

Effect of Methylamine and Monodansylcadaverine on the Susceptibility of McCoy Cells to *Chlamydia trachomatis* Infection

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We used inhibitors of receptor-mediated endocytosis to study the mechanism of infectivity, especially the uptake mechanism, of *Chlamydia trachomatis* for cultured cells. The effect of methylamine and monodansylcadaverine on the different stages of the chlamydial growth cycle in McCoy cells was examined. There was a dose-related decrease in the number of chlamydial inclusions in the presence of these agents. Monodansylcadaverine also decreased the chlamydia-dependent uptake of radioactive amino acids. The agents did not affect the attachment of chlamydiae to the cells, but they increased the protease-removable fraction of cell-bound chlamydiae. The amines reduced the number of inclusions when added at different times during the first 24 h of infection. However, this effect was influenced by host cell density, so that the effect of the amines at the early infectious phase was nullified in confluent monolayers, whereas, during later phases, the effect was comparatively independent of host cell density. This indicates that the amines have different modes of action at different infectious stages. The effect of the amines was reversible, and they had no effect on the infectivity of pretreated chlamydial elementary bodies. These experiments suggest that methylamine and monodansylcadaverine inhibit both the internalization of chlamydiae into McCoy cells and their intracellular development. These results are consistent with the hypothesis that chlamydiae utilize a constitutive cellular process, such as receptor-mediated endocytosis, to enter cells.

Chlamydiae are obligate intracellular parasites with a unique growth cycle (1). Their attachment to susceptible cells is influenced by proteins or glycoproteins on the host cell surface and by carbohydrates and amino groups on the surface of the parasite (12). In some studies, specific receptor sites on the host cells for different chlamydial agents have been suggested (17, 18). In intestinal epithelial cells, the cytoplasm of the cell beneath the site of chlamydia attachment develops an increase in electron density, and the chlamydiae are ingested through invaginations of the host cell membrane, which later form intracellular vesicles (4). The uptake of chlamydiae by cultured cells or infectious phases closely linked to uptake are inhibited by glycolytic and oxidative inhibitors (7, 16) but not by cytochalasin B (10). This would suggest that the ingestion is a microfilament-independent endocytosis. Once inside the host cell, chlamydiae are segregated into endosomes. Initially, no fusion with host cell lysosomes takes place with viable chlamydiae, but endosomes containing chlamydiae inactivated by heat or neutralized by antiserum fuse with lysosomes (7). However,

lysosomal activity may participate in host cell cytotoxicity and the release of mature chlamydiae (15, 24).

In several systems, nonprofessional phagocytes have been shown to use endocytosis to internalize proteins, e.g., low-density lipoprotein and polypeptide hormones that have become bound to cell surface receptors (8). The endocytosis of influenza virus (20) and Semliki Forest virus (13) closely resembles this process. This type of endocytosis is generally termed receptor-mediated or adsorptive, depending on the nature of the ingested prey (19). By using a biochemical approach, we examined whether a similar mechanism could be operative in the uptake of chlamydiae by cultured cells.

MATERIALS AND METHODS

Chlamydiae. *Chlamydia trachomatis* serotypes E and L1 (a lymphogranuloma venereum strain) were kindly supplied by P.-A. Mårdh, University of Lund, Sweden. They were grown in embryonated eggs, and yolk sac suspensions subsequently were passaged fewer than 10 times in McCoy cells.

Mammalian cell culture. McCoy cells, a heteroploid

mouse fibroblast line, were used as host cells. They were grown in Eagle minimal essential medium as described previously (23).

Preparation of unlabeled and ^{14}C -labeled *C. trachomatis*. Preparation of *C. trachomatis* has been described in detail previously (23). Essentially, McCoy cells were grown on cover slips in plastic tubes, inoculated with *C. trachomatis* serotypes E or L1, and centrifuged ($3,000 \times g$ for 60 min) to infect 50 to 100% of the cells. Three hours after inoculation, 2 μg of cycloheximide per ml–0.5% glucose was added (for unlabeled chlamydiae). To obtain ^{14}C -labeled chlamydiae, emetine at a final concentration of 0.5 $\mu\text{g}/\text{ml}$ was added after 24 h, and, after another 6 h, 1 to 2.5 μCi of a ^{14}C -labeled mixture of amino acids (^{14}C -L-amino acid mixture; New England Nuclear Corp., Boston, Mass.) was added. After 72 h, the chlamydiae were harvested by sonication and purified by DNase, RNase, and trypsin; the ^{14}C -labeled chlamydiae were finally centrifuged in a 20 to 54% Renografin gradient (Renografin-76; E. R. Squibb & Sons, Princeton, N.J.) for 4 h at $100,000 \times g$. The fractions closest to a density of 1.20 g/ml, at which inclusion-forming units (IFU) and peak radioactivity coincided, were used in subsequent experiments.

