Influence of Cysteine Deprivation on Chlamydial Differentiation from Reproductive to Infective Life-cycle Forms

By I. ALLAN,¹† T. P. HATCH² AND J. H. PEARCE¹*

¹ Department of Microbiology, University of Birmingham, Birmingham B15 2TT, UK

² Department of Microbiology and Immunology, Center for the Health Sciences, University of Tennessee, Memphis, Tennessee 38163, USA

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The effects of omission of individual amino acids from growth medium on the differentiation of *Chlamydia trachomatis* DK-20 (serotype E) during infection of cycloheximide-treated McCoy cells are described. As judged by inclusion body staining with acridine orange, omission of cysteine from the medium severely retarded differentiation of reproductive reticulate body (RB) to infective elementary body (EB) forms. The effect appeared specific to cysteine in that omission of other amino acids had little or no effect on differentiation once RBs appeared. On restoration of cysteine, culture infectivity increased and inclusions contained organisms which, by cytochemical and morphological criteria, were differentiating to infective forms, indicating that cysteine deprivation did not irreversibly inhibit differentiation. Impairment of RB to EB differentiation in cysteine-less medium was also observed for three strains of *Chlamydia psittaci* and 10 other strains of *C. trachomatis*. It is suggested that the effect arises via the biosynthetic requirement for cysteine for provision of three cysteine-rich proteins, whose synthesis and insertion into the outer membrane have previously been shown to accompany RB to EB differentiation of *C. psittaci* 6BC and *C. trachomatis* 434 (serotype L₂). Synthesis of cysteine-rich outer membrane proteins during differentiation may thus be common to all chlamydiae.

INTRODUCTION

Chlamydiae are obligate intracellular bacteria that undergo a unique developmental cycle within the host cell. Following endocytosis the small, rigid, infectious elementary body (EB) differentiates to the large, fragile, metabolically active reproductive or reticulate body (RB). After a period of binary fission, the latter redifferentiate to infectious forms and host cell lysis and release of progeny occurs.

The structural differences between RBs and EBs and their functional relationships with physiological properties of the two life-cycle forms are poorly understood. Nor is it known how the EB to RB to EB morphogenetic cycle is regulated. For transmissible infection the latter must ensure both division of reproductive forms and redifferentiation of these to infectious EBs.

Tamura & Manire (1967), studying the CAL-10 strain of *Chlamydia psittaci*, reported that envelope preparations of RBs differed from those of EBs in that they contained neither cysteine nor methionine. Failing to detect muramic acid in chlamydial envelopes (Tamura & Manire, 1967), they suggested that disulphide bonding might contribute to the structural rigidity of EBs that is lacking in the fragile RBs. Data supporting this proposal have been obtained by Hatch *et al.* (1981), who showed that solubilization of outer membrane proteins of the 6BC strain of *C.*

† Present address: Department of Paediatrics, John Radcliffe Hospital, University of Oxford, Headington, Oxford OX3 9DU, UK.

Abbreviations: EB, elementary body; RB, reticulate body; FBS, foetal bovine serum; HBSS, Hanks' balanced salts solution; i.f.u., inclusion-forming unit; p.i., post-infection.

psittaci required both detergent and reducing agent. Further, Newhall & Jones (1983) have reported the presence of disulphide cross-linked polypeptide complexes in outer membrane preparations of *C. trachomatis* UW94 (serotype E).

Stirling *et al.* (1983) presented evidence from metabolic studies that deprivation of cysteine during the growth of *C. trachomatis* DK-20 (serotype E) severely retarded differentiation of RB to EB forms. This led to the demonstration, for both *C. psittaci* 6BC and *C. trachomatis* 434 (serotype L₂), that RB to EB differentiation is associated with the synthesis of three extraordinarily cysteine-rich outer membrane proteins which appear to be important for the structural rigidity of EBs (Hatch *et al.*, 1984) and which may well influence, via surface structure, physiological properties unique to the infective EB. Here we have examined the specificity of amino acid deprivation on differentiation of strain DK-20 and assessed the effect of cysteine deprivation on differentiation of 13 other chlamydial strains.

