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Endocytic Mechanisms Utilized by Chlamydiae and Their Influence on Induction of Productive Infection

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The microfilament-disrupting drug cytochalasin D and, initially, inoculation at 20°C were used to differentiate between phagocytosis (sensitive to both treatments) and pinocytosis (resistant to both treatments) to assess whether chlamydial uptake into McCoy cells occurred by one or both mechanisms and whether each could contribute to productive infection. Both treatments suppressed the infectivity of *Chlamydia trachomatis* L2/434/Bu and *C. psittaci* GPIC (the guinea pig inclusion conjunctivitis strain) following static inoculation by only 50%, indicating that there was simultaneous operation of both phagocytosis and pinocytosis during uptake that led to productive infection. Measurement of the entry of organisms by two separate assays established that both strains predominantly used a cytochalasin D-resistant (pinocytic) mechanism, implying that phagocytic uptake was coupled to a higher frequency of productive infection. Integration of the data on infectivity and entry allowed the potential for an organism to infect a host cell to be quantified. This synthesis revealed that for both strains the infectivity potential following phagocytic entry was ca. 10-fold greater than that following pinocytic entry. However, both entry mechanisms were exploited more efficiently by strain L2/434/Bu than by strain GPIC (unless the latter was inoculated with centrifugation), indicating that intrinsic strain properties are more important for infectivity potential than the endocytic mechanism utilized.

The genus *Chlamydia* contains two major species of parasitic bacteria, *Chlamydia trachomatis* and *C. psittaci*. Both of these species have been shown to enter host cells by endocytosis (10), avoid lysosomes (4, 10), and initiate their replication cycle in 1 to 6 h (4, 6, 10), leading to development of characteristic inclusion bodies within infected cells. Not all endocytosed organisms go on to multiply in host cells (12, 16, 18), and it has been proposed that events during entry are pivotal in determining the potential for multiplication (16).

For lymphogranuloma venereum-causing *C. trachomatis* 434, which is highly infectious for cell cultures, evidence has been presented that entry can occur by phagocytosis—with the central findings that treatment with the microfilament inhibitor cytochalasin D (CD) inhibited entry by 50% and that association with clathrin-coated pits could not be detected (24). However, morphological evidence for uptake by coated-pit pinocytosis was presented by Wyrick and colleagues for *C. psittaci* CAL-10, which, like strain L2/434/Bu (hereafter called strain 434) is highly infectious for cell cultures (8). The finding that strain 434 showed only partially reduced entry after CD treatment led, in conjunction with the morphological evidence, to the proposal that chlamydiae may be capable of entry by both endocytic mechanisms (13).

Subsequent research in Wyrick's laboratory has provided morphological evidence for coated-pit entry by both strain 434 and *C. trachomatis* E/UW-5/CX—a strain which, like other trachoma strains, is poorly infectious for cell cultures in the absence of centrifugation—and drawn attention to the importance of the cell attachment substratum for visualization of coated-pit structures (7, 26). Studies on poorly infectious *C. psittaci* GPIC (the guinea pig inclusion conjunctivitis strain) have indicated that uptake was resistant to CD (i.e., pinocytic) and coupled to a low frequency of productive infection but that inoculation with centrifugation switched entry to a dominantly phagocytic mechanism coupled to increased productive infection (16). Further characterization of the pinocytic entry mechanism using specific physiological inhibition by cytosol acidification (17, 20) confirmed utilization of coated pits by both strains 434 and GPIC, with additional evidence for uncoated-membrane uptake (14) by strain 434 (17). Taken together, these observations suggest that pinocytosis is an uptake mechanism common to all chlamydiae. However, they leave unresolved the question of whether poorly infectious chlamydiae, as instanced by strain GPIC, are capable of induction of phagocytosis in the absence of centrifugation (16, 26) and whether, in turn, the endocytic mechanism or intrinsic strain properties are more important for infectivity differences between strains.

To answer these questions, CD and, initially, inoculation at 20°C were used to restrict strains GPIC and 434 to a pinocytic mode of entry and the effects of these treatments on the subsequent infectivity of both strains were assessed. Entry was then quantified in the presence and absence of CD by two separate techniques. The results obtained allowed the potential for an organism to multiply to be evaluated for a given entry mechanism in terms of the number of organisms that must enter a cell for an inclusion to develop. The data reveal the simultaneous utilization of phagocytosis and pinocytosis by the two strains during entry into McCoy cells in static incubation, with pinocytosis coupled to a lower and phagocytosis to a higher infectivity potential. However, strain characteristics were shown to be the dominant influence on productive infection in that, irrespective of the entry mechanism, strain 434 had a higher infectivity potential than strain GPIC.

MATERIALS AND METHODS

Growth and purification of chlamydiae. Radiolabeled and unlabeled *C. psittaci* GPIC and *C. trachomatis* 434 were

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grown in McCoy cells (Flow Laboratories) and purified by the Urografin batch procedure as previously described (17).