Inclusion counting. McCoy cells were grown in plastic tubes (15 by 40 mm) (Luckham Ltd., Sussex, England) with a circular glass cover slip (13-mm diameter) (Chance Propper Ltd., Warley, England) at the bottom. Each tube was seeded with McCoy cells in 1 ml of growth medium. After approximately 24 h, the growth medium was decanted, and the monolayer was infected with *C. trachomatis*. The chlamydiae were suspended in a sucrose-phosphate buffer (9). A 0.5-ml amount of this suspension plus 0.5 ml of Earle balanced salt solution (EBSS) supplemented with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) was added to each tube. The chlamydiae were diluted so as to obtain approximately 200 IFU per cover slip diameter after 48 h of incubation, when the McCoy cell concentration was 2×10^5 cells per ml. This dilution gave about 200 IFU per cover slip diameter with 5×10^5 McCoy cells per ml and about 35 IFU with 5×10^4 McCoy cells per ml. The tubes were incubated while stationary at 37°C in 5% CO_2 –95% air. After 3 h, the medium was replaced with growth medium containing 2 μg of cycloheximide per ml and 0.5% glucose (21). After approximately 48 h, the medium was decanted, the monolayers were fixed for 10 min in methanol and stained with 5 and 2.5% iodine solutions for 10 min each, and the cover slips were mounted. The number of IFU was counted on one cover slip diameter at a magnification of 400, i.e., about 35 visual fields. This was a satisfactory area to count to assess changes in IFU achieved by modifications of McCoy cells with different agents (see below), since similar results were also obtained by counting IFU on the entire cover slip area, i.e., about 23 diameters. An incubation time of 48 h was chosen, since this regularly gave about twice as many IFU as incubation for 72 h.

Measurement of association of ^{14}C -labeled *C. trachomatis* with McCoy cells. Monolayers of McCoy cells in 50-mm-diameter plastic tissue culture petri dishes (Flow Laboratories, Irvine, Scotland) that had been seeded 24 h previously with 4 ml of 10^6 McCoy cells per ml were inoculated with 0.3 ml of ^{14}C -labeled *C.*

trachomatis serotypes E or L1 suspended in sucrose-phosphate buffer plus 0.3 ml of EBSS. The dishes were incubated during gentle rocking (80 strokes per min) at 37°C for 3 h, washed three times in 2 ml of phosphate-buffered saline (PBS) (pH 7.3), and incubated with 2 ml of 1% sodium dodecyl sulfate. The lysed cells were transferred to scintillation vials, and the petri dishes were washed three times in 2 ml of PBS and added to scintillation vials. After 10 ml of Aquasol (New England Nuclear Corp., Boston, Mass.) was added, the samples were counted in a beta scintillation counter (Isocap 300; Searle-Nuclear, Chicago, Ill.). About 50% of a labeled *C. trachomatis* serotype L1 inoculum and about 15% of a serotype E inoculum were associated with the McCoy cells. Where indicated, 0.5 mg of proteinase K (Boehringer, Mannheim, Germany) in 0.5 ml of ice-cold PBS was added instead of sodium dodecyl sulfate and incubated at 4°C for 45 min. The cells were removed by adding 3 ml of 4°C PBS and scraping with a rubber policeman, transferred with 2 ml of PBS wash to a conical tube, and centrifuged at $300 \times g$ for 5 min at 4°C (Wifug Clinic, AB Winkelcentrifug, Stockholm, Sweden). The supernatant was added to a scintillation vial, and the McCoy cells were washed twice in 2 ml of PBS at $300 \times g$ for 5 min at 4°C; the PBS wash was transferred to scintillation vials, and 2 ml of 1% sodium dodecyl sulfate was added to the pellet and incubated on a shaker at 37°C for 30 min. The sodium dodecyl sulfate-lysed cells were added to scintillation vials with a 2-ml PBS wash. A 10-ml amount of Aquasol was added to each vial, and the samples were counted in the beta scintillation counter to obtain proteinase K-resistant and -sensitive radioactivity.