METHODS

Organisms. Chlamydiae were produced by growth in irradiated McCoy cell monolayers; inoculation, incubation and preparation were as previously described (Allan & Pearce, 1983*b*). The following strains were grown. *C. trachomatis* strains A/SA-1/OT (Wang & Grayston, 1971), B/TW-5/OT, C/UW-1/OT, D/CAL-8/ON (Treharne *et al.*, 1977), E/MRC-4/IC (Collier & Smith, 1967), E/DK-20/ON, F/MRC-301/U, G/IOL-238/R, H/UW-4/Cx, I/UW-12/U and L₂/434/Bu (Treharne *et al.*, 1977): the classification is serotype/strain/origin; OT, ocular trachoma; ON, ophthalmia neonatorum; IC, inclusion conjunctivitis; U, urethra; R, rectum; Cx, cervix; Bu, bubo. *C. psittaci* strains enzootic abortion of ewes (EAE; Stamp *et al.*, 1950), guinea pig inclusion conjunctivitis (GP-IC; Murray, 1964), and meningopneumonitis (CAL-10; Francis & Magill, 1938).

Cell culture and monolayer preparation. These were as previously described for irradiated McCoy cells (Allan & Pearce, 1983*b*).

Assessment of the effect on chlamydial development of deletion of amino acids from the growth medium. Suspensions of chlamydiae in Hanks' balanced salts solution (HBSS) containing 5% (v/v) foetal bovine serum (FBS) were centrifuged with monolayers of McCoy cells irradiated one day earlier (Griffiths *et al.*, 1976). Subsequently the inoculum was removed and control or test growth medium added. Growth media were prepared from a 'select amine kit' (Gibco Biocult, Paisley, UK) as previously described (Allan & Pearce, 1983*a*) and were supplemented with streptomycin (100 µg ml⁻¹) and cycloheximide (2 µg ml⁻¹); FBS was dialysed before supplementation to 5% (v/v). Monolayers were incubated at 35 °C in air/CO₂ (95:5, v/v) and sampled at intervals for assessment of one or more of the parameters considered below.

Numbers of infected cells. These were counted by microscopic examination of infected monolayers after fixation and Giemsa staining.

Yield of infectious progeny. This was determined as previously described (Allan & Pearce, 1983*a*).

Cytochemical composition of developing inclusions. This was assessed by microscopic examination of infected monolayers after fixation with cold glacial acetic acid:ethanol (1:2, v/v) and staining with acridine orange fluorescence (Pollard & Tanami, 1962). Infected monolayers were scanned (500× magnification) using an oil immersion planapochromat objective (40×; Carl Zeiss) for the presence of inclusions containing red RB and lime-green EB forms; these had been verified by electron microscopic examination of infected monolayers (Stirling *et al.*, 1983).

In all experiments with *C. trachomatis* DK-20, except where stated, one cytochemical form appeared to predominate, both in individual inclusions and in infected cell populations. In a few instances (see text) inclusions contained significant numbers of forms intermediate in fluorescence between RBs and EBs, although appearing EB-like in size; these were recorded as intermediate forms. In two experiments a range of strains of *C. trachomatis* and *C. psittaci* were examined (Table 3). Here, although individual inclusions contained largely one cytochemical form, variation between infected cells was apparent. Inclusions contained largely either RBs or EBs and were subjectively graded 0, 1, 2, 3 or 4, corresponding, approximately to 0, 25, 50, 75 or 100% of inclusions scanned being of one or other form. Thus, monolayers graded '2, 2' contained approximately equal numbers of inclusions in predominantly RB or EB form; monolayers graded '3, 1' or '1, 3' contained 75% and 25%, respectively, of RB-dominant inclusions.