Measurement of chlamydial particle numbers. Chlamydial particle numbers were measured as previously described (17). Briefly, poly-L-lysine-coated coverslips in 7-ml glass bottles were centrifuged with dilutions of chlamydiae and the organisms adhering to the coverslips were counted following immunofluorescence staining. The proportion of the inoculum attaching to the coverslip was determined by using radiolabeled chlamydiae.

Preparation of McCoy cell monolayers and infection with chlamydiae. McCoy cells were grown in complete Eagle minimal essential medium as previously described (17). Monolayers were produced by seeding 2×10^5 cells onto 12-mm-diameter coverslips in glass bottles or into 24-well plastic trays (17).

For infection, suspensions of chlamydiae were inoculated onto monolayers either statically or by centrifugation (1,580 $\times$ *g*) at 35°C for 1 h or by centrifugation at 2°C for 30 min, followed by entry into host cells during either static incubation or centrifugation at 35°C for 1 h. In certain experiments, the microfilament inhibitor CD (Sigma; the stock solution was 500 $\mu g\ ml^{-1}$ in dimethyl sulfoxide) was present in the medium from 1 h prior to inoculation until 4 h postinoculation.

Measurement of infectivity. Infected monolayers were incubated at 37°C for 24 to 48 h in complete Eagle minimal essential medium containing cycloheximide (1 $\mu g\ ml^{-1}$) and then either fixed in acid alcohol (33% glacial acetic acid in ethanol) for 10 min and stained with 0.01% acridine orange (21) or fixed in methanol (10 min) and stained by indirect immunofluorescence. In most of the experiments, 10 to 15% of the cells produced inclusions, ensuring that few cells in a monolayer received more than one inclusion-forming unit (IFU).

Measurement of chlamydial uptake. Entry of ^3H-labeled chlamydiae into McCoy cells was assessed at 4 h postinoculation by two methods: trypsin assay and immunofluorescence assay. The trypsin assay was done as previously described (17). Briefly, monolayers were treated with 0.25% trypsin (GIBCO) to remove extracellular attached radiolabeled chlamydiae, the detached chlamydiae and cells were separated by differential centrifugation in 1.5-ml microcentrifuge tubes (Sarstedt), and the radioactivity in each was determined. The immunofluorescence assay used was a modification of a previous method (15). Monolayers were rinsed with ice-cold Dulbecco phosphate-buffered saline (pH 7.2) and then fixed in paraformaldehyde (1.5% in phosphate-buffered saline) for 10 min on ice. Paraformaldehyde fixation did not permeabilize the cells to antibody (judged by the inability to stain inclusions in fixed infected monolayers immunofluorescently) and hence allowed selective staining of external attached chlamydiae by indirect immunofluorescence. The total number of chlamydiae associated per monolayer was determined by using factors derived from the particle and radioactivity counts of the ^3H-radiolabeled chlamydial inocula. Subtraction of the number of external attached chlamydiae from the total value gave the number of internalized organisms.

Cell loss. Exposure of McCoy cell monolayers to CD at 0.1 to 10 $\mu g\ ml^{-1}$ caused detachment of cells. In most of the experiments, the drug was used at 0.5 $\mu g\ ml^{-1}$ since this concentration gave nearly maximal inhibition of infection and entry (see Results) with negligible cell contraction and reduced cell detachment. Cell loss was corrected for by counting the numbers of cells remaining attached in both drug-treated and untreated control monolayers after counterstaining with rhodamine (Difco; diluted 1/100 in phosphate-buffered saline).

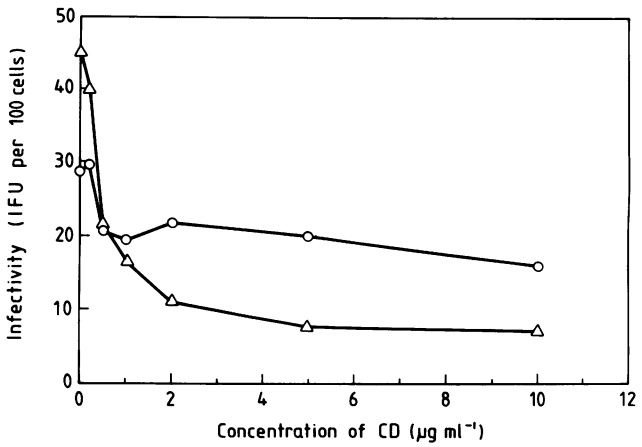

FIG. 1. Effect of an increased CD concentration on the infectivity of strains GPIC (○) and 434 (△). Following inoculation at 2°C, media containing CD were added and McCoy cell monolayers were maintained on ice for 1 h. After a further 4 h of incubation at 35°C, the media were replaced with complete Eagle minimal essential medium containing cycloheximide and the monolayers were incubated for inclusion development. The values are means from three monolayers corrected for cell loss; the standard deviations were less than 13% of the mean values.