Modifications of McCoy cells. Methylamine hydrochloride (MA) and monodansylcadaverine (MDC) were purchased from Sigma Chemical Co., St. Louis, Mo. MA was diluted in EBSS, and MDC was prepared in methanol and HCl, adjusted to pH 7.3, and diluted in EBSS. When chlamydial inclusions were to be scored, five experimental protocols were used. (i) Monolayers were pretreated for 30 min with MA or MDC; chlamydiae were added, together with agent, for 3 h (0 to 3 h); monolayers were washed twice with growth medium and subsequently incubated for 48 h for inclusion development. (ii) Monolayers were washed twice in EBSS, incubated with chlamydiae for 3 h, washed twice in EBSS, incubated for 3 h with MA or MDC (3 to 6 h), washed twice with growth medium, and incubated for 48 h for inclusion development. (iii) Monolayers were washed twice in EBSS, incubated with chlamydiae for 3 h, washed twice in growth medium, incubated for 3 h without agent, washed twice in EBSS, incubated with MA or MDC for 3 h (6 to 9 h), washed twice with growth medium, and incubated for 48 h for inclusion development. (iv) Monolayers were treated as in (iii), but with MA or MDC added at 24 to 27 h after inoculation with chlamydiae. (v) Monolayers were pretreated with MA and MDC for 30 min or 3 h, washed twice in EBSS, incubated with chlamydiae without agent for 3 h, and subsequently handled as in (i).

When association of ^{14}C -labeled chlamydiae was to be scored, monolayers were pretreated for 30 min with MA or MDC, chlamydiae were added with agent for 3 h, and monolayers were processed as described above.

Modifications of chlamydiae. Suspensions of *C. trachomatis* serotype E were incubated with MA or MDC at 37°C for 3 h, centrifuged twice in EBSS at 12,000 × g for 30 min in a Sorvall centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.), and titrated in McCoy cells for IFU as described above.

Chlamydial amino acid utilization. The effect of MDC on the ability of McCoy cells to support chlamydial growth was also assessed by measuring the chlamydia-dependent utilization of radioactive amino acids (25). McCoy cells were grown overnight to subconfluent monolayers in 24-well tissue culture trays with an area of 2 cm² per well (Flow Laboratories, Irvine, Scotland). After being washed in EBSS, the monolayers were incubated for 2 h at 37°C in minimal essential medium containing 1.0 µg of emetine hydrochloride per ml (Sigma). After this medium was decanted, the monolayers were incubated with 1.0 µg of emetine per ml in EBSS with or without 250 µM MDC for 30 min. After further washings in EBSS, 200-µl volumes of either sucrose-phosphate buffer (uninfected control) or *C. trachomatis* serotype E suspended in sucrose-phosphate buffer, plus 200 µl of EBSS with or without MDC (final concentration, 250 µM), were added to indicated wells. The trays were incubated during gentle rocking at 37°C for 3 h, washed in EBSS, further incubated in minimal essential medium supplemented with 0.5 µg of emetine and about 0.2 µCi of a ¹⁴C-labeled mixture of amino acids per ml, and incubated for about 48 h after chlamydial inoculation. The monolayers were then washed twice in PBS, solubilized in 1% sodium dodecyl sulfate at 37°C overnight, and transferred to vials with 2 ml of wash fluid and 10 ml of Aquasol for liquid scintillation counting. The monolayers were treated with MDC at different stages of the chlamydial growth cycle (described above), i.e., 0 to 3, 3 to 6, 6 to 9, and 24 to 27 h after inoculation with chlamydiae. The radiolabeled amino acids for each protocol were added to monolayers after treatment with MDC. The controls were emetine-treated uninfected cells with and without MDC to ensure that MDC did not affect the protein synthesis in McCoy cells and uninfected cells with and

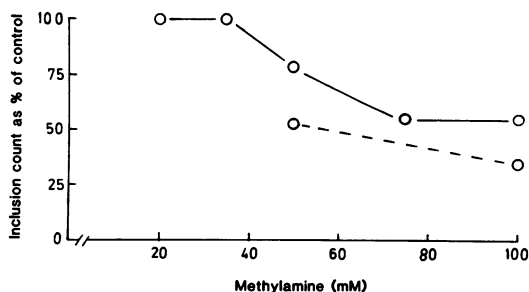


FIG. 1. Effect of MA on the number of inclusions of *C. trachomatis* serotype E in McCoy cells. The cells were pretreated with agent for 30 min (○—○) or 90 min (○- - -○), and the agent was subsequently present with chlamydiae during the first 3 h of infection. The McCoy cell density was 2×10^5 cells per cover slip. An inclusion count of 100% corresponds to about 200 IFU per cover slip diameter.