RESULTS

Specificity of effect of amino acid deprivation on development of life-cycle forms of C. trachomatis DK-20

Deprivation of cysteine from growth medium markedly delayed the differentiation of RB to EB forms within inclusions in cells infected with *C. trachomatis* (oculogenital strain DK-20,

serotype E) as judged by both acridine orange staining and electron microscopy (Stirling *et al.*, 1983). This might have arisen as a result of a general inhibition of inclusion body formation, as seen in earlier studies following deprivation of glutamine, histidine, leucine, phenylalanine or valine (Allan & Pearce, 1983*b*). These experiments with strain DK-20 were therefore repeated with acridine orange staining so that the RB and EB content of inclusions, and thus possible interference with differentiation, could be assessed (Table 1). Observation was extended to 72 h post-infection (p.i.), which, for the irradiated treated cell monolayers under study, approached the maximum time over which infected fully-supplemented cultures could be examined unobscured by cell deterioration.

Of those amino acids whose omission did not impair infection only absence of cysteine markedly retarded RB to EB development (Table 1). Deprivation of leucine, which inhibited infection by G, H, I but not D, E, F serotypes of oculogenital strains (Allan & Pearce, 1983*b*), delayed appearance of inclusions to 48 h p.i.; at this time these were RB in content but by 72 h were entirely EB.

For amino acids whose omission inhibited infection, and which defined nutritional biotypes of *C. trachomatis* (Allan & Pearce, 1983*b*), the effect of deprivation upon development was similar to the above. Thus, for glutamine and phenylalanine, inclusion formation was absent at 24 h p.i., small RB-containing inclusions were evident at 48 h and these had converted to EB by 72 h. Deprivation of histidine or valine inhibited appearance of inclusions until 72 h p.i. For valine, the latter were RB-containing but further incubation to test for differentiation to EB was not feasible.

Effect of cysteine deprivation and restoration on differentiation of C. trachomatis DK-20 to infectious life-cycle forms

One explanation for the retardation of RB to EB differentiation during cysteine deprivation was that structurally or metabolically abnormal forms were induced which were incapable of differentiation to EB forms. To test this possibility the effect of restoration of cysteine on chlamydial differentiation and appearance of infectivity was examined, following a period of deprivation.

Inclusions in infected monolayers 48 h p.i. and under cysteine deprivation contained RB forms, but with a low background of infectivity which had declined by 72 h p.i. (Table 2). Restoration of cysteine for 24 h led to a significant increase in infectivity over that in control infected monolayers still deficient in cysteine or to which cysteine and chloramphenicol (for inhibition of chlamydial protein synthesis) had been added (Table 2). It thus appeared that chlamydiae present after deprivation of cysteine for 48 h were capable of differentiation to infective forms, at least to some extent, on addition of cysteine.

Effect of amino acid deprivation on the development of life-cycle forms of C. trachomatis and C. psittaci

Having established the specificity of cysteine deprivation on RB to EB differentiation for strain DK-20, it was important to assess the general validity of the effect. In addition, the earlier work of Tamura & Manire (1967) had suggested that differences in the content of both methionine and cysteine existed between the envelopes of RB and EB forms of *C. psittaci* CAL-10. We therefore compared cysteine, methionine and combined cysteine and methionine deprivation on the developmental cycle of a range of strains of *C. trachomatis* and *C. psittaci*. Since from earlier data deprivation of either arginine, isoleucine, lysine, threonine, tryptophan or tyrosine had not impaired infection by oculogenital strains of *C. trachomatis* and certain strains of *C. psittaci* (Allan & Pearce, 1983*b*), the effect of *en bloc* omission of these was also examined.