RESULTS

Effects of phagocytosis inhibitors on chlamydial infectivity. When chlamydiae were attached to McCoy cells at 2°C and then the monolayers were exposed to CD during organism entry, there was marked reduction in infectivity for both strains as the CD concentration was increased to ca. 0.5 $\mu g\ ml^{-1}$ (Fig. 1). Strain 434 showed a further infectivity reduction as the CD concentration was raised to 2 $\mu g\ ml^{-1}$ (Fig. 1). However, a concentration of 0.5 $\mu g\ ml^{-1}$ was adopted for subsequent experiments because this concentration minimized cell loss and appeared to suppress the entry of both strains maximally (see below).

When the effects of exposure of McCoy cells to CD at 0.5 $\mu g\ ml^{-1}$ or inoculation at 20°C on chlamydial infectivity were compared, similar patterns of infectivity suppression were observed (Table 1). With both treatments, there was a ca. 50% reduction in the infectivity of strains 434 and GPIC; for strain GPIC only, this was further reduced to ca. 80% by centrifugation of the inoculum.

Interpretation of the data (Table 1) with respect to the influence of the entry mechanism on the infectivity of chlamydiae was impeded by a number of alternative effects that treatment with CD during entry and inoculation at 20°C may have had. (i) Both treatments may have affected organism viability or caused impaired attachment to McCoy cells, leading to reduced infectivity. (ii) Some organisms may have entered after the restrictive conditions imposed by the drug or the low-temperature treatment had been withdrawn (late entry), so that the true contribution to infectivity of the drug and low-temperature-sensitive entry pathway was underassessed. (iii) Although host cell loss was corrected for, it was assumed that the loss would be random. However, if selective detachment of infected host cells occurred in treated

TABLE 1. Effects of phagocytosis inhibitors on the infectivity of strains GPIC and 434

Inhibitor	Strain	Mode of inoculation	Mean no. of IFU 2,000 cells^{-1} (SD)[a]				Cell loss (%)	Reduction in IFU (%)
			Without CD	With CD	35°C	20°C		
CD[b]	GPIC	Static	233 (17)	112 (17)			3.0	52
		Centrifuged	287 (32)	48 (9)			4.4	83
	434	Static	394 (58)	260 (23)			17.3	34
		Centrifuged	351 (24)	237 (22)			11.0	32
Inoculation at 20°C[c]	GPIC	Static			810 (81)	445 (34)	4.0	45
		Centrifuged			825 (178)	216 (3)	0	74
	434	Static			882 (35)	458 (20)	6.5	48
		Centrifuged			694 (51)	413 (23)	0	41

[a] Values are means from three replicate monolayers. Both sets of data are for single experiments representative of two.
[b] Monolayers were inoculated at 35°C. Where indicated, CD (0.5 μg ml^{-1}) was present for 1 h postinoculation and for a further 4 h.
[c] Monolayers were inoculated at 35 or 20°C and kept at that temperature for 1 h. The lower temperature was maintained for a further 4 h before warming to 35°C.

monolayers it might have accounted for the observed reduction in inclusion number.

Investigation of the above possibilities was confined to experiments with CD, since the drug was thought to be a potentially more specific inhibitor of phagocytosis than inoculation at 20°C. Treatment of purified organisms with CD at 0.5 and 5 μg ml^{-1} for 4 h at 35°C did not appear to affect their viability significantly (the following values are in mean IFU per 2,000 cells ± the standard deviation, where CD was absent or present at 0.5 or 5.0 μg ml^{-1}, respectively: strain 434, 247.2 ± 3.1, 211.5 ± 29, and 232.9 ± 18.1; strain GPIC, 216.3 ± 33.5, 270 ± 53, and 300 ± 48). Moreover, irrespective of the strain or inoculation mode, CD had no detrimental effect on chlamydial association with McCoy cells (Table 2; $P > 0.1$; Student's t test). Indeed, in most instances CD caused an increase in organism association (see also Tables 4 and 5).

To assess the contribution of the late entry of organisms to the inclusion number, experiments were done in which CD was present throughout the inoculation and incubation periods. Initial experiments demonstrated that addition of CD (0.5 μg ml^{-1}) to medium after inoculation with strain GPIC or 434, either statically or with centrifugation, had no effect on the inclusion number compared with untreated monolayers ($P > 0.1$; Student's t test; the following values are in mean IFU per 2,000 cells ± the standard deviation, where CD was absent or present at 0.5 μg ml^{-1}, respectively: strain 434, static, 520.3 ± 45.6 and 512.1 ± 12.1, and centrifuged, 497.5 ± 6.5 and 521.5 ± 36.4; strain GPIC, static, 212 ± 15.1, and 196.1 ± 12.2 and centrifuged, 216.7 ± 22.3 and 243.7 ± 27.6). When the drug was present during both inoculation and development, the infectivity values obtained (Table 3) were similar to those obtained when CD was present only during entry (Table 1), implying that late entry of organisms by the CD-sensitive pathway did not occur to a significant