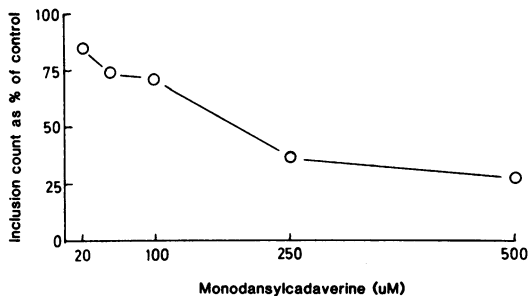


FIG. 2. Effect of MDC on the number of inclusions of *C. trachomatis* serotype E in McCoy cells. The cells were pretreated with agent for 30 min, and the agent was subsequently present with chlamydiae during the first 3 h of infection. The McCoy cell density was 2×10^5 cells per cover slip. An inclusion count of 100% corresponds to about 200 IFU per cover slip diameter.

without emetine to ensure that the host cell protein synthesis was blocked.

RESULTS

Effect of MA and MDC on the number of inclusions of *C. trachomatis* in McCoy cells. Both MA and MDC inhibited the number of chlamydial inclusions in a dose-response relationship in which the McCoy cells were pretreated with MA or MDC and the agents were mixed with chlamydiae for 3 h (Fig. 1 and 2). When the McCoy cells were pretreated with MA for 90 min instead of 30 min and MA was mixed with chlamydiae for the next 3 h, the inhibition of IFU increased 20 to 25% (Fig. 1).

MA and MDC also inhibited the number of inclusions when added to 2×10^5 McCoy cells at different times after withdrawal of the chlamydial inoculum (Table 1). When 75 mM MA was present for 3 consecutive h after chlamydial withdrawal (3 to 6 h), the effect was similar to that which occurred when 75 mM MA was mixed with chlamydiae (0 to 3 h): 51 and 54% of IFU developed, respectively. When 75 mM MA was present for 6 to 9 h after the addition of chlamydiae, the number of IFU decreased significantly, from 100 to 76% ($P < 0.05$; Student's *t* test) compared with untreated control cells, but results were similar to those with 75 mM MA present for 0 to 3 h: 76 and 54%, respectively ($P < 0.1$; Student's *t* test) (Table 1). When 75 mM MA was present 24 to 27 h after the addition of chlamydiae, only 17% of IFU developed, significantly less than that for both untreated control cells ($P < 0.005$; Student's *t* test) and cells treated during other periods ($P < 0.05$; Student's *t* test).

When 250 µM MDC was added to 2×10^5 McCoy cells at different times in relation to the addition of chlamydiae, there was a significant

TABLE 1. Effect of MA and MDC on the number of inclusions of *C. trachomatis* serotype E in McCoy cells^a

Time ^b (h)	% Control \pm SEM ^c	
	MA (75 mM)	MDC (250 μ M)
0-3	54 \pm 4.9 ($P < 0.002$)	37 \pm 2.2 ($P < 0.02$)
3-6	51 \pm 4.8 ($P < 0.05$)	59 \pm 4.1 ($P < 0.05$)
6-9	76 \pm 1.5 ($P < 0.05$)	54 \pm 1.6 ($P < 0.005$)
24-27	17 \pm 0.5 ($P < 0.005$)	52 \pm 4.4 ($P < 0.02$)

^a Determined at a McCoy cell density of 2×10^5 cells per cover slip.

^b Time at which the agent was added and withdrawn, respectively. At 0 to 3 h, McCoy cells were pretreated with agent for 30 min. Chlamydiae were present at 0 to 3 h.