For all strains examined, deprivation of cysteine retarded differentiation of RBs to EBs (Table 3); in no case, however, was this enhanced by the combined deprivation of cysteine and methionine. Methionine omission alone did not retard, except marginally for strain CAL-10, at 24 h p.i. Absence of six other amino acids as a group was also without effect. Overall, it appeared

Table 1. *Effect of single amino acid omissions on the infectivity and development of life-cycle forms of C. trachomatis DK-20 in cycloheximide-treated monolayers*

Suspensions of strain DK-20 in HBSS supplemented with 5% FBS were centrifuged with irradiated McCoy cell monolayers and, after removal of inoculum, growth medium containing 12 of the 13 amino acids of Eagle's minimal essential medium, 5% dialysed FBS and 2 µg cycloheximide ml⁻¹ was added. Monolayers 48 h p.i. were fixed and stained with Giemsa for counts of infected cell numbers and monolayers treated in parallel (24 and 48 and 72 h p.i.) were fixed and stained with acridine orange for assessment of the cytochemical composition of inclusions. In control monolayers, which were incubated with complete growth medium, approx. 95% of cells were infected. The results are for one experiment; individual values are the mean of duplicate monolayers with approx. 800 cells per monolayer scanned after Giemsa staining and 1000 cells after acridine orange staining.

Omitted amino acid	Infection*	Dominant cytochemical form within inclusion bodies		
		24 h p.i.	48 h p.i.	72 h p.i.
—	100	RB	EB	EB
Arginine	96	RB	RB > IF†	EB
Cysteine	95	RB	RB	RB
Glutamine	18	0	RB‡	EB‡
Histidine	0	0	0	EB‡§
Isoleucine	101	RB	EB > IF†	EB
Leucine	97	0	RB‡	EB
Lysine	97	RB	IF†	EB
Methionine	96	RB	EB	EB
Phenylalanine	4	0	RB‡	EB
Threonine	96	RB	IF†	EB
Tryptophan	98	RB	EB > IF†	EB
Tyrosine	95	RB	EB > IF†	EB
Valine	0	0	0	RB‡§

* Infection is expressed as a percentage of the number of infected cells in the control receiving complete medium.

† Organisms appearing intermediate in fluorescence colour between typical RBs and EBs, although EB-like in size, were recorded as IF (intermediate forms). In some instances inclusions contained both RB and IF or IF and EB forms.

‡ Inclusions were markedly smaller than those in cells in complete medium.

§ Less than 1% of cells in the monolayer contained inclusions.

Table 2. *Effect of restoration of cysteine on the differentiation of C. trachomatis DK-20 to infective forms in cell monolayers*

Infected monolayers (approx. 95% cells infected) were incubated for 48 h p.i. in medium with cysteine (Cys) present or absent. Cysteine-less cultures were denied cysteine for a further 24 h or replenished with cysteine, with or without the addition of chloramphenicol (80 µg ml⁻¹). Infected monolayers were assessed at these times for the cytochemical composition of inclusions in infected cells and, in parallel monolayers, for their yield of infective progeny. Results are the mean of two experiments; estimates of cytochemical composition are from examination of duplicate monolayers, 1500 cells per monolayer; infectivity yields were titrated from four monolayers pooled after sonication and inclusion-forming units (i.f.u.) per sonicated cell were calculated from mean inclusion counts from duplicate monolayers after scanning 2500 cells per monolayer (approx. 10% cells infected).

Infected monolayers: feeding schedule		Dominant cytochemical form in infected cells	Yield (i.f.u. per infected cell)
0-48 h	48-72 h		
+ Cys	—	EB	590
- Cys	—	RB	17.5
- Cys	- Cys	RB	0.2
- Cys	+ Cys + Cpl	RB	0*
- Cys	+ Cys	IF†	7.1

* Failure to detect infectivity in this sample may be due to contaminating chloramphenicol (Cpl).

† Intermediate forms (see Table 1).

Table 3. *Effect of omission of sulphur-containing or 'non-required' amino acids on the development of strains of C. trachomatis and C. psittaci in cell monolayers*

Infection conditions were as described in Table 1 except that media were deficient either in cysteine, methionine, cysteine and methionine or a 'non-required' group of six amino acids comprising arginine, isoleucine, lysine, threonine, tryptophan and tyrosine. At the times indicated p.i. monolayers were fixed and stained with acridine orange for assessment of cytochemical composition of inclusions. Approx. 1000 cells on each of two monolayers were scanned; individual inclusions were predominantly RB or EB and the proportion in one or other form was graded 0, 1, 2, 3 or 4 corresponding to 0 to 100% (see Methods). In monolayers prepared in parallel, strain infectivity in complete medium was assessed by staining of monolayers (800 cells scanned on each of duplicate monolayers) 48 h p.i. (*C. trachomatis*) or 24 h p.i. (*C. psittaci*) and compared with the values for deficient media. Results are for one experiment (cysteine deprivation data are typical of results for two experiments); between 60 and 95% of cells were infected by the strain inocula tested.

