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Effect of Cytochalasin D on *Toxoplasma gondii* Cell Entry

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Cytochalasin D did not prevent attachment but did prevent entry of *Toxoplasma gondii* into peritoneal macrophages and bladder tumor 4934 cells. Inhibition of entry of *T. gondii* into peritoneal macrophages by cytochalasin D was dose related and comparable to inhibition of phagocytosis. Prevention of entry of *T. gondii* into bladder tumor 4934 cells by cytochalasin D followed a dose response identical to that observed with peritoneal macrophages. After removal of the medium containing cytochalasin D, its effect was completely reversible, and the kinetics of the loss of inhibition followed a similar time course for both phagocytic and "nonphagocytic" cell types. The studies support the concept that the host cells actively participate in the process by which *T. gondii* gains entry into cells.

Toxoplasma gondii is an obligate intracellular parasite capable of infecting all mammalian phagocytic and "nonphagocytic" cells. However, the mode of entry of this organism into cells has been the subject of considerable controversy (12). A number of investigators interpret their data as showing that phagocytosis is the primary process by which this parasite enters cells (9, 18, 23), whereas others have presented data which they interpret as showing active penetration as the mechanism by which *T. gondii* gains an intracellular position (5, 11, 13, 24).

The cytochalasins, a group of congeneric secondary fungous metabolites (6), affect a number of cellular functions, one of which is the suppression of phagocytosis (16). We used this group of compounds to study the means by which *Toxoplasma* gains entry into mononuclear phagocytes and into cells generally not considered to be phagocytes.

MATERIALS AND METHODS

***Toxoplasma* preparation.** Purified preparations of trophozoites of the RH strain of *T. gondii* were obtained from the peritoneal cavity of 2-day-infected mice as described previously (19). The suspending medium was M199 (Grand Island Biological Co., Grand Island, N.Y.) containing 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 20% heat-inactivated (56°C for 30 min) Sabin-Feldman dye test-negative fetal calf serum (M199-20% FCS).

Macrophage monolayers. Unelicited mouse peritoneal macrophages (PM) were obtained from Swiss Webster mice (Simonsen Laboratories, Gilroy, Calif.). Cells were harvested, and monolayers were prepared in M199-20% FCS as described previously (20). At 4 h after plating, nonadherent cells were removed from the monolayers, fresh M199-20% FCS was added, and the monolayers were incubated for an additional 20 h before infection in an atmosphere containing 5% CO_2. Cell viability was determined by the exclusion of trypan blue dye.

Continuous cell line. Monolayers of bladder tumor cell line 4934 (B 4934) were prepared as previously described (10). Each chamber of a four-chambered tissue culture slide (Lab-Tek Products, Div. Miles Laboratories Inc., Westmont, Ill.) was seeded with 1 ml of M199-10% FCS containing 4×10^5 B 4934 cells and incubated for 24 h before infection at 37°C in an atmosphwere containing 5% CO_2.

Infection of monolayers. Monolayers of macrophages and B 4934 cells were incubated for 60 min with 2×10^6 trophozoites in 0.5 ml of M199-20% FCS. Additional monolayers of PM were incubated in parallel with 2×10^6 heat-killed *Candida albicans*. After this incubation period, monolayers were washed with warm (37°C) Hanks balanced salt solution, and fresh M199-20% FCS was added to the chambers. At various intervals thereafter, slides were fixed in 0.4% aminoacridine and stained with Giemsa stain. The preparations were evaluated for the number of trophozoites attached to the cells, the number of cells with intracellular organisms, the morphology and staining characteristics of the trophozoites, and the average number of trophozoites per vacuole.