TABLE 2. Effect of CD on the association of strains GPIC and 434 with monolayers[a]

Strain and inoculation mode	CD	Mean dpm/monolayer (SD)[b]; no. of associated organisms/cell with inoculation at:	
		2°C[c]	35°C[d]
GPIC[e]			
Static	−	542 (77); 3.4	455 (74); 2.9
	+	593 (38); 3.7	447 (22); 2.9
Centrifuged	−	1,107 (125); 6.9	239 (17); 1.5
	+	1,137 (184); 7.1	192 (35); 1.2
434[e]			
Static	−	681 (78); 6.0	2,183 (160); 14.4
	+	781 (50); 6.8	2,204 (68); 14.5
Centrifuged	−	856 (16); 7.5	1,162 (75); 7.7
	+	963 (119); 8.4	1,193 (67); 7.9

[a] Monolayers inoculated at 2°C with centrifugation were warmed to 35°C to allow entry, with or without centrifugation at 35°C for 1 h. For both sets of experiments, association of organisms was assessed at 4 h postinoculation.
[b] The values shown are means from four monolayers.
[c] Results of one experiment representative of three (GPIC) or four (434) are shown.
[d] Data from individual experiments are shown.
[e] Chlamydia particle/disintegrations-per-minute ratios were as follows: for inoculation at 2°C, 1,250:1 (GPIC) and 1,750:1 (434); for inoculation at 35°C, 1,276:1 (GPIC) and 1,317:1 (434).

TABLE 3. Effect of the presence of CD during inoculation and development on the infectivities of strains GPIC and 434

Strain and inoculation mode	Expt no.	Mean no. of IFU[a] 2,000 cells^{-1} (SD)		Cell loss (%)	Reduction in IFU no. (%)
		Without CD	With CD[b]		
GPIC[c]					
Static	1	338 (22)	166 (28)	28	51
	2	27 (2)	14 (4)	31	48
Centrifuged	1	171 (21)	23 (3)	22	86
	2	49 (7)	2 (0.5)	22	96
434[c]					
Static	1	520 (57)	247 (16)	36	53
	2	388 (41)	208 (26)	46	47
Centrifuged	1	398 (26)	140 (16)	32	65
	2	341 (28)	232 (14)	32	32

[a] The values shown are means from four replicate monolayers.
[b] CD (0.5 μg ml^{-1}) was present throughout inoculation and incubation for inclusion development.
[c] Inoculated at 35°C and incubated at that temperature for 1 h.

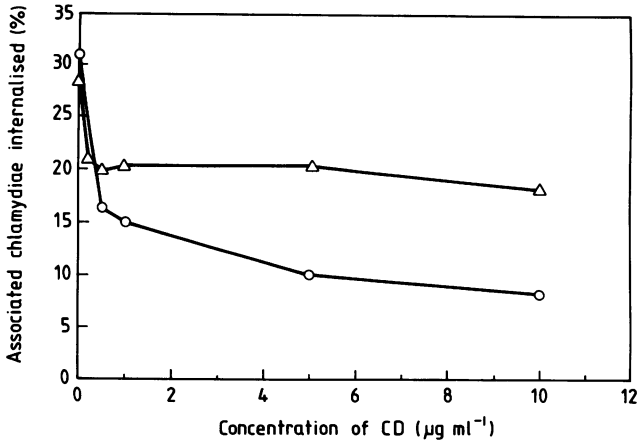

FIG. 2. Effect of an increased CD concentration on the entry of strains GPIC (○) and 434 (△) into McCoy cells. Inoculation was done as detailed in the legend to Fig. 1, with 45 to 50 radiolabeled chlamydiae associated per cell. After 4 h of incubation at 35°C, uptake of chlamydiae was determined by using the trypsin assay (see Materials and Methods). The values for internalized chlamydiae are means from seven replicate monolayers (the standard deviations of original radioactivity values were less than 15% of the mean values). The batches of chlamydiae used had particle/disintegrations-per-minute ratios of 442:1 (GPIC) and 1,438:1 (434).