^c Results are expressed as percentage of control receiving EBSS alone for each treatment period. P values, Student's t test, compared with nontreated control cells.

reduction in the number of IFU compared with untreated cells ($P < 0.05$; Student's t test), whereas the effect on the number of IFU in McCoy cells treated for different periods was somewhat similar (Table 1).

The effect of MA and MDC on the number of inclusions was dependent on McCoy cell density (Tables 2 and 3). At high cell density, 5×10^5 McCoy cells per ml, giving confluent monolayers, the inhibiting effect of 75 mM MA was abolished when MA was added to chlamydiae at 0 to 3 h, but MA retained its effect when it was added later in the infectious process (Table 2). Also, when 75 mM MA was added at 0 to 3 h to confluent monolayers, the effect was significantly less than the corresponding effect in subconfluent (2×10^5 cells per ml) ($P < 0.005$; Student's t test) and sparse (5×10^4 cells per ml) ($P < 0.002$; Student's t test) monolayers. However, when 75 mM MA was added later in the infectious process, the effect was similar for confluent, subconfluent, and sparse cultures ($P < 0.5$;

Student's t test), except in sparse cultures, in which the inhibitory effect was less pronounced when MA was added at 24 to 27 h (Tables 1 and 2). A 250- μ M amount of MDC had a statistically significant inhibitory effect on the number of IFU in confluent monolayers only when added at 6 to 9 h. However, the number of IFU decreased significantly, from 75% in confluent to 37% in subconfluent monolayers, when 250 μ M MDC was added at 0 to 3 h ($P < 0.01$; Student's t test) (Table 3). When MDC was added to McCoy cells later in the infectious cycle, the effect was, as with 75 mM MA, similar for confluent and subconfluent monolayers. Pretreatment of McCoy cells for 30 min or 3 h or chlamydiae for 3 h with MA or MDC did not significantly ($P < 0.5$; Student's t test) affect the number of chlamydial inclusions (Table 4).

The agents had no effect on the number of McCoy cells that excluded trypan blue when monolayers were treated with 75 mM MA or 250 μ M MDC for 3 h and then stained with trypan blue.

Effect of MDC on the chlamydia-dependent uptake of radiolabeled amino acids. Emetine (1 μ g/ml) inhibited the incorporation of amino acids in uninfected McCoy cells by more than 95% (data not shown). However, emetine has no effect on amino acid utilization in chlamydiae (25). MDC significantly decreased the incorporation of amino acids in chlamydia-infected compared with nontreated cultures. However, MDC had no effect on amino acid utilization in uninfected cells (Fig. 3). Thus, MDC selectively affects the ability of McCoy cells to support chlamydial growth. This effect is expressed at least during the first 24 h after chlamydial inoculation.

Effect of MA and MDC on the association of *C. trachomatis* with McCoy cells. There was no significant effect on the association of *C. trachomatis* serotypes E and L1 with McCoy cells when the monolayers were pretreated for 30 min with 75 mM MA or 250 μ M MDC and subse-

TABLE 2. Effect of MA on the number of inclusions of *C. trachomatis* serotype E at different McCoy cell densities

Time (h) ^a	% Control \pm SEM ^b		P^c
	5×10^5 McCoy cells	5×10^4 McCoy cells	
0-3	96 \pm 5.5 ($P < 0.8$)	39 \pm 6.3 ($P < 0.01$)	<0.002
3-6	48 \pm 3.3 ($P < 0.002$)	55 \pm 4.6 ($P < 0.2$)	<0.5
6-9	67 \pm 7.9 ($P < 0.001$)	51 \pm 5.6 ($P < 0.05$)	<0.5
24-27	19 \pm 3.9 ($P < 0.001$)	41 \pm 1.3 ($P < 0.05$)	<0.05

^a Time at which 75 mM MA was added and withdrawn, respectively. At 0 to 3 h, McCoy cells were pretreated with agent for 30 min. Chlamydiae were present at 0 to 3 h.

^b Results are expressed as percentage of control receiving EBSS alone for each treatment period and cell density. P values, Student's t test, compared with nontreated control cells.

^c Student's t test; comparison between 5×10^5 and 5×10^4 McCoy cells treated with MA for the same period.