CD. A stock solution of cytochalasin D (CD; A grade, lot 400606; Calbiochem, La Jolla, Calif.) was prepared by dissolving 1 mg of CD in dimethyl sulfoxide (DMSO) and adjusting the concentration of CD to 100 μg/ml in M199. This was stored at −20°C. Final DMSO concentrations never exceeded 0.1% (vol/vol). Medium from monolayers that had been cultivated for 24 h was removed, and M199-20% FCS containing the desired concentration of CD per 0.5 ml of M199-20%

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FCS was added to the chambers. Controls were untreated monolayers and monolayers to which was added medium containing DMSO alone. The monolayers were then divided into two groups. In one group, untreated monolayers or monolayers which had been preincubated with varying concentrations of CD or DMSO for 60 min were washed and immediately thereafter challenged with *Toxoplasma* for varying intervals. In the other group, *T. gondii* was added directly to the monolayers without prior removal of the medium containing CD or DMSO. CD was used in this study rather than cytochalasin B (CB) because CD does not interfere with membrane transport of sugars as does CB (15).

RESULTS

Effect of CD on the entry of *T. gondii* into PM. When *T. gondii* was added directly to PM monolayers in the presence of CD, a dose-dependent inhibition of entry of these parasites into PM was observed (Fig. 1). As little as 1 μg of CD per ml significantly decreased ($P < 0.05$) the number of intracellular *Toxoplasma* in PM compared with untreated control monolayers or with monolayers exposed to DMSO alone. No intracellular organisms were observed in PM which had been treated with 10 μg of CD per ml. Despite the ability of CD to prevent entry of *Toxoplasma* into PM, it did not prevent attachment of parasites to these cells. As the concentration of CD was increased and the number of intracellular organisms decreased, there was a concomitant increase in the number of trophozoites attached to PM. CD was the agent that prevented entry of *Toxoplasma* into PM, as demonstrated by the fact that the number of intracellular organisms in PM exposed to DMSO did not differ significantly from the number in untreated PM.

To determine the effect of CD on the uptake of an agent about which there is no controversy regarding direct invasion versus phagocytosis, monolayers of PM were incubated with heat-killed *Candida* in the presence of CD in parallel with monolayers incubated with *Toxoplasma*. The concentration of CD that produced complete inhibition of uptake of *Candida* by PM was comparable to the concentration of CD that resulted in the complete inhibition of entry of *Toxoplasma* into these cells, and the dose response was similar (Fig. 1).

To determine whether the effect of CD that we observed was due to an effect of this agent on *Toxoplasma*, 10^8 trophozoites were incubated for 14 min at 37°C in 1 ml of M199–20% FCS containing 10 μg of CD or containing DMSO alone. After this incubation period, M199–20% FCS (at 37°C) was added to effect a final concentration of 2×10^6 trophozoites per ml. (In separate experiments, the concentration of CD [0.2 μg/ml] in this final suspension had no inhibitory effect on entry of *T. gondii* into PM.) A 1-ml volume was added to each monolayer of PM and incubated for varying time intervals. In two separate experiments, we were unable to demonstrate any effect of the preincubation of *Toxoplasma* with CD alone on the entry or uptake of *Toxoplasma* trophozoites by PM.