TABLE 4. Effect of CD on entry[a] of strains GPIC and 434 as measured by immunofluorescence assay

Strain and inoculation mode	CD	Mean no. of chlamydiae associated/cell (SD); % inside cells in:		
		Expt 1[b]	Expt 2[b]	Expt 3[c]
GPIC				
Static	−	3.46 (0.49); 41	11.0 (0.77); 49	4.66 (0.57); 97
	+	3.79 (0.24); 38	16.5 (0.32); 46	3.74 (1.39); 94
Centrifuged	−	7.05 (0.8); 57	9.87 (0.76); 61	3.94 (0.31); 67
	+	7.25 (1.2); 12	15.0 (2.05); 31	4.53 (0.72); 35
434				
Static	−	2.85 (0.29); 48	2.74 (0.13); 52	4.36 (0.63); 79
	+	2.9 (0.14); 36	4.34 (0.46); 49	3.83 (0.57); 67
Centrifuged	−	2.97 (0.26); 52	3.09 (0.68); 49	2.67 (0.4); 41
	+	3.38 (0.17); 23	5.0 (0.3); 37	3.18 (0.27); 27

[a] Entry was assayed at 4 h postinoculation; the values shown are means from four replicate monolayers.
[b] Inoculation at 2°C with centrifugation and then warming to 35°C with or without centrifugation for 1 h.
[c] Inoculation at 35°C and incubation at that temperature for 1 h.

extent. Furthermore, selective loss of infected cells from CD-treated monolayers did not appear to have occurred, since cell counts from these monolayers were similar to those from drug-treated, uninfected monolayers ($P > 0.1$; Student's t test). This series of experiments confirmed that the effect of CD on the infectivity of the two strains was due to effects of the drug on McCoy cells during the process of organism entry.

Effect of CD on chlamydial uptake. In an initial experiment, the concentration of CD required to prevent organism entry was determined for both strains by using the trypsin assay (Fig. 2). As for the study of infectivity reduction (Fig. 1), suppression of entry was nearly maximal at a CD concentration of approximately 0.5 μg ml^{-1}.

The effect of CD (0.5 μg ml^{-1}) on the entry of strains GPIC and 434 was further studied by using two distinct assays employing either trypsin removal of attached extracellular organisms or their enumeration following immunofluorescence staining (see Materials and Methods). The results were similar for both procedures (Tables 4 and 5). For each of the strains, the proportion of organisms entering McCoy cells during static inoculation was reduced by only ca. 10% in the presence of CD. However, if the inoculum was centrifuged, the drug caused a ca. 50% reduction in the entry of both strains. These observations indicated that both strains entered cells predominantly by a CD-resistant mechanism in static inoculation but that CD-sensitive uptake could be greatly enhanced by centrifugation. The extents of entry were different for the two assay methods, with those from the trypsin assay consistently lower.

Entry mechanism and potential for productive infection. The relationship between entry mechanism and productive infection can be quantified as the number of organisms that must be internalized for an inclusion to be produced. The infectivity data (Tables 6 and 7) were abstracted from Tables 1 and 3. The experiments were not accompanied by parallel measurements of attachment and entry. Therefore, the attachment values determined in a separate experiment (Tables 6 and 7), together with entry estimates abstracted from Table 4, were applied to the data on infectivity. Infectivity potentials were calculated from the combined infectivity, attachment, and entry data (Tables 6 and 7).

In static inoculation, CD-sensitive entry for both strains was ca. 10-fold more likely to lead to multiplication than was CD-resistant entry; however, strain 434 exploited both entry mechanisms more effectively than did strain GPIC (Tables 6 and 7). Following centrifugation, differences in infectivity potential between the two entry mechanisms were less marked. Centrifugation enhanced strain GPIC infectivity over that in static inoculation but had little effect on strain 434.

DISCUSSION

Overall, the data on infectivity and entry establish that two distinct endocytic mechanisms, microfilament-dependent phagocytosis and pinocytosis, operate simultaneously during the entry of both strains GPIC and 434 into McCoy cells and that the infectivity potentials of the two strains are modulated by the entry mechanisms. In addition, while centrifugation promoted phagocytic entry by both strains it enhanced the infectivity potential of only strain GPIC.

For both the infectivity and entry experiments, it was important that CD treatment did not interfere with pinocytic uptake and that inhibition of phagocytosis did not markedly divert organisms to the pinocytic pathway. There is evidence that cytochalasins can interfere with constitutive fluid uptake (23) and coated-pit vesiculation (19). However, these early studies used cytochalasins that are less specific than CD (1) and the effects occurred at high drug concentrations (5 to 20 μg ml^{-1}). CD at the concentration used here (0.5 μg ml^{-1}) has no effect on fluid-phase pinocytosis by McCoy cells (17).

