated cytolysis, probably via impaired function of microfilaments.

(a) It has been reported that target cell lysis involves motility of effector lymphocytes, both movement from one target to another and amoeboid movement during their attachment to a certain target cell (Ginsburg, 1970). The inhibition of cell movement may abolish the lytic process. Lymphocyte mobility is of particular importance in our system in which the lymphocyte : target cell ratio is 4 : 1, a low ratio compared to 100:1 in the study of Plaut *et al.* (1973). In order to obtain a high level of $^{51}Cr$ release in our system, one effector cell has to lyse more than one target cell, which indeed has been demonstrated (Ginsburg, 1970). Such a mechanism requires movement of effector cells from one target cell to another. Thus, cytochalasin B, which affects motility, inhibited the lysis in our system even when added after the first interaction between lymphocytes and target cells had been established.

(b) It has been suggested that membrane modulation is a prerequisite for antigen recognition (Diener and Paetrau, 1972). In that respect it was suggested that microfilaments are involved in the rearrangement of immunoglobulin and con A receptors on the lymphocyte membrane, both of which are inhibited by cytochalasin B (Taylor *et al.*, 1971; Loor *et al.*, 1972; de Petris, 1974). It is tempting to suggest that pokeweed receptors behave similarly and that their rearrangement is also inhibited by the drug so that the necessary modulation of the lymphocyte membrane does not occur and adherence is prevented. Some observations in favour of this notion were obtained from the electron microscopical studies. Since it is clear that the typical attachment of PWM-secondary lymphocyte and target cells via pseudopods is not obtained when cytochalasin B is added, one can assume that this is due to lack of rearrangement of the pokeweed receptors, this affecting the morphology of the contact region. However, we cannot exclude the possibility that the drug mainly affects the cell movement, for which the absence of pseudopods provides support.

(c) Our results do not exclude the possibility that, in addition to the initial step of adhesion, cytochalasin B also affects a later stage in lysis. This is in accordance with the known facts that cytochalasin B also affects cytoplasmic streaming (Bradley, 1973), macromolecular secretion (Schofield, 1971; Thomas *et al.*, 1974) and lymphotoxin production (Yoshinaga, Waksman and Malawista, 1972). Such a notion is valid if one assumes a lytic mechanism which involves transport of cytotoxic agents from the adherent lymphocyte to the target cell, as was suggested by several investigators (Plaut *et al.*, 1973; Sellin, Wallach and Fischer, 1971). Our electron microscopic studies do not support this notion, as no

morphological signs of secretion could be seen in the vicinity of the contact region, i.e. vacuoles, vesicles, Golgi elements. However, another secretory mechanism connected with lysis, which is inhibited by cytochalasin B, cannot be excluded.

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