TABLE 3. Effect of MDC on the number of inclusions of *C. trachomatis* serotype E at different McCoy cell densities

Time (h) ^a	% Control \pm SEM ^b		P ^c
	5 \times 10 ⁵ McCoy cells	2 \times 10 ⁵ McCoy cells	
0-3	75 \pm 1.3 (P < 0.1)	37 \pm 2.2 (P < 0.02)	<0.01
3-6	62 \pm 1.4 (P < 0.1)	59 \pm 4.1 (P < 0.05)	<0.8
6-9	61 \pm 3.5 (P < 0.005)	54 \pm 1.6 (P < 0.005)	<0.5
24-27	75 \pm 3.9 (P < 0.1)	52 \pm 4.4 (P < 0.02)	<0.2

^a Time at which 250 μ M MDC was added and withdrawn, respectively. At 0 to 3 h, McCoy cells were pretreated with agent for 30 min. Chlamydiae were present at 0 to 3 h.

^b Results are expressed as percentage of control receiving EBSS alone for each treatment period and cell density. P values, Student's *t* test, compared with nontreated control cells.

^c Student's *t* test; comparison between 5 \times 10⁵ and 2 \times 10⁵ McCoy cells treated with MDC for the same period.

quently incubated for 3 h with chlamydiae and agents (Table 5).

When ¹⁴C-labeled *C. trachomatis* serotype E was allowed to associate with McCoy cells in EBSS, about 45% of the radioactivity was proteinase K resistant, i.e., was not removed by 1 mg of proteinase K per ml at 4°C for 45 min (Fig. 4). The corresponding proteinase K resistance for serotype L1 was about 64%. When chlamydiae were allowed to associate with McCoy cells in the presence of 75 mM MA or 250 μ M MDC for 3 h before treatment with proteinase K, the proteinase K-resistant radioactivity decreased for serotype E and especially for serotype L1 with both agents (Fig. 4). By calculating proteinase K-resistant chlamydial fractions in the presence of agent as a percentage of proteinase K-resistant fractions in the absence of agent (in EBSS), the data in Table 6 were obtained. Thus, both agents significantly increased the proteinase K-removable fraction of cell-bound chlamydiae, with the exception of serotype E and MA.

DISCUSSION

Both MA and MDC inhibited the inclusion formation and MDC also inhibited the amino

acid uptake of *C. trachomatis* serotype E in McCoy cells (Fig. 1 to 3). This inhibition was expressed at various phases of the infectious process (Tables 1 to 3). Experiments were therefore designed to try to elucidate at what stage or stages in the infectious cycle the inhibition was exerted. The infection of McCoy cells with *C. trachomatis* may schematically be separated into the following stages: (i) attachment of chlamydiae to the cell surface, (ii) internalization, (iii) intracellular development of chlamydiae, and (iv) release of mature chlamydiae.

MA and MDC did not show any cytotoxic effect toward McCoy cells as assessed by exclusion of trypan blue or by the effect of MDC on amino acid uptake (Fig. 3). Neither MA nor MDC exerted any residual activity, since pretreatment of cell monolayers with MA or MDC followed by addition of chlamydiae in the absence of agent did not affect the number of IFU (Table 4). A corresponding reversible effect has also been shown for dansylcadaverine in the uptake of vesicular stomatitis virus (22) and for

TABLE 4. Effect on the number of inclusions of *C. trachomatis* serotype E after pretreatment of McCoy cells or chlamydiae with MA or MDC^a

Pretreatment of:	% Control \pm SEM	
	MA	MDC
McCoy cells (30 min) ^b	105 \pm 3.9	101 \pm 1.2
McCoy cells (3 h) ^b	99 \pm 6.0	99 \pm 9.9
Chlamydiae (3 h) ^c	104 \pm 1.8	87 \pm 5.8

^a Determined at a McCoy cell density of 2 \times 10⁵ cells per cover slip. Results are expressed as percentage of control receiving EBSS.

^b 75 mM MA or 250 μ M MDC.

^c 100 mM MA or 500 μ M MDC.

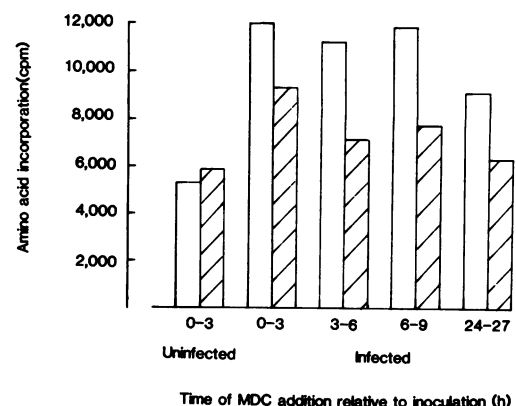


FIG. 3. Effect of MDC on the amino acid incorporation in *C. trachomatis* serotype E-infected and uninfected McCoy cells. □, Untreated (EBSS) cultures; ▨, 250 μ M MDC-treated cultures.