If diversion to pinocytosis occurred, then drug treatment would always result in underestimation of phagocytic entry. However, when pinocytic entry was prevented by cytosol acidification with amiloride (17, 20, 25) there was no signif-

TABLE 5. Effect of CD on entry [a] of strains GPIC and 434 as measured by trypsin assay

Strain[b] and inoculation mode	CD	Mean no. of chlamydiae associated/cell (SD); % inside cells in:		
		Expt 1	Expt 2	Expt 3
GPIC				
Static	−	14.7 (0.85); 13.8	7.5 (0.1); 8.7	7.2 (0.4); 12.4
	+	18.4 (1.2); 11.1	9.85 (0.2); 8.9	8.7 (0.25); 9.2
Centrifuged	−	18.3 (1.55); 26.5	10.25 (0.95); 10.6	8.35 (0.35); 19.3
	+	21.3 (4.05); 13.2	12.4 (0.65); 8.9	10.1 (0.55); 8.8
434				
Static	−	19.5 (1.4); 19.0	16.9 (0.65); 9.5	14.1 (0.57); 26.9
	+	20.7 (0.1); 18.0	18.85 (0.7); 9.1	17.0 (2.2); 26.1
Centrifuged	−	21.5 (1.1); 17.0	20.0 (0.45); 6.7	17.6 (1.6); 26.6
	+	23.9 (1.1); 11.3	21.8 (1.05); 5.3	20.65 (1.85); 18.8

[a] Inoculated at 2°C and then warmed to 35°C with or without centrifugation. Entry was assayed at 4 h postinoculation. The values shown are means from six monolayers.

[b] Chlamydia particle/disintegrations-per-minute ratios were 442:1 for GPIC and 1,438:1 for 434.

icant difference in CD-suppressible entry compared with an unacidified control. The mean percentages of intracellular organisms ± the standard deviation were as follows: unacidified monolayers, 16.9 ± 1.9 without CD and 15.1 ± 1.6 with CD (hence, the CD-suppressible component of entry was 1.8); acidified monolayers, 5.3 ± 0.6 without CD and 3.3 ± 0.8 with CD (hence, the CD-suppressible component of entry was 2.0).

Initial conclusions from the infectivity experiments concerning endocytic mechanism were similar for both the CD and 20°C inoculation experiments. Although interpretation of the 20°C inoculation data was hindered by alternative explanations, additional experiments (Results; Tables 2 and 3) ruled them out for CD treatment, so it seems reasonable to conclude that in both treatments lowered infectivity resulted from selective suppression of phagocytosis.

Assessment of the relative efficiencies with which the two mechanisms mediated productive infection required measurement of entry. The proportions of chlamydial uptake by each of the two endocytic mechanisms were comparable for both the immunofluorescence and trypsin entry assays. However, the trypsin assay gave lower estimates of total uptake in most instances. This difference was not related to organism multiplicity, because when similar numbers of organisms were associated, uptake was still lower (Tables 4

TABLE 6. Estimated efficiencies of chlamydial multiplication following entry by CD-resistant and -sensitive mechanisms[a]

Strain and expt no.	No. of organisms inoculum^{-1} (10^5); no. of IFU monolayer^{-1} (10^3) without/with CD[b]	
	Static inoculation	Centrifuged inoculation
GPIC		
1	6,280; 33.84/16.58	6.98; 17.06/2.33
2	1,956; 2.7/1.39	2.3; 4.91/0.195
3	15,730; 23.26/11.15	26.25; 28.68/4.836
434		
1	59.76; 4.52/2.75	4.95; 6.19/3.1
2	62.96; 11.91/4.37	6.35; 11.5/3.28
3	84.05; 34.14/12.89	8.47; 27.49/7.17

[a] Original data are from experiments in which CD was applied throughout the development cycle (experiments 1 and 2 for both strains, Table 3) or only at entry (experiment 3: GPIC, Table 1; 434, data not previously shown). In all experiments except no. 3 with strain 434, inoculation was at 35°C. Between 10 and 15% of cells were infected, ensuring that few cells received more than 1 IFU.

[b] The association and entry values for strains GPIC and 434 that were applied to the infectivity data shown were as follows. For the static and centrifuged-inoculation modes, respectively, 0.6% of GPIC and 4.25% of strain 434 organisms inoculated were associated with the monolayer. With static inoculation, 50% of the organisms of both strains associated with the monolayer were internalized without CD versus 45% with CD, and with centrifuged inoculation, the corresponding proportions were 50 and 25%.

TABLE 7. Frequency of productive infection in terms of calculated numbers of chlamydiae internalized per inclusion detected[a]

Strain and expt no.	No. of chlamydiae internalized/inclusion (CD resistant[b]/sensitive[c])	
	Static inoculation	Centrifuged inoculation
GPIC		
1	102.3/10.9	26.3/4.1
2	380.3/44.7	113.4/4.7
3	381.3/39.0	47.5/9.6
434		
1	54.3/5.3	19.7/11.6
2	27.6/1.8	17.7/7.0
3	12.5/0.84	10.8/3.8

[a] Derived from the values shown in Table 6.

[b] Calculated from the mean number of chlamydiae internalized (derived from inoculum, association, and entry data) and the mean numbers of inclusions formed in CD-treated monolayers.