Effect of CD on the entry of *T. gondii* into

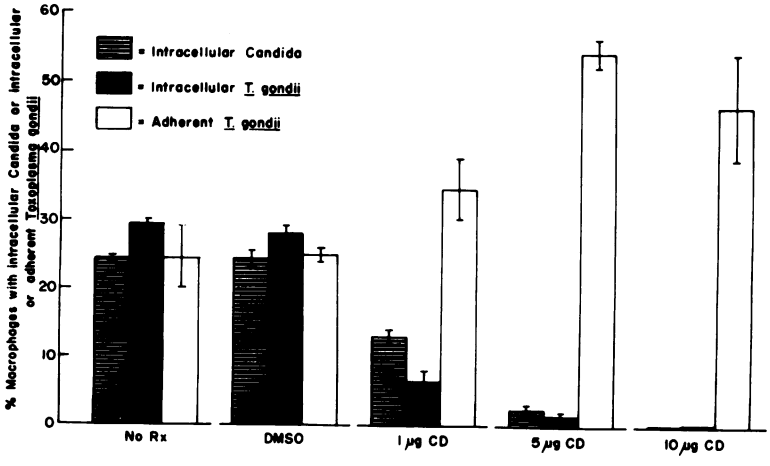

FIG. 1. *Effect of increasing concentration of CD on uptake of heat-killed Candida by PM and on entry of Toxoplasma into these cells. Monolayers were pre-incubated for 60 min with varying concentrations of CD (1, 5, or 10 μg of CD) in 0.5 ml of M199–20% FCS. Immediately thereafter, 2×10^6 Toxoplasma or heat-killed Candida was added directly to the medium containing CD, and the monolayers were incubated for an additional 60 min. Controls consisted of untreated (No Rx) monolayers or monolayers treated with DMSO. The concentration of DMSO was equivalent to the amount present in a medium containing 10 μg of CD per 0.5 ml. The results represent the mean of two experiments and the standard error. Each experiment was run in duplicate.*

B 4934 cells. To determine the effect of CD exposure on the entry of *Toxoplasma* into nonphagocytic cells, trophozoites were added to monolayers of B 4934 cells in the presence of CD. The B 4934 cells had been preincubated for 60 min with CD. Figure 2A shows that the dose-dependent inhibition of *Toxoplasma* entry into these cells was comparable to that observed in parallel experiments using PM (Fig. 2B). In addition, as was observed with PM, CD did not prevent the attachment of these organisms to B 4934 cells (Fig. 2A).

Effect of CD on the morphology of PM and B 4934 cells. Exposure of PM and B 4934 cells to CD resulted in morphological changes. With PM, this consisted primarily of a rounding in more than 90% of the cells, but no changes in the density of the monolayers exposed to CD were observed when compared with untreated monolayers or monolayers exposed to DMSO. The change in B 4934 cells consisted of a retraction of the cytoplasm toward the nucleus, leaving dendritic processes extending from the cell. Morphological changes were noted in 4 to 6% (mean ± standard error of the mean, 5 ± 1%) of B 4934 cells exposed to 10 μg of CD per ml for 2 h; when the concentration of CD was increased to 20 μg/ml, more than 90% of these cells exhibited morphological changes after a 2-h exposure.

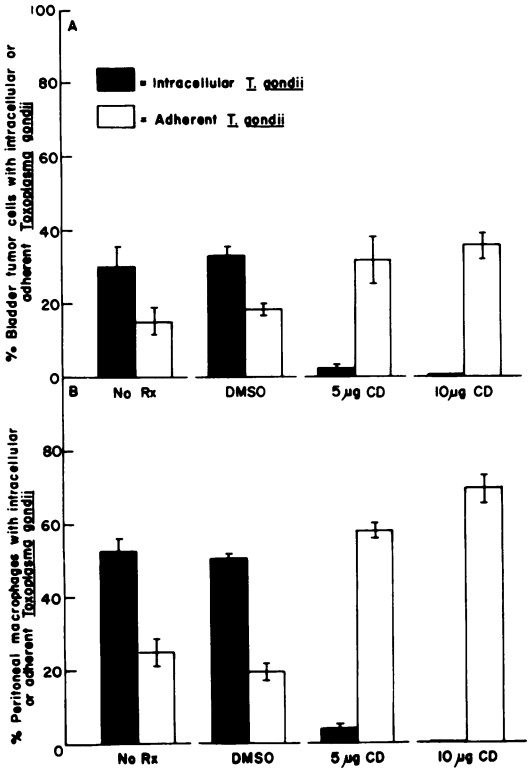

FIG. 2. *Effect of CD on the percentage of B 4934 cells (A) and PM (B) with intracellular or adherent Toxoplasma. Organisms were added directly to each chamber containing 5 or 10 μg of CD in 0.5 ml of M199-20% FCS. Monolayers were treated with CD for 60 min before the addition of Toxoplasma. Controls consisted of untreated monolayers (No Rx) or monolayers exposed to DMSO alone. The results represent the mean ± standard error of the mean for two experiments, and each experiment was run in duplicate.*

Reversibility of the effect of CD on PM and B 4934 cells. Parasites that were attached to PM and B 4934 cells were noted to be intracellular after the removal of CD. Under these circumstances it could not be determined whether the reversal of the inhibitory effect that was observed after the removal of CD was due to a loss of its effect on the parasite, on the cell, or on both.