TABLE 5. Effect of MA and MDC on the association of *C. trachomatis* serotypes E and L1 with McCoy cells^a

Agent	cpm \pm SEM ^b	
	Serotype E	Serotype L1
EBSS	952 \pm 22.3	2,418 \pm 87.3
MA (75 mM)	1,066 \pm 22.3 ($P < 0.2$)	2,093 \pm 121.3 ($P < 0.5$)
MDC (250 μ M)	1,039 \pm 2.1 ($P < 0.2$)	2,254 \pm 97.2 ($P < 0.5$)

^a Agents were present 30 min before and during 3 h of incubation with chlamydiae.

^b Results are expressed as cpm associated with 4×10^6 McCoy cells. About 6,500 cpm of serotype E and about 5,000 cpm of serotype L1 were added to the McCoy cells, corresponding to 5.9×10^6 and 1.5×10^6 IFU, respectively, when the number of IFU was titrated in McCoy cells with the aid of centrifugation. P values, Student's t test, compared with nontreated control cells.

MA in receptor-mediated internalization of *Pseudomonas* toxin (6). Pretreatment of chlamydiae with MA or MDC did not affect the number of IFU (Table 4). Thus, the agents exerted their effect on the ability of McCoy cells to support chlamydial growth or, possibly, on intracellular developmental forms of chlamydiae. No effect of the agents was observed on the association of radiolabeled chlamydiae with McCoy cells (Table 5). We regard this association after 3 h of incubation as reflecting both attached and internalized chlamydiae and observe that the attachment was not affected by the agents. To distinguish between the chlamydiae on the cell surface and those inside the cell, we used

proteinase K, a nonspecific protease which has been previously applied to remove proteins external to membranes (26). The data in Fig. 4 show that the fraction of proteinase K-resistant chlamydiae decreased in the presence of MA and MDC; i.e., these agents inhibited at the internalization step of chlamydial infection. Furthermore, MA and MDC also inhibited the number of IFU when they were added after the removal of the chlamydiae (Table 1). Thus, we conclude that these agents inhibit the infectivity of *C. trachomatis* toward McCoy cells during the chlamydial uptake process and the intracellular development of chlamydiae.

To rule out the possibility that the inhibition of the number of IFU was unique to the iodine-staining procedure, we confirmed the results with MDC by measuring chlamydia-dependent uptake of amino acids (Fig. 3). The results show that the effect of MDC is a metabolic effect inhibiting chlamydial growth and not an effect of glycogen accumulation. Furthermore, the magnitude of inhibition in the two assays was somewhat similar: 63% for IFU and 49% for amino acid utilization when infected cells were treated at 0 to 3 h with MDC (Table 1 and Fig. 3). Also, the number of IFU in cultures treated with MDC was identical when the cultures were stained with iodine or Giemsa (data not shown).

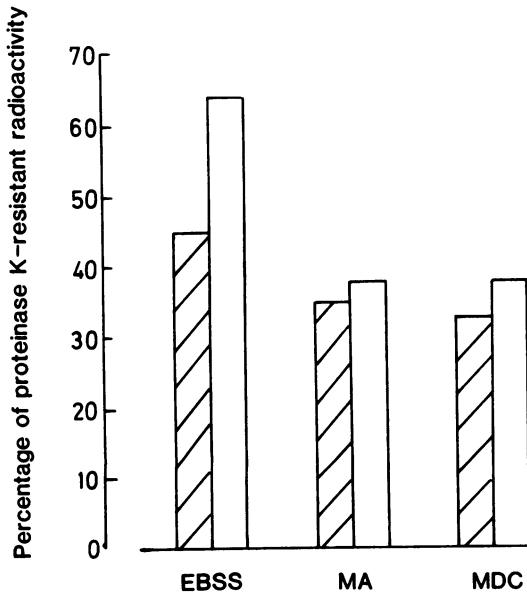


FIG. 4. Effect of MA and MDC on the proteinase K-resistant fraction of McCoy cell-associated radiolabeled *C. trachomatis* serotype E (▨) and L1 (□). An association of 100% corresponds to about 1,000 cpm/ 4×10^6 McCoy cells for serotype E and about 2,500 cpm for serotype L1.