[c] The numbers of chlamydiae internalized and inclusions formed in untreated monolayers were calculated as described in footnote *b*, and the numbers of internalized organisms per inclusion were derived. By using this derived value and the values for CD-resistant efficiency of productive infection, the efficiency of CD-sensitive productive infection was determined on the basis of the example below. If 1,000 chlamydiae (arbitrary value) enter cells in a monolayer, the efficiency of productive infection following CD-sensitive entry is given by: $(1{,}000 \times b)/\{(1{,}000/B) - [(1{,}000 \times a)/A]\}$, where A is the number of internalized chlamydiae per inclusion in CD-treated monolayers, B is the number of internalized chlamydiae per inclusion in untreated monolayers, a is the proportion of entry that is CD resistant, and b is the proportion of entry that is CD sensitive.

and 5, experiments 2). It seems likely that the trypsin assay underestimated entry because when infectivity and entry of strain GPIC were measured in parallel monolayers, a greater number of inclusions was seen than the number of organisms determined to be intracellular (unpublished observations). The reason for the underestimate is not clear, but it may result from trypsin-induced cytoskeletal disruption (5) leading to exocytosis of organisms. Whatever the reason for the difference in total-uptake measurement between the two assays, suppression of entry was clearly comparable for both strains in each inoculation mode. The amount of CD-sensitive entry found for strain 434 was considerably less than that reported for HeLa cells (24), but this may relate to the different cell line used or to the time allowed for uptake (30 min versus the 4 h used here) if early entry was predominantly phagocytic.

The chlamydial uptake values allow the frequency of productive infection following entry by each of the two endocytic mechanisms to be evaluated. In static inoculation of strain 434 or GPIC, CD-induced suppression of infectivity indicated that ca. 50% of inclusions were from CD-sensitive (phagocytic) entry and ca. 50% were from CD-resistant (pinocytic) entry (Tables 1 and 3). However, uptake measurements revealed that most (75 to 97%; Tables 4 and 5) of the organisms entered pinocytically, indicating that for both strains the infectivity potential was much greater following phagocytic entry.

The relationship between entry mechanism and infectivity is presented quantitatively in Tables 6 and 7. Two key points emerged. (i) Phagocytic entry increased the infectivity potential over pinocytic entry for both strains. (ii) Strain 434 exploited both mechanisms more efficiently than did strain GPIC, unless the latter was centrifuged. Therefore, the poor infectivity of strain GPIC is related not to inability to induce phagocytic entry but to its low infectivity potential irrespective of the entry mechanism. Overall, then, it appears that in this cell culture model intrinsic strain properties are dominant over the endocytic mechanism for productive infection.

How can the entry mechanism affect the intracellular destination? Current evidence suggests that the intracellular fate of simple ligands is related to properties of the internalizing vesicle membrane (11, 22). Intracellular routing of chlamydiae to lysosomes has been shown to correlate with differences in the composition of the vesicle membrane surrounding endocytosed chlamydiae (27), implying a process of membrane selection or modification during entry. It is possible that such changes in vesicle membrane properties also determine access to host cell nutrients required for parasite multiplication (14).

Whether these two entry mechanisms involve the same or different host cell receptors is not known. The observation that following attachment at 4°C subsequent entry at 35°C is diverted from pinocytosis to phagocytosis by centrifugation (Tables 4 and 5) suggests that the entry mechanism is not decided by initial attachment events. On the other hand, the finding that inhibition of phagocytosis by CD and prevention of coated-pit uptake have little effect on one another (Tables 4 and 5; 17) suggests that the two endocytic mechanisms occur independently, implying that at some stage during attachment commitment to one or the other endocytic pathway takes place.

This increased complexity of interaction events during bacterial invasion of host cells is not unique; there is evidence for multiple ligand-receptor pathways of phagocytosis for a number of facultative intracellular bacteria (2, 3, 9).