In an attempt to solve this problem, PM were preincubated with CD. Monolayers were then washed, and the cells were incubated for varying periods of time with *T. gondii* or with heat-killed *Candida* in a medium without CD, in an attempt to determine whether the inhibitory effect on the cell entry of *T. gondii* persisted when *Toxoplasma* were not exposed to a medium containing CD. Figure 3 shows that the inhibitory effect of CD on *T. gondii* entry was not immediately reversible after washing of the monolayers. In addition, the loss of the inhibitory effect on the entry of *T. gondii* into PM closely paralleled the residual inhibitory effect on the ability of PM to phagocytize heat-killed *Candida* when compared with controls. The inhibitory effect of CD was completey gone when PM were examined at 60 min after the removal of CD, as was the inhibitory effect on the entry of *T. gondii* into B 4934 cells. Furthermore, *T. gondii* attached to PM and B 4934 cells in the presence of CD rapidly gained an intracellular position after the medium containing CD was replaced by a medium without this agent. CD exposure did not have an adverse effect on these organisms, as the number of infected cells, the number of *Toxoplasma* per cell, and their subsequent multiplication after internalization were comparable to controls. Also, morphological alteration of PM and B 4934 cells exposed to CD was reversed within 60 min after removal of this agent.

DISCUSSION

The results described above demonstrate that CD inhibits the entry of *T. gondii* both into phagocytes and into cells not generally considered to be phagocytic. In the case of PM, the concentrations of CD that produced increasing inhibition of phagocytosis of heat-killed *Can-*

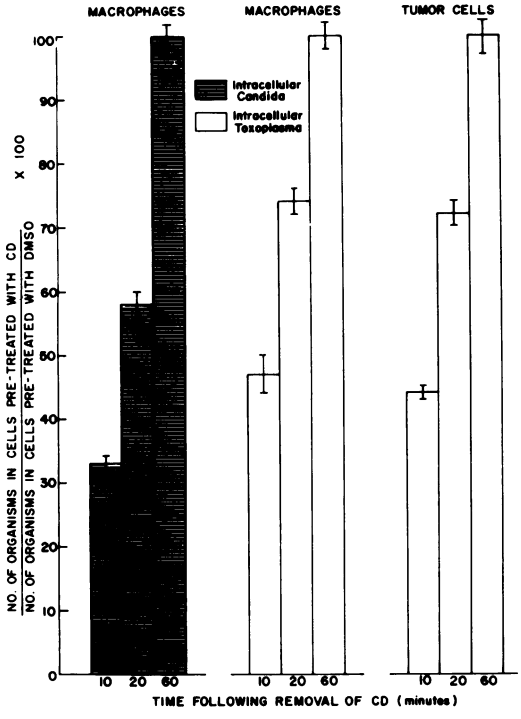

FIG. 3. *Reversibility of the inhibitory effect of CD on the entry of T. gondii into PM and B 4934 cells and on the uptake of heat-killed Candida by PM. PM were preincubated with medium containing CD or DMSO for 60 min, washed, and then incubated in CD-free medium with 2 × 10^6 Toxoplasma or heat-killed Candida for varying periods (10, 20, or 60 min). Tumor cells were similarly exposed to CD or DMSO, washed, and incubated with T. gondii for various time intervals. Each bar graph is expressed as a percentage of the results obtained in control (DMSO-treated) monolayers of PM or B 4934 cells. Each bar graph represents the results of two experiments, and each experiment was run in duplicate.*

dida were identical to those that produced increasing inhibition of entry of *T. gondii* into these cells. Excluding an effect of CD on the parasite, this suggests that endocytosis is the primary mechanism whereby *Toxoplasma* enter macrophages. In studies of *Leishmania mexicana* by Alexander (4) and of *Trypanosoma cruzi* by Nogueira and Cohn (17), the authors came to similar conclusions about the primary mechanism of entry of these protozoa into PM. In their studies, CB, an agent closely related to CD but less potent (14), prevented entry but not attachment of these parasites to PM. Unfortunately, in our study as well as in theirs, total inhibition of parasite entry into PM only occurred in the presence of cytochalasin, making it impossible to exclude an inhibitory effect of this agent on the organism as the reason for the lack of entry.