TABLE 6. Fractions of proteinase K-resistant radioactivity from *C. trachomatis* associated with McCoy cells in the presence of MA or MDC

Agent	% Radioactivity \pm SEM ^a	
	Serotype E	Serotype L1
MA (75 mM)	77 \pm 5.1 ($P < 0.1$)	59 \pm 4.4 ($P < 0.05$)
MDC (250 μ M)	73 \pm 1.5 ($P < 0.002$)	59 \pm 0.04 ($P < 0.001$)

^a Calculated from Fig. 4: (% proteinase K-resistant fraction in presence of agent)/(% proteinase K-resistant fraction in presence of EBSS) \times 100. P values, Student's t test, compared with nontreated control cells.

Chlamydiae are obligate intracellular parasites and should therefore have an extraordinary capability to infect mammalian cells. Byrne and Moulder (2) have shown that the uptake of chlamydiae by nonprofessional phagocytes is 10 to 100 times greater than the uptake of *Escherichia coli*. They suggest that the chlamydiae have evolved surface structures with a high affinity for normal, ubiquitously occurring structures on the host cell surface. Thus, chlamydiae enter host cells by parasite-specified phagocytosis.

Several physiological substances, such as low-density lipoprotein and α_2 -macroglobulin, are taken up by host cells by a process termed receptor-mediated endocytosis (8). The transport of some such substances into cells is reduced by several amines, e.g., MA and dansylcadaverine (3). This inhibition may occur at the internalization step because MA and dansylcadaverine are transglutaminase inhibitors and interfere with the clustering of receptors (3) or change receptor recycling or insertion of newly synthesized receptors (14).

The effect of MA on the number of chlamydial inclusions was abolished in confluent monolayers when the agent was present with chlamydiae during the first 3 h of the infectious process (Table 2). Analogously, MA seems to inhibit the clustering of α_2 -macroglobulin to a higher extent in sparse cultures of CHO cells compared with dense cultures (3). However, MA retained its effect on the number of IFU when it was added to confluent monolayers later during infection. This effect was less obvious with MDC, but the number of IFU decreased in subconfluent compared with confluent monolayers when MDC was present during the first 3 h of infection; the number of IFU was independent of cell density when MDC was added later (Table 3). This indicates that the agents had different modes of operation at various stages in the infectious cycle. In confluent monolayers, the number and distribution of exposed sites in the cell membrane to which chlamydiae attach may be different compared with subconfluent monolayers. This difference could be crucial for the early effect of MA and MDC, whereas it would be indifferent for their later effect on the intracellular development of chlamydiae. On this basis, and because of the shift of proteinase K-resistant chlamydial radioactivity in the presence of MA or MDC, we suggest that these substances may inhibit the internalization of chlamydiae.

Many of the amines mentioned earlier are also lysosomotropic agents that influence phagosome-lysosome fusion (11). This observation is of special interest with regard to chlamydiae, since endosomes containing viable chlamydiae do not fuse with lysosomes (5, 7). Since the

magnitude of the inhibition of the number of IFU was essentially independent of host cell density when the agents were added in later infectious phases (later than 3 h after inoculation of chlamydiae), we suggest that this may reflect their lysosomotropic properties. However, MA and MDC may also affect intracellular developmental forms of chlamydiae without activation of lysosomes. Furthermore, the effect of MA was greatest when it was added 24 h after infection, when chlamydial metabolism is at its height (1) (Table 1). The amines had a greater effect on the number of IFU and the uptake of amino acids than on the shift of proteinase K-resistant radioactivity (Tables 1 and 6 and Fig. 3). It is therefore possible that they influence both uptake and intracellular development when added early in the infectious process.

In conclusion, MA and MDC inhibit the infectivity of *C. trachomatis* toward McCoy cells. This inhibition may operate at the internalization step and during intracellular development of chlamydiae. These results are compatible with the hypothesis that chlamydiae utilize a constitutive cellular process, such as adsorptive (receptor-mediated) endocytosis, to enter cells.

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