Strain	Omitted amino acids	Proportion of inclusions in RB- or EB-dominant form	
		48 h p.i. RB, EB	72 h p.i. RB, EB
<i>C. trachomatis</i> (serotype)			
CAL-8(D)	Cys, Met	2, 2	2, 2
	Cys	2, 2	2, 2
	Met	0, 4	0, 4
	Arg, Ile, Lys, Thr, Try, Tyr	0, 4	0, 4
	None	0, 4	0, 4
MRC-4(E)*	Cys, Met	2, 2	0, 4
	Cys	2, 2	0, 4
	Met	0, 4	0, 4
DK-20(E)*	Cys, Met	4, 0	3, 1
	Cys	4, 0	3, 1
	Met	0, 4	0, 4
MRC-301(F)*	Cys, Met	2, 2	2, 2
	Cys	2, 2	2, 2
	Met	0, 4	0, 4
IOL-238(G)*	Cys, Met	2, 2	1, 3
	Cys	2, 2	1, 3
	Met	0, 4	0, 4
UW-4(H)*	Cys, Met	4, 0	1, 3
	Cys	4, 0	1, 3
	Met	0, 4	0, 4
UW-12(I)*	Cys, Met	4, 0	2, 2
	Cys	4, 0	2, 2
	Met	0, 4	0, 4
SA-1(A)†	Cys	3, 1	ND
TW-5(B)†	Cys	3, 1	ND
UW-1(C)†	Cys	3, 1	ND
434(L ₂)†	Cys	2, 2	ND
		24 h p.i. RB, EB	48 h p.i. RB, EB
<i>C. psittaci</i>			
GP-1C*	Cys, Met	4, 0	1, 3
	Cys	4, 0	1, 3
	Met	2, 2	0, 4
CAL-10*	Cys, Met	2, 2	1, 3
	Cys	3, 1	1, 3
	Met	2, 2	0, 4
EAE†	Cys	4, 0	ND

ND, Not determined.

* Results for complete medium or omission of the 'non-required' group of amino acids were identical to those for omission of methionine. Omissions did not reduce infectivity compared with that in complete medium.

† In complete medium all inclusions were EB dominant (RB, EB = 0, 4) at 48 h p.i. (*C. trachomatis*) or contained equal numbers of RB and EB (RB, EB = 2, 2) at 24 h p.i. (*C. psittaci* EAE); omissions did not reduce infectivity compared with that in complete medium.

that only cysteine deprivation significantly affected differentiation. Interestingly, the extent of retardation appeared to vary between strains, with DK-20, the strain first examined for these effects, apparently the most affected.

DISCUSSION

For strain DK-20, omission of cysteine from the medium severely retarded RB to EB differentiation without impairment of infectivity (Table 1). Deletion of leucine, glutamine, histidine, phenylalanine or valine (Table 1) reduced inclusion size and/or infected cell number and thus substantially interfered with growth; nevertheless, with the possible exception of valine, conversion of RB to EB forms was not impaired. We conclude, therefore, that retardation of differentiation is specific to deprivation of cysteine and does not occur as a result of generalized slowing of chlamydial protein synthesis and RB division.