The mechanism of entry of *T. gondii* into cells which are not generally considered phagocytic has long been the subject of controversy (12). Although *T. gondii* is contained in a cytoplasmic vacuole once it gains an intracellular position, the real issue is the question of whether it achieves entry by invaginating the cell membrane without the active participation of the host cell or whether this parasite gains an intracellular position by inducing phagocytosis, as suggested by Jones et al. (9). The observation in our study that inhibition of entry of *T. gondii* into B 4934 cells by CD exactly paralleled the inhibition of entry of *T. gondii* into PM and of phagocytosis of killed *Candida* by PM supports the hypothesis of an active participation of the host cell in the entry process of *T. gondii*, probably through some as yet undefined stimulus from the parasite. That the lack of cell entry might be attributed to an effect of CB on the organism rather than on the cell was the conclusion of Jensen and Edgar (8), who studied the entry process of *Eimera magna* into Marvin Darby bovine kidney cells. In the presence of cytochalasin, the motility of this parasite was inhibited as documented under phase microscopy. However, in that study, an inhibitory effect on the cell, which may participate actively in cell entry by *Eimera*, could not be excluded.

The persistence of the inhibitory effect of CD on the entry of *T. gondii* into PM and B 4934 cells after removal of the medium containing CD suggests that CD prevents entry by acting on the target cells. The mechanism by which CD produces this inhibitory effect has not been elucidated (14), but the similarity in the kinetics of the loss of the inhibitory effect of CD on PM and B 4934 cells suggests that CD might exert its effect at a common location in both cell types. Microfilaments are likely candidates for this common site of action in preventing entry of *T. gondii* into cells. These subcellular structures are contractile proteins composed of actin and myosin polymers, which are present in most, if not all, mammalian cells (1, 2). The subunits of these proteins, namely, actin and actomysin, appear to be the major binding sites for CD (22). Furthermore, it has been noted with electron microscopy that, when *T. gondii* enters both phagocytes and cells not generally considered phagocytic, micropseudopods are frequently seen partially enveloping this organism as it gains an intracellular position (3, 9). The suggestion by Stossel (21) of the importance of contractile proteins in the formation of these pseudopods and his observation that the interaction

of these protein subunits results in actin filament gelation, which can be prevented by cytochalasin (7), support the hypothesis that microfilaments are the most likely subcellular site of CD action in the prevention of *T. gondii* entry.

The results of this study suggest that *T. gondii* acts on the nonphagocytic cell in some undefined way, inducing phagocytosis rather than utilizing the cell merely as a passive agent during the entry process. If this were not the case, it would be unlikely that the inhibition of entry of *T. gondii* and its reversibility after withdrawal of CD from the medium to which the cells were exposed would exactly parallel inhibition and recovery of *T. gondii* entry into PM, as well as phagocytosis of heat-killed *Candida* by macrophages. Our studies do not preclude the concept that *T. gondii* actively gains entry into nonphagocytic cells without active participation by the host cell; however, we consider this a less likely mechanism. For example, CD might render the cell membrane resistant to the parasite's efforts to gain an intracellular position. To establish whether either one or both mechanisms are operative requires development of methods to differentiate between the effect of CD on the host cell membrane and the effect of CD on *T. gondii* itself. Although we were unable to demonstrate an effect of CD on *Toxoplasma* that might account for inhibition of cell entry, the results of our experiments do not eliminate this possibility.

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