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REFERENCES

1. **Cooper, J. A.** 1987. Effects of cytochalasin and phalloidin on actin. J. Cell Biol. **105**:1473–1478.
2. **Elsinghorst, E. A., L. S. Baron, and D. J. Kopecko.** 1989. Penetration of human intestinal epithelial cells by *Salmonella*: molecular cloning and expression of *Salmonella typhi* invasion determinants in *Escherichia coli*. Pro. Natl. Acad. Sci. USA **86**:5137–5177.
3. **Finlay, B. B., and S. Falkow.** 1989. *Salmonella* as an intracellular parasite. Mol. Microbiol. **3**:1833–1841.
4. **Friis, R. R.** 1972. Interaction of L cells and *Chlamydia psittaci*: entry of the parasite and host responses to its development. J. Bacteriol. **110**:706–721.
5. **Furcht, L. T., and G. Wendelschafer-Crabb.** 1978. Trypsin-induced coordinate alterations in cell shape, cytoskeleton and intrinsic membrane structure of contact-inhibited cells. Exp. Cell Res. **114**:1–14.
6. **Hatch, T. P., M. Miceli, and J. E. Sublett.** 1986. Synthesis of disulfide-bonded outer membrane proteins during the developmental cycle of *Chlamydia psittaci* and *Chlamydia trachomatis*. J. Bacteriol. **165**:379–385.
7. **Hodinka, R. L., C. H. Davis, J. Choong, and P. B. Wyrick.** 1988. Ultrastructural study of endocytosis of *Chlamydia trachomatis* by McCoy cells. Infect. Immun. **56**:1456–1463.
8. **Hodinka, R. L., and P. B. Wyrick.** 1986. Ultrastructural study of mode of entry of *Chlamydia psittaci* into L-929 cells. Infect. Immun. **54**:855–863.
9. **Isberg, R. R.** 1989. Mammalian cell adhesion functions and cellular penetration of enteropathogenic *Yersinia* species. Mol. Microbiol. **3**:1449–1453.
10. **Lawn, A. M., W. A. Blyth, and J. Taverne.** 1973. Interactions of TRIC agents with macrophages and BHK-21 cells observed by electron microscopy. J. Hyg. **71**:515–528.
11. **Mellman, I. S., R. Fuchs, and A. Helenius.** 1986. Acidification of the endocytic and exocytic pathways. Annu. Rev. Biochem. **98**:663–700.
12. **Monnickendam, M. A., and J. H. Pearce.** 1983. Immune responses and chlamydial infections. Br. Med. Bull. **39**:187–193.
13. **Pearce, J. H.** 1986. Early events in chlamydial infection. Ann. Inst. Pasteur/Microbiol. (Paris) **137A**:325–332.
14. **Pearce, J. H.** 1988. Chlamydial invasion mechanisms: implications for parasite growth and host restraint. Proc. Eur. Soc. Chlamydia Res. **1**:109–112.
15. **Prain, C. J., and J. H. Pearce.** 1985. Endocytosis of chlamydiae by McCoy cells: measurements and effects of centrifugation. FEMS Microbiol. Lett. **26**:233–237.
16. **Prain, C. J., and J. H. Pearce.** 1989. Ultrastructural studies on the intracellular fate of *Chlamydia psittaci* (strain guinea pig inclusion conjunctivitis) and *Chlamydia trachomatis* (strain lymphogranuloma venereum 434): modulation of intracellular events and relationship with endocytic mechanism. J. Gen. Microbiol. **135**:2107–2123.
17. **Reynolds, D. J., and J. H. Pearce.** 1990. Characterization of the cytochalasin D-resistant (pinocytic) mechanisms of endocytosis utilized by chlamydiae. Infect. Immun. **58**:3208–3216.
18. **Ripa, K. T., and P.-A. Mardh.** 1977. Cultivation of *Chlamydia trachomatis* in cycloheximide-treated McCoy cells. J. Clin. Microbiol. **6**:328–331.
19. **Salisbury, J. L., J. S. Condeelis, and P. Satir.** 1980. Role of coated vesicles, microfilaments and calmodulin in receptor-mediated endocytosis by cultured B lymphoblastoid cells. J. Cell Biol. **87**:132–141.
20. **Sandvig, K., S. Olsnes, O. W. Petersen, and B. Van Deurs.** 1987. Acidification of the cytosol inhibits endocytosis from coated pits. J. Cell Biol. **105**:679–689.
21. **Starr, T. J., M. Pollard, Y. Tanami, and R. W. Moore.** 1960. Cytochemical studies with psittacosis virus by fluorescence

microscopy. Tex. Rep. Biol. Med. **18**:501–514.
22. **Ukkonen, P., V. Lewis, M. Marsh, A. Helenius, and I. Mellman.** 1986. Transport of macrophage Fc receptors and Fc receptor-bound ligands to lysosomes. J. Exp. Med. **163**:952–971.
23. **Wagner, R., M. Rosenberg, and R. Estensen.** 1971. Endocytosis in Chang liver cells. Quantitation by sucrose-^{3}H uptake and inhibition by cytochalasin B. J. Cell Biol. **50**:804–817.
24. **Ward, M. E., and A. Murray.** 1984. Control mechanisms governing the infectivity of *Chlamydia trachomatis* for HeLa cells: mechanisms of endocytosis. J. Gen. Microbiol. **130**:1765–1780.
25. **West, M. A., M. S. Bretscher, and C. Watts.** 1989. Distinct endocytotic pathways in epidermal growth factor-stimulated human carcinoma A431 cells. J. Cell Biol. **109**:2731–2739.
26. **Wyrick, P. B., J. Choong, C. H. Davis, S. T. Knight, M. O. Royal, A. S. Maslow, and C. R. Bagnell.** 1989. Entry of genital *Chlamydia trachomatis* into polarized human epithelial cells. Infect. Immun. **57**:2378–2389.
27. **Zeichner, S. L.** 1983. Isolation and characterization of macrophage phagosomes containing infectious and heat-inactivated *Chlamydia psittaci*: two phagosomes with different intracellular behaviors. Infect. Immun. **40**:956–966.