Only a moderate rise in infectivity occurred when cysteine-deprived monolayers were replenished with cysteine (Table 2). It is possible that absence of cysteine led to the formation of a proportion of morphologically and metabolically abnormal RBs. However, the presence in such inclusions 72 h p.i. of what were predominantly intermediate forms suggests that the low infectivity appearing subsequent to cysteine restoration was due to uncompleted differentiation of the majority of organisms present rather than to failure of the majority to differentiate from a state of irreversible inhibition. As in the earlier experiment with valine deprivation (Table 1), it was not possible to extend the period of examination because of monolayer deterioration. The latter, via inactivation, may have contributed to the apparent decline in recoverable infectivity over 48 to 72 h (17.5 versus 0.2; Table 2). Inhibition by chloramphenicol of the appearance of infectivity, despite the presence of cysteine, provides further evidence that a true increase had occurred on cysteine restoration, emphasizing that RB to EB differentiation requires protein synthesis in the presence of cysteine and does not occur merely by reorganization from existing RB components.

Extension of cysteine deprivation studies to other strains of *C. trachomatis* and *C. psittaci* indicated that differentiation was retarded for all 13 strains examined (Table 3). This was significant in each case compared with strain behaviour in complete medium or in medium lacking the six amino acids arginine, isoleucine, lysine, threonine, tryptophan and tyrosine. It is of interest that, in a previous analysis of *C. psittaci* GP-IC (Allan & Pearce, 1983*b*), only a marginal reduction of infectivity occurred on cysteine deprivation for inclusions harvested at 40 h p.i., which presumably retained similar perceptible numbers of RBs as observed here at 48 h (RB, EB = 1, 3; Table 3).

We have shown elsewhere that for *C. psittaci* 6BC and *C. trachomatis* LGV 434 RB to EB differentiation is accompanied by the synthesis and appearance in outer membrane complexes of three cysteine-rich proteins (Hatch *et al.*, 1984), one or more of which may be similar to the keratin-like spore coat proteins in cysteine content (Aronson & Fitz-James, 1968). It is possible therefore that there is a considerable biosynthetic demand for cysteine during differentiation and this may account for the retardation which we have now shown to be cysteine-specific. Supportive evidence from estimates of molar ratios and cysteine contents of envelope proteins of RB and EB forms is not yet available. It is, however, clear from comparative radiolabelling experiments that the cysteine-rich proteins contain cysteine in amounts very significantly above either that in total RB proteins or the major outer membrane protein, which contains both cysteine and methionine and is common to both RB and EB envelopes (Hatch *et al.*, 1984).

Although cysteine deprivation, following general nutrient starvation, can inhibit inclusion formation (Karayiannis & Hobson, 1981), omission here did not appear to prevent RB division – which entails incorporation of cysteine into RB proteins. Thus, if the biosynthetic demand hypothesis is correct, it is the fulfilment of cysteine-rich protein synthesis during differentiation, rather than total protein synthesis, which is impaired by cysteine deprivation. In one other instance where the cysteine level influences differentiation the effect on metabolism is more general: transition of *Histoplasma capsulatum* from the mycelial to the yeast form requires cysteine to reactivate mitochondrial metabolism via shunt pathways which bypass components of the cytochrome system lost during the transition (Sacco *et al.*, 1983).

As discussed elsewhere, the failure of Tamura & Manire (1967) to detect cysteine and methionine in RB outer membrane preparations may have resulted from exposure of RBs to 0.5% SDS which, for RBs of strains 6BC and LGV 434, extracted a large proportion of envelope proteins (Hatch *et al.*, 1984). The absence of significant retardation of differentiation following methionine deprivation (Table 3) is in accord with our observations on [35 S]methionine and [35 S]cysteine labelling of polypeptides in strains 6BC and LGV 434. These showed that the major outer membrane protein is the major methionine-containing protein and that it is present in both RB and EB envelopes, probably in quantitatively similar amounts (Hatch *et al.*, 1984).

The fact that all 14 strains were retarded during cysteine deprivation suggests that the strategy of biosynthesis of cysteine-rich proteins during RB to EB differentiation is common to all chlamydiae. It is of interest that DK-20, the *C. trachomatis* strain first examined, was the one most markedly impaired (Table 3). It is possible that such variations could reflect significant differences of detail, between chlamydiae, in envelope content of cysteine-rich proteins and deployment of disulphide bond cross-linking.

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